Enhancement of Neutrophil Function by Granulocyte-Macrophage Colony-Stimulating Factor Involves Recruitment of a Less Responsive Subpopulation

By Mark P. Fletcher and Judith C. Gasson

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) enhances numerous functions of mature neutrophils (PMN) including phagocytosis, superoxide responses to chemotaxins, antibody-dependent cellular cytotoxicity, and expression of complement receptors. A central question concerns whether the mechanism of enhancement involves quantitative increases in the response of all cells or subpopulation recruitment. The effects of GM-CSF on individual cell light scatter changes, membrane potential, and oxidant responses induced by the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (FMLP) were assessed by flow cytometry and by scoring individual cells for nitroblue tetrazolium dye (NBT) reduction. GM-CSF produced a dose- and time-dependent shift in forward light scatter that was very similar in character to that seen with FMLP or leukotriene B4 stimulation. Although not capable of depolarizing the cells directly, GM-CSF primed PMNs for enhanced membrane potential responses to FMLP by significantly increasing the proportion of depolarizing cells when compared with diluent-treated controls after a 60-minute incubation at 37°C (79.4% ± 3.4% vs. 29.5% ± 4.7%) GM-CSF vs diluent, mean ± SE, P < .005, n = 11). Subpopulation recruitment by GM-CSF treatment was also demonstrated by the FMLP-elicited NBT test. Taken together, these results indicate that GM-CSF can modulate the function of mature PMN by enhancing the proportion of responsive cells.

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Materials. Hanks' balanced salt solution (GIBCO, Grand Island, NY) supplemented with 0.01% bovine serum albumin (HBSS/BSA) or calcium- and magnesium-free HBSS plus BSA, pH 7.4, was used for all assays except as noted in the text. Cytochalasin B (Cyto B) and the chemoattractant peptide FMLP (Sigma Chemical Co, St Louis) were dissolved in dimethyl sulfoxide (Sigma) to make appropriate stock solutions that were further diluted such that the final concentration of dimethyl sulfoxide in the reaction mixtures was 0.1% (vol/vol) or less, a concentration found not to affect cell function. Trypan blue exclusion and lactic dehydrogenase release were assessed as previously described.

Recombinant GM-CSF. Recombinant GM-CSF was purified to homogeneity from the conditioned medium of monkey COS cells transiently transfected with GM-CSF cDNA in the expression vector pSV1023 (B) by sequential gel-filtration chromatography and reverse-phase, high-performance liquid chromatography as previously described.

PMN isolation. PMN were isolated from the peripheral blood of normal subjects by Ficoll-Hypaque density gradient centrifugation followed by dextran sedimentation and hypotonic lysis of RBCs as previously described.

Flow cytometric assay of light scatter and membrane potential...
responses to FMLP with or without GM-CSF. Flow cytometry was performed at 37°C with an Ortho 50H Cytosfluorograf (Ortho Diagnostics, Westwood, MA) equipped with an argon ion laser emitting 400 mW at 488 nm (Spectra Physics, Mountain View, CA) as previously described.13,15 Briefly, forward light scatter (FWD-SC) and orthogonal (right-angle) light scatter (90°-SC) were collected on all cells and allowed discrimination of viable from nonviable cells as well as the different white cell classes. Contaminating RBCs plus debris (non-viable cells) and mononuclear cells could then be excluded from the fluorescence analysis by appropriate gating of the scatter parameters, and only the green di-O-C(3) fluorescence of those cells whose scatter parameters corresponded to viable PMN was observed. The data were analyzed with Ortho's model 2150 Data Handling System.

Before assay, the neutrophils were stored on ice in the absence of calcium or magnesium to minimize PMN self-aggregation as well as spontaneous secretion of granule contents and generation of superoxide anion. In preparation for assay PMN were suspended at 1.0 to 1.5 x 10⁶ cells/mL in HBSS/BSA and incubated at 37°C with diluent or GM-CSF for the times and with the concentrations indicated. The fluorescent probe di-O-C(3)(25 nmol/L) was then added with continuous stirring for five minutes to allow the dye to equilibrate with the cells and demonstrate a stable fluorescence pattern before stimulating with FMLP. In those experiments in which the direct effects of GM-CSF were assessed, PMN were preequilibrated with the membrane potential probe at 37°C for 15 to 20 minutes before the addition of GM-CSF. A minimum of 10,000 PMN were analyzed for each specimen.

