

REGULATION OF MHC CLASS II GENE EXPRESSION
IN MACROPHAGES BY HEMATOPOIETIC
COLONY-STIMULATING FACTORS (CSF)

Induction by Granulocyte/Macrophage CSF and Inhibition by CSF-1

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The class II MHC molecules expressed on antigen-presenting cells are highly polymorphic, heterodimeric cell surface glycoproteins that bind and present proteolytic fragments of foreign antigens to T cells to initiate the immune response (1). MHC class II (Ia) molecules are composed of a 33–36-kD α subunit noncovalently associated with a 24–28-kD β subunit. A third nonpolymorphic invariant chain (Ii) of 31 kD may be found associated with Ia molecules in the cytoplasm and on the cell surface. In contrast to the constitutive expression of Ia in B cells, the expression of Ia and Ii in different macrophage populations has been reported to be induced by IFN- γ (2, 3), IL-4 (4), and granulocyte/macrophage CSF (GM-CSF)¹ (5, 6). Using highly purified macrophages derived from in vitro bone marrow cultures, we have determined that the recombinant hematopoietic colony-stimulating factors CSF-1 and GM-CSF differently regulate Ia and Ii gene and protein expression in macrophages.

Materials and Methods

Bone Marrow Cultures. Bone marrow macrophages (BMM) were derived from short term in vitro cultures of DBA/2 or C3H/HEJ murine bone marrow cells cultured at 2×10^4 nucleated cells/ml in α -MEM medium supplemented with L929 cell-conditioned medium containing CSF-1 (LCM) and 10% FCS (HyClone Laboratories, Logan, UT) as previously described (7). After 6 d, the adherent macrophages in these cultures were washed in PBS and were then stimulated with fresh α -MEM supplemented with either recombinant CSF-1 (>95% pure by SDS-PAGE; used in our experiments at 1,000 CFU/ml; Cetus Corp., Emeryville, CA), recombinant murine GM-CSF (obtained either from Immunex, Seattle, WA [>95% pure; 10^8 CFU/mg protein] or the kind gift of J. Gasson, University of California, Los Angeles [derived from COS cell supernatants; >95% pure] used at 500–1,000

This work was supported by a National Institutes of Health Physician Scientists Award to C. L. Willman (DK-01284) and by NIH grants AI-19490, CA-22105, and HD-17013. V. Miller's present address is Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024. T. B. Tomasi and C. C. Stewart's is Roswell Park Memorial Institute, Buffalo, NY 14236. Address correspondence to Dr. Cheryl L. Willman, Department of Cell Biology, UNM Cancer Center, 900 Camino de Salud N. E., Albuquerque, NM 87131.

¹ *Abbreviations used in this paper:* BMM, bone marrow-derived macrophages; CSF-1, monocyte CSF; GM-CSF, granulocyte/macrophage CSF; LCM, L-929 cell-conditioned medium.

CFU/ml in our experiments); recombinant murine IFN- γ (1.3×10^7 U/mg protein; used at 20 U/ml; Genentech, San Francisco, CA), IFN- α/β (250 U/ml; Lee Biomolecular, San Diego, CA), or IL-3 (1,000 U/ml; Genzyme, Boston, MA). All media and supplements contained <0.1 ng/ml of endotoxin as determined by the limulus amoebocyte lysate assay.

RNA Isolation and Hybridization Assays. Total cellular RNA was isolated, electrophoresed, transferred to nitrocellulose, and hybridized as previously described (17). DNA probes used in this study included invariant chain (Ii), 1.17-kb Pst I fragment (8); Ia-A β ^d, 1.7-kb Hind III fragment (9); and pHII2a, an MHC class I (H-2K^k) probe/2.0-kb Hba I-Sac I fragment (10).

