

# Functional Switching of Macrophage Responses to Tumor Necrosis Factor- $\alpha$ (TNF $\alpha$ ) by Interferons

## Implications for the Pleiotropic Activities of TNF $\alpha$

Fiona R. Lake,\* Paul W. Noble,† Peter M. Henson,\* and David W. H. Riches\*‡§

\*Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine; †Division of Pulmonary Sciences, Department of Medicine; and ‡§Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80206

### Abstract

Recent work conducted in our laboratory has been directed towards understanding the role of TNF $\alpha$  in stimulating the synthesis of two macrophage gene products, namely IGF-1, a growth factor implicated in wound repair and fibrosis, and complement component factor B (Bf), an alternative pathway complement component. The expression of these proteins is induced by hyaluronic acid and poly(I:C), respectively, although TNF $\alpha$  plays a requisite role in the expression of both proteins. The objective of this study was to determine the mechanism governing the dichotomy in the expression of IGF-1 and Bf by TNF $\alpha$ . First, we questioned if the diversity in IGF-1 and Bf synthesis was regulated at the level of TNF receptor usage. Second, based on earlier findings that IFNs contribute to the initiation of Bf expression, we determined if IFNs modulate the response of macrophages to TNF $\alpha$ . Our data show that differences in TNF receptor usage cannot fully explain the dichotomy in the expression of IGF-1 and Bf. However, prior exposure to IFN- $\beta$  or IFN- $\gamma$  was found to be a dominant factor controlling the expression of these proteins, suppressing IGF-1, and enhancing Bf. These findings indicate that IFNs mediate a functional "switch" in the response of macrophages to TNF $\alpha$  and suggest that the pattern of cytokine expression by diverse macrophage stimuli is an important determinant of the eventual responses of macrophages to TNF $\alpha$ . (*J. Clin. Invest.* 1994. 93:1661–1669.) **Key words:** tumor necrosis factor • macrophages • interferons • inflammation • TNF receptors • insulin-like growth factor-1

### Introduction

The secretion of TNF $\alpha$  by mononuclear phagocytes can be initiated by a plethora of stimuli ranging from bacteria and viruses (1) to cytokines (1, 2), immune complexes (3), and components of connective tissue matrices (4). After its release

into the extracellular milieu, TNF $\alpha$  is recognized by specific cell surface receptors that initiate the expression of an equally expansive array of cellular responses. Diversity in responsiveness is reflected first at the level of the host organism, where TNF $\alpha$  has been implicated in protection against pathogenic bacteria and parasites (2), as well as in the pathology of a number of human and experimental conditions such as cachexia, septic shock (1, 2, 5), pulmonary fibrosis, the adult respiratory distress syndrome, and graft-vs.-host disease (6, 7). Diversity in responses to TNF $\alpha$  is also reflected at the cellular level and, in particular, within the mononuclear phagocyte system, where TNF $\alpha$  has been reported to be involved in precursor cell differentiation (8, 9), the expression of cytotoxic and microbicidal activity (10–14), and the expression of a number of cytokines and growth factors (13) that regulate the activities of mononuclear phagocytes themselves as well as other cell types.

In view of the heterogeneity in cellular responses to TNF $\alpha$ , recent interest has focused on determining how this heterogeneity is governed. The finding that TNF $\alpha$  is recognized by two structurally distinct receptors, TNFR1 (p55) and TNFR2 (p75) (15–18), has led to the development and application of receptor-specific monoclonal and polyclonal antibodies (16, 18, 19), to the cloning of their respective cDNAs (17, 20–22), and to the expression of each receptor in surrogate cell lines (18, 21). The results of these investigations have indicated that most cells express both types of TNF receptor (R) (16, 23) and that certain responses are regulated in a receptor-restricted fashion. For example, ligation of TNFR1 initiates cytolysis in a variety of cell types (19, 24, 25), whereas ligation of TNFR2 stimulates the proliferation of thymocytes (18) and upregulates GM-CSF secretion in a mouse–rat T cell hybridoma transfected with the human TNFR2 (26). However, while these findings shed important new light into the mechanism of pleiotropism in TNF $\alpha$  action, such a binary system of recognition cannot be the sole determinant of such marked heterogeneity, and thus it would seem likely that additional factors contribute to the diversity of cellular responses to TNF $\alpha$ .

Recent work conducted in our laboratory has been directed towards understanding the role of TNF $\alpha$  in stimulating the synthesis of two macrophage gene products, namely complement component factor B (Bf),<sup>1</sup> a component of the alternative pathway of complement activation whose expression coincides with the acquisition of nonspecific macrophage cytotoxic activity (27, 28), and IGF-1, a progression-type growth factor that is thought to contribute to the role of the macrophage in wound repair (29) and pulmonary fibrosis (30). The expression of these two proteins is induced by different stimuli (e.g.,

Address correspondence to Dr. D. W. H. Riches, Department of Pediatrics, Neustadt, Room D405, National Jewish Center for Immunology and Respiratory Medicine, 2400 Jackson Street, Denver, CO 80206.

F. R. Lake's present address is the Department of Medicine, University of Western Australia, Perth, Australia. P. W. Noble's present address is the Division of Pulmonary and Critical Care, the Johns Hopkins University School of Medicine, Baltimore, MD.