NBT reduction test. NBT was dissolved in PBS at 10 mg/mL; this prewarmed stock was diluted by the addition of an equal volume of diluent or GM-CSF–pretreated (60 minutes, 37°C) PMN in HBSS/BSA at 10 x 10⁶/mL and the reactions maintained in a stirred heating module. Buffer or Cyto B in a final concentration of 5 μg/mL was then added to the appropriate tubes and allowed to incubate for five minutes; buffer or FMLP (10⁻⁶ mol/L) was then added for a final 15-minute incubation. The cells were cooled in an ice bath for five minutes, fixed, and scored as previously described.13 A cell was considered positive if it contained easily identified formazan granules within the cytoplasm.

Statistical analysis. The proportion of depolarizing PMN was determined by analyzing a minimum of 10,000 cells and measuring the percentage of cells in the null peak after a five-minute incubation with the stimulus.13 Because the peaks often overlapped, the fluorescent channel at the nadir between the two peaks was used as the dividing point for the two populations. This estimate yielded a variability of less than 2% on repeat estimates of the nadir point. We also used an alternate method to assess the proportion of depolarizing cells by electronically subtracting the prestimulus histogram from the post-FMLP histogram on a channel-by-channel basis, with the number of cells remaining used to calculate the percentage of depolarized cells. By this more objective method the calculated proportion of depolarizing cells in any experiment was slightly reduced, but the qualitative differences between the treatment groups remained equally significant. Although certain experiments did not technically allow duplicate reactions to be simultaneously run, in general, the mean error for the proportion of depolarizing cells on duplicate samples from the same donor was 3.8% (range, 0.4% to 13.6%; n = 15).13 Group means for control and GM-CSF–treated cells were compared by using the paired-sample t test or Student's t test for summary data, where appropriate.

RESULTS

Effects of GM-CSF on PMN light scatter as assessed by flow cytometry and FMLP-induced membrane potential
changes. In Fig 1A and B, purified PMN exposed to GM-CSF (100 pmol/L) for 20 minutes at 37°C demonstrated a shift in light scatter that is very similar in character to that recently described with activation by FMLP\textsuperscript{15} or leukotriene B\textsubscript{4} (LTB\textsubscript{4}).\textsuperscript{13} The change involved predominantly an increase in the average FWD-SC of the cells (along with an increase in the coefficient of variation of the PMN population) that corresponds to a ruffling response that accompanies receptor-mediated activation\textsuperscript{13} and that we have confirmed by electron microscopy (data not shown). Figure 1C demonstrates the concentration dependence of GM-CSF (0 to 1,000 pmol/L) incubation on PMN and FWD-SC. GM-CSF produced a time- and dose-dependent shift in FWD-SC comparable in magnitude to that induced by activation with FMLP. A brief incubation of five minutes with GM-CSF alone produced only minimal FWD-SC changes (○—○); 20 minutes of preincubation produced a maximal response between 100 and 1,000 pmol/L (●—●). The addition of FMLP after a 20-minute preincubation with GM-CSF (▲—▲) did not further enhance FWD-SC. In addition, although the PMNs' average FWD-SC increased, the 90\textdegree-SC was unaffected by exposure to increasing concentrations of GM-CSF (data not shown).