Flow Cytometric Analysis of Class II Antigen Expression. Viable BMM were centrifuged and resuspended in PBS supplemented with 0.1% sodium azide and 0.5% BSA (PAB) at a concentration of 2×10^7 cells/ml and kept on ice. Membrane expression of Ia was determined using a rat anti-mouse Ia with specificity for IA^{bdq} and IE^{dk} (IgG2b, TIB120; American Type Culture Collection, Rockville, MD). Because of this broad specificity, a haplotype control was not used in these experiments. To prevent nonspecific Fc binding of the rat anti-Ia, 10^6 BMM (in 50 μ l) were first preincubated with 10 μ g (in 10 μ l) of purified mouse normal Ig (Sigma Chemical Co., St. Louis, MO) for 10 min. We have previously determined that this preincubation inhibits the binding of directly fluoresceinated mouse IgG2a myeloma protein to BMM (data not shown). Without washing, 1 μ g (in 10 μ l) of the rat anti-Ia was added to the cells and the cells were incubated for 15 min. After washing in 4 ml PAB, the cells were incubated for 15 min with 1 μ g (in 10 μ l) of fluoresceinated goat anti-rat Ig (with no crossreactivity for mouse Ig; Caltag, San Francisco, CA). All antibody reagents used were >90% Ig protein. After a final wash, the cells were resuspended in 1 ml PAB containing 2 μ g of propidium iodide (to exclude dead cells from analysis) and were analyzed by flow cytometry. A single argon laser operating at 488 nm was used to measure low angle light scatter and to excite fluorescein and propidium iodide. Emission of fluorescein was detected at 530 ± 15 nm, while propidium iodide emission was detected above 590 nm.

Cell Cycle Analysis. To measure the fraction of proliferating BMM in S phase, 30 μ M bromodeoxyuridine (BrdU; Sigma Chemical Co.) was added to the culture medium 30 minutes before cell harvest. Cells were collected, centrifuged, resuspended in 0.5 ml saline, and fixed in 5 ml of 70% ethanol in water for 24 h. Fixed cells were centrifuged and the pellet was resuspended in 5 ml of 4 N HCl with 0.5% Tween 20. After vortexing, cells were incubated 30 min at 37°C; washed with 2.5 ml of 0.1 M sodium borate, pH 8.5; and the pellet was resuspended in 30 μ l PBS with 0.5% Tween 20. Cells were then stained with fluoresceinated anti-BrdU (Becton Dickinson & Co., Mountain View, CA) for 30 min at room temperature, washed, counterstained with 20 μ g/ml propidium iodide containing 50 μ g/ml RNase, and analyzed by flow cytometry using the same parameters described above.

Results

Suppression of Ia and Ii Gene and Protein Expression by CSF-1. In studies designed to examine CSF-1 inducible genes in monocytic cells, we noted that BMM expressed variable basal levels of Ia mRNA. Adherent BMM, derived directly from day 6 bone marrow cultures supplemented with LCM (see Materials and Methods), contained essentially undetectable levels of Ia-A β and Ii mRNA (Fig. 1 A), as well as undetectable levels of Ia-A α mRNA (data not shown). When these day 6 adherent BMM were washed and refed with fresh medium containing serum only, Ia and Ii mRNA transcripts remained at essentially undetectable levels until after 24 h of culture, at which time there was an abrupt 20-fold increase in the basal levels of both Ia and Ii mRNA (Fig. 1 A). Ia and Ii mRNA could still be detected at 48 h (Fig. 1 A). BMM viability, assessed by dye exclusion, was maintained under these experimental conditions (data not shown). In contrast to Ia and Ii, the expression of MHC class I mRNA (H-2K^k) was unaltered during this experiment (Fig. 1 A).

To determine the effects of CSF-1 on basal levels of Ia expression in BMM, recom-

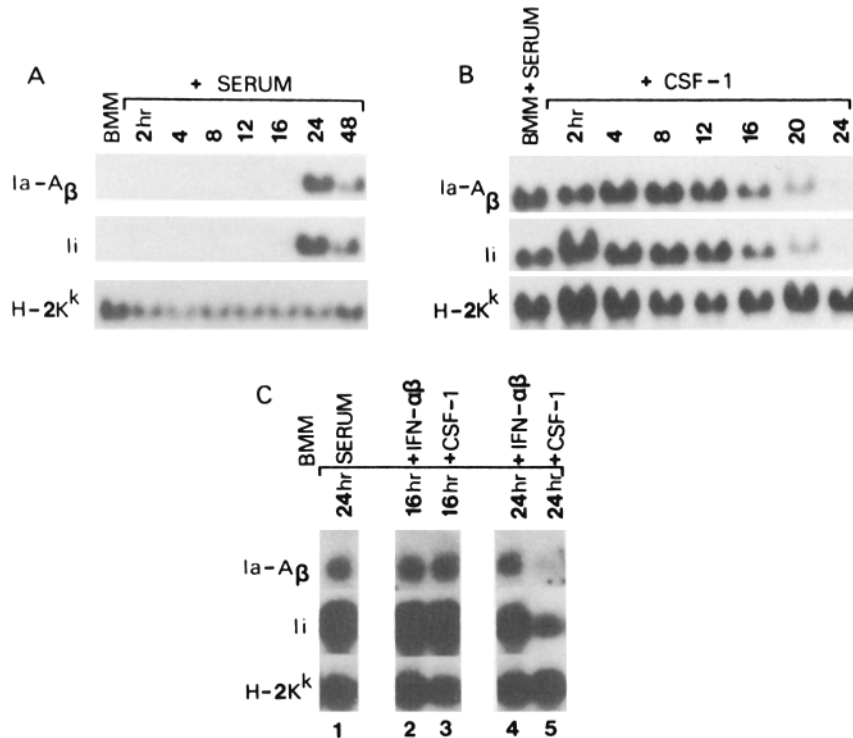


FIGURE 1. (A) Total RNA was isolated from BMM derived directly from day 6 *in vitro* cultures supplemented with LCM (BMM, lane 1) or from day 6 BMM that were washed, refed with medium containing serum alone, and then cultured for 2–48 h (lanes 2–8). 10 μ g of total RNA from each time point was hybridized in Northern analysis with probes for MHC class II Ia-A β^d , Ii, and MHC class I H-2K^k. (B) Day 6 BMM were cultured in serum alone for 24 h to allow maximal basal expression of Ia and Ii (BMM + serum, lane 1). These cells were then washed, refed with medium containing recombinant CSF-1, and cultured for 2–24 h (lanes 2–8). 10 μ g of total RNA from each time point was hybridized in Northern analysis with probes described in (A). (C) Total RNA was isolated from day 6 adherent BMM which had been cultured for 24 h in serum alone to allow maximal basal Ia expression (lane 1). These cells were then washed and recultured for 16 (lane 2) or 24 h (lane 4) in medium containing IFN- α/β ; or alternatively, in medium containing recombinant CSF-1 for 16 (lane 3) or 24 h (lane 5). 10 μ g of total RNA from each experimental variable was analyzed in Northern analysis with probes described in (A).

binant CSF-1 was added back to the cells that had been cultured in serum alone for 24 h and that now expressed maximal levels of Ia and Ii mRNA. Ia and Ii transcript levels were unaltered until after 16 h of culture with CSF-1, at which time there was a 25% decrease in mRNA expression (Fig. 1 B). By 24 h of culture with CSF-1, Ia and Ii transcripts were essentially undetectable (Fig. 1 B). Expression of H-2K^k mRNA was essentially unaffected.

Since the suppression of basal levels of Ia by recombinant CSF-1 occurred over a 24-h time course (Fig. 1 B), we wanted to determine whether this suppression might be mediated by the autocrine effects of IFN- α/β , or prostaglandin E₂ (PGE₂) induced in the BMM by CSF-1 (11–13). To test this possibility, day 6 BMM were first cultured for 24 h in fresh medium containing serum alone to allow maximal expres-