The effects of GM-CSF on individual PMN membrane potential responses was assayed by using the fluorescent probe di-O-C\textsubscript{5}(3)\textsuperscript{19}. GM-CSF alone did not directly induce a loss of the membrane potential dye fluorescence seen when cells are activated with FMLP\textsuperscript{11,15,19} (Fig 2). If PMN were incubated with GM-CSF and then stimulated with FMLP, however, there was a dose-dependent increase in the percentage of PMN that lost fluorescence (depolarized) without an increase in the degree of fluorescence loss by the responding cells when compared with the cells preincubated with diluent alone (Fig 2). Although there was considerable variability in the baseline proportion of responsive cells from donor to donor, as we have recently described,\textsuperscript{14} as well as variability in the time of incubation with GM-CSF to yield maximal enhancement, in four separate dose-response experiments 60 minutes of incubation with GM-CSF produced maximal enhancement in the proportion of depolarizing PMN at doses between 100 and 1,000 pmol/L (data not shown). In general, because maximal enhancement in the proportion of depolarizing PMN was consistently seen between 30 and 60 minutes of incubation (with older PMN requiring longer to reach a maximum) and a maximal respiratory burst response is seen at two hours,\textsuperscript{8} a 60-minute incubation was routinely used as a compromise to minimize the effects of cell aging on the control cells' response (see the following section).\textsuperscript{7} In 11 separate experiments, a 60-minute incubation with 100 pmol/L GM-CSF significantly increased the percentage of PMN that depolarize in response to FMLP (Fig 3), with an average of 79.4\% ± 3.4\% v 29.5\% ± 4.7\% of the cells responding after GM-CSF v diluent treatment (mean ± SE; \( P < .005 \), paired sample \( t \) test).

Kinetics of GM-CSF enhancement in FMLP-elicited membrane potential responses. Figure 4 plots a representative time course of the enhanced membrane potential responsiveness of GM-CSF--treated PMN and demonstrates that 30 minutes of exposure to 100 pmol/L GM-CSF significantly increased the proportion of depolarizing PMN over cells exposed to diluent. In three separate experiments, the enhancement was maximal between 30 and 60 minutes, fell off between 60 and 120 minutes, but was still significantly above the control cells' responsiveness, which in some experiments dropped considerably with the continued incubation of the cells at 37°C. Thus, it appears that GM-CSF is capable of both increasing the proportion of functionally
responsive PMN as well as protecting the cells from an
age-related decline in the proportionate response. Our data
support similar previous observations on GM-CSF's ability
to enhance oxidative responses and prolong PMN viability.7
In other experiments comparing the rates at which diluent-
and GM-CSF-treated PMN subpopulations depolarized in
response to FMLP activation, there was no significant differ-
ence in the rate of loss or recovery of the membrane potential
probe between the two groups over a 30-minute period (data
not shown).

Effects of cell washing and protein on GM-CSF-
mediated enhancement of FMLP-elicted membrane potential
responses. To determine whether the continuous pres-
ence of GM-CSF in the extracellular fluid was required for
the enhancing effects on membrane potential responsiveness,
PMN were incubated for varying times with 100 pmol/L
GM-CSF before its removal by centrifugation and resuspension
of the cells in fresh HBSS/BSA. All cells were then
incubated for a total of 90 minutes at 37°C and the propor-
tion of cells depolarizing to FMLP measured by flow cyto-
metry. Figure 5 indicates that the maximum enhancing effect of
GM-CSF was seen after only 20 minutes despite the lack of
continuous exposure. In other experiments in which cells
were exposed to GM-CSF for briefer periods, the maximum
enhancement was seen between ten and 20 minutes, although
some increase in membrane potential responsiveness was
seen after only one minute when doses of 100 to 1,000
pmol/L were used (data not shown).

Because high concentrations of serum proteins are known
to modify the cyanine dye distribution and the pattern of
membrane potential dye shifts with FMLP stimulation,19
protein is routinely excluded from the membrane potential
assay. In the present system, however, 0.01% BSA
was included to stabilize the GM-CSF and prevent its nonspecific
adherence to reaction tubes, especially at the lower concen-
trations. Therefore, experiments were performed to deter-
mine whether the presence of 0.01% BSA in the system
affected the enhancement of membrane potential responses
seen with GM-CSF pretreatment for 60 minutes. There was
no significant difference in the proportion or pattern of
depolarizing PMN in the presence or absence of 0.01% BSA
(86.6% vs 86.8% and 73.3% vs 74.4%, respectively, in two
separate experiments).