sion of basal levels of Ia and Ii. Fresh medium containing serum and either recombinant CSF-1, IFN- α/β , or PGE₂ was then added directly to the BMM. After 16 (Fig. 1 C, lane 2) or 24 (Fig. 1 C, lane 4) h of culture with IFN- α/β , no suppression of the basal levels of Ia or Ii mRNA was seen. A similar result was obtained when cultures were supplemented with PGE₂ (data not shown). In contrast, basal levels of Ia and Ii mRNA were inhibited by CSF-1, but not until after 24 h of culture (Fig. 1 C, lane 5), as previously shown (Fig. 1 B). The suppression of Ia and Ii gene expression by CSF-1 was not due to contaminating endotoxin (LPS) since medium and supplements contained <0.1 ng/ml endotoxin and similar experiments performed in LPS-hyporesponsive C3H/HeJ mice produced identical results (data not shown).

Correlating with these changes in Ia and Ii gene expression, very low levels of Ia glycoproteins could be detected on day 6 BMM that had been cultured in serum alone for an additional 24 h in the absence of CSF-1 (Fig. 2 B), while no expression of Ia could be detected on the cell surface of BMM derived directly from the day 6 cultures (data not shown) or on day 6 BMM which had been cultured with recombinant CSF-1 for an additional 24 h (Fig. 2 A).

Induction of Ia and Ii Gene Expression by GM-CSF. In contrast to the inhibitory effects of CSF-1, recombinant murine GM-CSF induced maximal levels of Ia-A β and Ii mRNA after 24 h of culture (Fig. 3 A, lane 3), although increased transcript levels could be detected as early as 8 h (data not shown). The levels of Ia and Ii mRNA induced by GM-CSF (1,000 U/ml) were similar to the levels induced with IFN- γ (20 U/ml) (Fig. 3 A, lane 4). No induction of Ia was seen with doses of GM-CSF <150 U/ml (data not shown) and no synergistic effect was noted with the simultaneous addition of GM-CSF and IFN- γ (data not shown). In contrast, IL-3 had no effect on Ia and Ii expression (data not shown). Additionally, no significant modulation of H-2K^k mRNA expression was noted (Fig. 3 A).

Paralleling the increases in Ia mRNA, both GM-CSF and IFN- γ significantly increased the expression of Ia proteins on the BMM cell surface (Fig. 2, C and D). Levels of Ia gene and protein expression induced by GM-CSF and IFN- γ in the BMM were ~10–20-fold higher than the maximal basal levels of expression (Fig. 3 A).

Suppression of the IFN- γ and GM-CSF Induction of Ia by CSF-1. To determine if recombinant CSF-1 could suppress the induction of Ia by GM-CSF and IFN- γ , as it had suppressed the basal levels of Ia expression, these factors were added simultaneously to BMM. Day 6 adherent BMM were washed and refed with fresh medium containing either GM-CSF, the combination of CSF-1 + GM-CSF, IFN- γ , or the combination of CSF-1 + IFN- γ , and cultured for 24 h. As before, both GM-CSF (Fig. 3 B, lane 1) and IFN- γ (Fig. 3 B, lane 3) induced high levels of Ia and Ii mRNA. However, when CSF-1 was also present, the Ia induction was markedly suppressed (Fig. 3 B, lanes 2 and 4). In multiple experiments, CSF-1 markedly inhibited the induction of Ia by GM-CSF, while the IFN- γ -mediated induction of Ia mRNA was suppressed by 50–90% and Ii mRNA levels were suppressed to a somewhat lesser degree (25–50%). CSF-1 (1,000 U/ml) was capable of suppressing the IFN- γ induction of Ia and Ii over a dose range of 10–100 U/ml of IFN- γ (data not shown). Lower doses of CSF-1 were not tested.

The CSF-1-mediated suppression of the IFN- γ and GM-CSF induction of Ia mRNA was paralleled by decreased expression of Ia glycoproteins on the BMM cell surface. Compared with the induced levels of Ia detected on BMM treated with IFN- γ alone

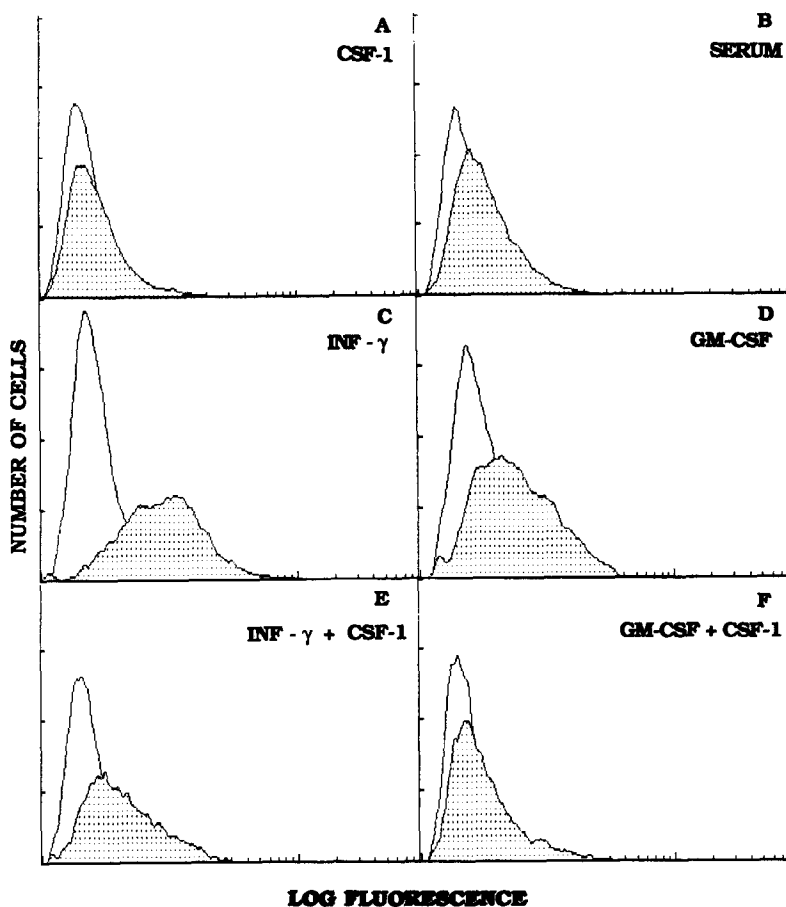


FIGURE 2. Day 6 adherent BMM were washed and recultured for an additional 24 h in medium containing recombinant CSF-1 (*A*), serum alone (*B*), IFN- γ (*C*), recombinant GM-CSF (*D*), the combination of IFN- γ + CSF-1 (*E*), or the combination of GM-CSF + CSF-1 (*F*). The cells were then harvested, stained with the anti-Ia reagents and analyzed by flow cytometry as described in Materials and Methods. The shift in log fluorescence due to specific binding of anti-Ia is displayed on the histograms as a filled histogram relative to the background control (fluorescence of the BMM stained with the fluoresceinated second step antibody alone) displayed as an unfilled histogram.

(Fig. 2 *C*), Ia levels in BMM cultured simultaneously with IFN- γ and CSF-1 were reduced by more than 50% (Fig. 2 *E*). Similarly, Ia expression in BMM treated with the combination of CSF-1 + GM-CSF was also suppressed (Fig. 2 *F*), when compared with Ia levels induced by GM-CSF (Fig. 2 *D*).

To determine if suppression or induction of cytoplasmic steady-state levels of Ia and Ii mRNA by CSF-1, GM-CSF, and IFN- γ were due to the production of new transcripts in the nucleus, or due to posttranscriptional mechanisms, *in vitro* nuclear transcriptional assays were performed. In triplicate experiments, no significant differences were noted in the level of transcription of Ia-A β when day 6 BMM were washed and recultured for 24 h in fresh medium containing serum alone, CSF-1,