Effects of GM-CSF on the dose response of FMLP-
induced membrane depolarization. To determine whether
the enhancing effect of GM-CSF on FMLP-elicited mem-
brane potential responses involved modulation of the FMLP
dose-response relationship, diluent or 100 pmol/L GM-CSF
was mixed with prewarmed PMN; aliquots were either
immediately removed, equilibrated with the membrane
potential probe for 20 minutes and stimulated with varying
concentrations of FMLP, or incubated for 60 minutes with
GM-CSF before equilibration with the probe and assessment
of the FMLP-induced response. Figure 6 indicates that
GM-CSF pretreatment produced a time- and concentra-

Fig 4. Kinetics of GM-CSF-induced enhancement in the propor-
tion of FMLP-induced depolarizing cells. PMN were incubated
with diluent (O—O) or 100 pmol/L GM-CSF (— ) for the times
indicated; this was followed by a ten-minute incubation with the
membrane potential probe and stimulation with FMLP (10−8 mol/
L). One of two representative experiments is shown. In general,
the SEM for duplicate determinations under the same conditions is
less than 4%.

Fig 5. Effects of cell washing on GM-CSF-induced enhance-
ment of the membrane potential response. In two separate
experiments, PMN were incubated at 37°C with diluent or 100
pmol/L GM-CSF for the times indicated; this was followed by
centrifugation and washing of the cells to remove extracellular
GM-CSF. The cells were then further incubated for a total time of
90 minutes at 37°C; this was followed by assessment of FMLP-
induced membrane depolarization as previously described. Data
represent the mean ± range for duplicate reactions, with 20,000
cells counted for each determination.

Fig 6. Effects of GM-CSF on the dose response of FMLP-
induced membrane depolarization. PMN were mixed with diluent
(circles) or 100 pmol/L GM-CSF (squares) and either immedi-
ately equilibrated with di-O-C3(3) or preincubated for 80 minutes
at 37°C before equilibration and stimulation (open symbols). The
data represent single determinations on one cell preparation.
Three experiments yielded qualitatively comparable results,
although the proportion of depolarizing PMN in the control group
varied.
dependent increase in the proportion of depolarizing PMN at every concentration of FMLP where a response was measurable, thus indicating an absolute increase in the percentage of cells depolarizing rather than merely a shift of the dose-response curve. Although a slight shift in the dose response at low FMLP concentrations (ie, \( 5 \times 10^{-4} \) mol/L) was seen in two of the three experiments, it was not statistically significant.

**GM-CSF-induced enhancement in the proportion of oxidatively responsive PMN.** Because previous data have indicated that GM-CSF is capable of priming PMN for enhanced oxidative responsiveness to chemotactic agents including FMLP,\(^7\)\(^9\) we assessed the FMLP-mediated oxidative burst capabilities of individual PMN in response to GM-CSF priming by scoring individual cells for NBT reduction to FMLP after a 60-minute incubation with either 100 pmol/L GM-CSF or diluent. Table 1 indicates that GM-CSF significantly enhanced the proportion of NBT-positive PMN after FMLP stimulation \((P < .05)\) without directly promoting NBT reduction above control levels, although there was a trend for increased NBT reduction by GM-CSF-treated cells in some experiments, especially in the presence of Cyto B. The slight increase in NBT-positive PMN with Cyto B alone likely reflects age-related priming of the cells by endogenously generated chemotactic factors and some enhancement of this effect by the presence of GM-CSF because the freshly isolated PMN not incubated for 60 minutes at 37°C demonstrated no increase in NBT reduction when exposed to Cyto B (data not shown).

**DISCUSSION**

GM-CSF is one of a family of specific humoral glycoproteins involved in the proliferation and maturation of irreversibly committed progenitor cells.\(^30\) With the availability of highly purified natural as well as recombinant materials, the biochemistry and biology of the individual factors are being rapidly enumerated. Although most of the work characterizing the structure and function of these molecules has been focused on the selectivity of their proliferative signals for progenitor cells, recent data from a number of laboratories indicate that colony-stimulating factors may have significant potentiating effects on the functional capabilities of mature phagocytic cells.\(^1\)\(^3\)\(^4\)\(^-\)\(^12\) The present studies were carried out to address the question of whether the mechanism(s) by which GM-CSF was capable of enhancing the functional response of PMN as previously described\(^1\)\(^4\) might involve recruitment of a subpopulation of PMN into a more responsive state.