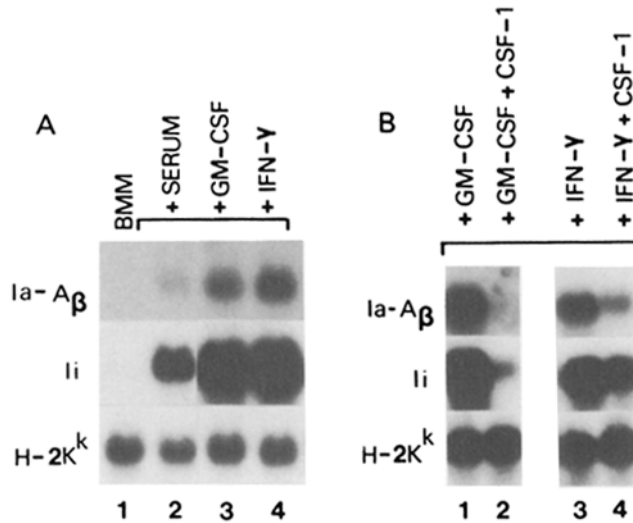


FIGURE 3. (A) Total RNA was isolated directly from day 6 adherent BMM (lane 1) or from BMM that had been washed and refed with medium containing either serum alone (lane 2), GM-CSF (lane 3), or IFN- γ (lane 4) and cultured for an additional 24 h. 10 μ g of total RNA from each variable was hybridized in Northern analysis with probes for Ia, Ii, and H-2K^k. (B) 10 μ g of total RNA was isolated from day 6 BMM cultured for an additional 24 h in GM-CSF (lane 1), GM-CSF + CSF-1 (lane 2), IFN- γ (lane 3), or IFN- γ + CSF-1 (lane 4) and was hybridized in Northern analysis with the probes described in A.

or GM-CSF (data not shown). In response to IFN- γ , the BMM displayed only a 1-1.7-fold increase in the level of transcription of class Ia-A β after 24 h of culture, (data not shown), similar to recent reports (14). No significant changes in the level Ia transcription were observed when BMM were cultured with these factors for shorter time periods of 30 min and 8 h (data not shown).

Relationship of the Suppression and Induction of Ia to the Proliferative Status of BMM. Previous studies by Calamai et al. (15) suggested that the induction of macrophage proliferation by LCM led to a cell cycle-dependent inhibition of Ia expression. To determine if there was a relationship between the proliferative status of the BMM and Ia expression, we determined the S phase fraction of BMM at each time point in which Ia expression had been assessed. Day 6 adherent BMM, derived directly from cultures supplemented with LCM, had 32% of cells in S phase and had no evidence of Ia gene or protein expression (Figs. 1 A and 2 A). Similarly, BMM cultured for an additional 24 h with recombinant CSF-1 had 29% of cells in S phase and had essentially undetectable Ia expression (Figs. 1, B and C; 2 A).

In contrast, day 6 BMM cultured for an additional 24 h in serum alone in the absence of CSF-1, or day 6 BMM cultured for 24 h with GM-CSF or IFN- γ alone were not in cell cycle (with <2%, <1%, and <4% of cells in S phase, respectively); and, the nonproliferating BMM derived from each of these conditions expressed both Ia and Ii mRNA (Fig. 3) and proteins (Fig. 2). Culture of the BMM for 24 h with CSF-1 + IFN- γ or CSF-1 + GM-CSF maintained the proliferative state of the macrophages (with 30% and 22% of the cells in S phase, respectively). These conditions were associated with suppression of the IFN- γ and GM-CSF-mediated induction of Ia expression (Figs. 2, E and F; 3 B). Thus, in every case in which CSF-1 was present and proliferation was induced, Ia expression was markedly inhibited.

Discussion

In our studies, hematopoietic CSF were shown to modulate the levels of Ia expression in BMM. We determined that BMM have low basal levels of Ia and Ii gene

and protein expression. While IL-3 had no effect, both GM-CSF and IFN- γ increased the basal levels of Ia expression 10–20-fold. In contrast, recombinant CSF-1 suppressed both the basal levels of Ia expression and inhibited the induction Ia by GM-CSF and IFN- γ . Our preliminary studies investigating the molecular mechanisms that regulate Ia gene expression in BMM suggested that both transcriptional and posttranscriptional mechanisms may be operative. Only a 1–1.7-fold increase in transcription of Ia-A β^d was noted in response to IFN- γ , at a time in which steady-state levels of Ia-A β^d mRNA transcripts in the cytoplasm increased >20–30-fold. Whether these changes in the level of transcription can account solely for the large increase in cytoplasmic mRNA (without involving additional posttranscriptional mechanisms) remains to be determined.

The delay in the CSF-1-mediated suppression of basal levels of Ia suggested an indirect mechanism. We determined that suppression was not due to the autocrine production of either IFN- α/β or PGE₂. Although IFN- α/β was previously shown to inhibit the IFN- γ -mediated induction of Ia (13), no previous data has been reported on the effects of IFN- α/β on basal levels of Ia in macrophages. In agreement with our experiments, recent studies have also reported that PGE₂ has no effect on the basal levels of Ia expression in macrophages (6), even though PGE₂ may inhibit the induction of Ia by IFN- γ (12). Our work has determined that the inhibition of Ia expression by CSF-1 is correlated with the induction of cellular proliferation, extending the earlier work of Calamai et al. (15). To insure that the adherent BMM were still in cell cycle on day 6, initial bone marrow cultures were plated at extremely low densities; when plated at high initial densities, day 6 BMM may be confluent and quiescent, due to consumption of all of the CSF-1 in the culture medium (7). When day 6 BMM derived from the low density cultures are rendered quiescent by removal of LCM and are then stimulated to proliferate by the readdition of recombinant CSF-1, the cells enter the S phase of the cell cycle by 20–24 h (7). This time frame of S phase entry correlates precisely with the time of inhibition of Ia expression by CSF-1 (Fig. 1 B). Whether the suppression of Ia is due directly or indirectly to CSF-1 or is a consequence of the induction of cellular proliferation remains to be determined.

Even though CSF-1 may inhibit Ia expression through cell cycle-dependent mechanisms, Ia expression can certainly be inhibited in macrophages by other cell cycle-independent mechanisms, such as those initiated by LPS (16) and TNF- α (Adams, D., personal communication). TNF- α may also be induced in BMM by CSF-1 (Willman, C., unpublished data). These data thus suggest that the combinatorial effects of the different stimulatory (IFN- γ , GM-CSF, IL-4) and inhibitory (CSF-1, TNF- α) factors produced by resident and infiltrating cells in specific tissue microenvironments may ultimately determine the level of Ia expression in resident macrophages, thereby regulating the ability of the macrophage in that tissue to present antigen and initiate the immune response.

Summary

CSF-1 and granulocyte/monocyte CSF (GM-CSF) were shown to modulate the levels of Ia gene and protein expression in bone marrow-derived macrophages (BMM). Recombinant GM-CSF induced high levels of Ia expression, similar to the levels induced by INF- γ , while IL-3 had no effect. In contrast, recombinant CSF-1 not

only suppressed the basal levels of Ia gene and protein expression in BMM, but also inhibited the induction of Ia by IFN- γ and GM-CSF. Basal levels of Ia were not inhibited by recombinant CSF-1 until after 16–24 h of culture, suggesting an indirect mechanism of suppression. IFN- α/β and PGE₂ were shown not to be involved in the CSF-1 inhibition of basal levels of Ia expression. However, the CSF-1-mediated suppression of both the basal levels of Ia expression and the induction of Ia in BMM by IFN- γ and GM-CSF did correlate with the induction of cellular proliferation. These data imply that in addition to regulating hematopoiesis, CSFs may regulate the initiation of the immune response through their effects on Ia expression in macrophages.

We thank Dr. David Urdahl of Immunex for recombinant GM-CSF, Dr. Peter Ralph of Cetus for recombinant CSF-1, and Dr. Hsiu-san Lin for the purified nonrecombinant CSF-1 used in our initial studies.

Received for publication 21 February and in revised form 12 July 1989.

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