The concept of an FMLP-nonresponsive subpopulation is controversial; some laboratories find that all PMN in their preparations respond to FMLP.\(^21\) Data from this and other laboratories have demonstrated, however, that circulating and purified peripheral blood PMN are structurally and functionally heterogeneous\(^13\)\(^5\)\(^-\)\(^18\)\(^22\)\(^-\)\(^26\) and that their functional heterogeneity (eg, as assessed by membrane potential and NBT responses to FMLP) may be significantly different for a given individual and may be modified by intermittent inflammatory reactions.\(^16\)\(^26\) For example, we have recently shown that the endogenously generated inflammatory lipid LTb\(_2\) has a rapid and concentration-dependent recruiting effect on the proportion of neutrophils that will respond to FMLP with a depolarization response and with the proportion of neutrophils capable of reducing NBT.\(^13\) Although it is often difficult to measure "responding" \(\times\) "nonresponding" cells in an absolute sense because responding cells are defined by the sensitivity limit of any assay, the present studies using GM-CSF indicate that this important glycoprotein is capable of increasing the measurable proportion of depolarizing cells in response to FMLP in a time- and concentration-dependent fashion (Figs 2 to 4). Interestingly, GM-CSF, like LTb\(_2\), is not directly capable of depolarizing PMN but does produce a concentration-dependent change in the cells' FWD-SC (Fig 1).

The time course and dose response of the GM-CSF–induced scatter changes correlate well with the published data on GM-CSF binding to human PMN,\(^3\) which implies

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**Table 1. Effects of GM-CSF on FMLP-Stimulated NBT Dye Reduction**

<table>
<thead>
<tr>
<th>Pretreatment*</th>
<th>Experiment</th>
<th>Buffer</th>
<th>FMLP (10^{-4}) mol/L</th>
<th>Cyto B</th>
<th>Cyto B and FMLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent</td>
<td>1</td>
<td>0.5 ± 0.5</td>
<td>57.0 ± 1.0</td>
<td>12.0 ± 3.0</td>
<td>80.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5 ± 0.5</td>
<td>75.0 ± 0.0</td>
<td>6.0 ± 1.0</td>
<td>94.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.0 ± 0.0</td>
<td>38.5 ± 0.5</td>
<td>1.0 ± 1.0</td>
<td>86.0 ± 1.0</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td>0.3 ± 0.2</td>
<td>56.8 ± 10.5</td>
<td>6.3 ± 3.2</td>
<td>86.8 ± 4.2</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1</td>
<td>0.0 ± 0.0</td>
<td>95.0 ± 2.0†</td>
<td>25.0 ± 1.0†</td>
<td>96.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.0 ± 1.0</td>
<td>86.0 ± 1.0</td>
<td>9.5 ± 0.5</td>
<td>99.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.0 ± 3</td>
<td>66.0 ± 2.0</td>
<td>17.0 ± 1.0</td>
<td>90.0 ± 2.0</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td></td>
<td>5.0 ± 4.0†</td>
<td>82.3 ± 8.6†</td>
<td>17.2 ± 4.5†</td>
<td>95.3 ± 2.8†</td>
</tr>
</tbody>
</table>

Data are percentages of NBT-positive PMN. Pretreated PMN were incubated with \(10^{-4}\) mol/L FMLP or buffer for 15 minutes at 37°C before preparation of cytopreps, staining, and scoring as outlined in Materials and Methods. For each experiment, the percentage of NBT-positive PMN was the average of two 100-cell counts.

*Purified PMN were incubated with diluent or GM-CSF for 60 minutes at 37°C; buffer or Cyto B (5 μg/mL) was added and the cells incubated for an additional five minutes before stimulation.

†P < .05 by paired-sample t test when compared with diluent-pretreated cells.

‡Not significant when compared with diluent-pretreated cells.
that the changes are due to direct interaction of the GM-CSF with specific neutrophil membrane receptors. Unlike the LTB4-induced recruitment response, however, the effect of GM-CSF on FMLP-mediated membrane potential responses requires a significantly longer incubation time at optimal concentrations to achieve a maximal effect (Fig 4) and, in this way, is quite similar to the action of bacterial lipopolysaccharide (LPS). Although the maximum recruiting effect for LTB4 occurs within one minute of addition, the present studies indicate that between 30 and 60 minutes of continuous exposure to GM-CSF is required for a maximal response in freshly isolated cells (although some effect of GM-CSF is seen after one to two minutes of exposure when high concentrations are used) and between 30 and 60 minutes for LPS-induced PMN priming. The facts that LPS was not present in the GM-CSF preincubation in levels >0.1 ng/mL and that LPS is capable of priming phorbol ester-induced superoxide production whereas GM-CSF is not argue against the mechanism of GM-CSF enhancement being the same as that for LPS.

The potentiating effects of GM-CSF on PMN function are also similar to those of LTB4 in that a brief exposure (in this case between ten and 20 minutes) is all that is required for the maximal response to persist despite removal of the factor because washing the cells free of the GM-CSF after this time period does not reduce the level of enhancement seen (Fig 5). The data imply that both the potentiating effect on membrane potential responsiveness as well as the light scatter shifts relate to the time required for GM-CSF to bind to sites on the PMN membrane. The early enhancement in FMLP-mediated processes (ie, membrane potential and NBT responses) is likely related to a modification in both the affinity and number of FMLP binding sites available to the cells. The increased percentage of FMLP-responsive cells at all stimulus concentrations (Fig 6) without a significant increase in the degree of loss of the probe (Fig 2) indicates that the GM-CSF recruitment relates to increasing the proportion of cells reaching threshold without greatly affecting the degree of response. Although the shift of the FMLP dose-response curve at low FMLP concentrations (ie, <5 x 10^-4 mol/l; Fig 6) was not statistically significant in our studies, such a shift in the high-affinity binding range would be consistent with the data of Weisbart et al that show that GM-CSF-treated neutrophils demonstrate an increase in the number of high-affinity binding sites within five to 15 minutes of exposure. Interestingly, the GM-CSF effect on FMLP receptor function appears to differ from that of both LTB4 and LPS in that LTB4 does not modify FMLP binding (but likely works through a postreceptor site), whereas LPS has been reported to decrease the number of binding sites without changing the affinity.

In addition to the enhancing effect of GM-CSF on membrane potential responses, measurement of individual cell oxidative metabolism by using the NBT test was used to determine that GM-CSF is also capable of enhancing the respiratory burst of a subpopulation of PMN and thus supports the membrane potential data. Although the NBT dye reduction test has been used as a measure of oxidative metabolism, it is known to be somewhat nonspecific in reactivity, and scoring of NBT positivity is somewhat subjective. It does, however, allow the examination of individual cells, which bulk biochemical measurements do not. Thus, within these limitations and under the conditions of our system, GM-CSF is capable of increasing the oxidative response of a proportion of the PMN above the level of the diluent-treated cells.

In summary, the present data indicate that the enhanced functional capacity of GM-CSF-treated PMN may be in part by related to recruitment of a proportion of the cells to be functionally more responsive to inflammatory stimuli. Our data on recruitment are supported by previous work indicating that GM-CSF is capable of enhancing PMN phagocytosis by increasing the number of phagocytizing cells as well as the number of targets ingested by each cell. Although it is likely that the recruitment is related to modification of the affinity and/or number of available surface receptors on PMN subsets, the present studies do not rule out the possibility of nonspecific and/or postreceptor mechanisms that could subsequently affect the quantitative response of the activatable cells. Multiple mechanisms of action for GM-CSF are also supported by its ability to enhance some cell responses rapidly (eg, shape change and depolarization) but requiring more prolonged incubation for others (eg, superoxide production). Human colony-stimulating factors may have great clinical value in modulating the functional responsiveness of mature circulating neutrophils as well as stimulating the regeneration of hematopoietic tissue under conditions where a lack of significant numbers and/or quality of neutrophil function may compromise host defense.

ACKNOWLEDGMENT

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Enhancement of neutrophil function by granulocyte-macrophage colony-stimulating factor involves recruitment of a less responsive subpopulation

MP Fletcher and JC Gasson