Received for publication 2 July 1993 and in revised form 29 November 1993.

*J. Clin. Invest.*

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0021-9738/94/04/1661/09 \$2.00

Volume 93, April 1994, 1661–1669

1. Abbreviation used in this paper: Bf, complement component factor B.

the polyribonucleotide, poly(I:C) or LPS, and the connective tissue matrix component, hyaluronic acid, respectively) but, as we will show, the secretion and autocrine action of TNF $\alpha$  plays a requisite role in the induction of both responses. In view of these findings, the primary objective of this investigation was to determine the mechanism governing the dichotomy in the expression of these two TNF $\alpha$ -responsive gene products. First, we questioned if diversity in the synthesis of Bf and IGF-1 was regulated at the level of TNF $\alpha$  receptor usage. Second, based on earlier findings that an additional cytokine, IFN- $\beta$ , was involved in the initiation (27) and continuation (28) of Bf expression in response to poly(I:C), we determined if IFNs, either applied exogenously or induced by the primary stimulus and acting in an autocrine fashion, could influence the response of the macrophage to TNF $\alpha$ . Our data show that differences in TNF $\alpha$  receptor usage cannot fully explain the dichotomy in the expression of Bf and IGF-1. However, prior exposure to the cytokines IFN- $\beta$  and IFN- $\gamma$  was found to be a dominant factor controlling the response of the macrophage to TNF $\alpha$  in this system.

## Methods

Mice of strain C3H/HeJ were obtained from The Jackson Laboratory (Bar Harbor, ME) and were used throughout the study to avoid the possibility of stimulation of the cells by trace contamination of reagents with endotoxin (31). DME was obtained from Whittaker Bioproducts Inc. (Walkersville, MD). FBS was purchased from Irvine Scientific (Santa Ana, CA). 12-well (22-mm diameter) and 96-well (6.4-mm diameter) culture plates were obtained from Costar (Cambridge, MA) and 100-mm diameter culture petri dishes from Falcon Labware (Oxnard, CA). Mouse IFN- $\beta$  (sp act,  $1.3 \times 10^8$  international reference units/mg) was purchased from Lee Biomolecular Research Inc. (San Diego, CA). Recombinant mouse IFN- $\gamma$  (sp act,  $10^7$  U/mg), recombinant mouse TNF $\alpha$  ( $\sim 4 \times 10^7$  U/mg), recombinant human TNF $\alpha$  ( $\sim 2 \times 10^7$  U/mg), and polyclonal rabbit anti-mouse TNF $\alpha$  antibodies (neutralizing) were purchased from Genzyme (Boston, MA). Sheep antiserum to mouse L cell IFN and control antiserum were obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Monoclonal antibodies to mouse IFN- $\beta$  was obtained from the Yamasa Corporation through Seigagaku America (St. Petersburg, FL). Goat anti-human factor B IgG was obtained from Atlantic Antibodies (Scarborough, ME). Somatomedin-C (IGF-1) polyclonal antiserum was obtained from the National Hormone and Pituitary program (Baltimore, MD). L-[ $^{35}$ S]Methionine ( $> 1,000$  Ci/mmol) was purchased from New England Nuclear (Boston, MA).  $\alpha$ -[ $^{32}$ P]dCTP (3,000 Ci/mmol) was from ICN Biomedicals Inc. (Irvine, CA). Hyaluronic acid, poly[I:C], and actinomycin-D were obtained from Sigma Chemical Co. (St. Louis, MO). L929 cells (CCL 1) were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Rabbit polyclonal anti-TNFR1 and -TNFR2 antibodies were kindly provided by Drs. R. F. Weber and D. V. Goeddel (Genentech Inc. San Francisco, CA).

**Macrophage isolation and culture.** Bone marrow-derived macrophages were obtained using a modification of the technique described by Stewart (27, 32). DME containing 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, 0.37% (wt/vol) NaHCO $_3$ , 10% (vol/vol) heat-inactivated FBS, and 10% (vol/vol) L929 cell-conditioned medium as a source of M-CSF was used for the isolation, culture, and stimulation of the macrophages. Bone marrow cells were aseptically flushed from the dissected pelvises, femurs, and tibias of C3H/HeJ mice with a jet of complete medium directed through a 25-gauge needle. The bone marrow cells were adjusted to a density of  $\sim 3 \times 10^5$  cells/ml of medium and distributed in 2-ml aliquots into

22-mm diameter wells of 12-well tissue culture plates or 13-ml aliquots into 100-mm diameter culture dishes. The cells were maintained at 37°C under a 10% (vol/vol) CO $_2$  atmosphere for 5 d and thereafter the medium was changed on alternate days until the cells were used between days 5 and 7.

**Quantification of Bf and IGF-1 synthesis.** The level of synthesis of Bf and IGF-1 was determined by biosynthetic pulse labeling of macrophage monolayers followed by quantitative immunoprecipitation (27, 33). Cell monolayers were rinsed with HBSS and incubated for 1 h at 37°C with 100  $\mu$ Ci/well of L-[ $^{35}$ S]methionine dissolved in 1 ml of MEM (with Earle's salts) lacking methionine. Incorporation of isotope into IGF-1, Bf, and total protein was linear with respect to time for up to at least 3 h. After labeling, the supernatants were removed and the cells were lysed in 1 ml of a detergent/inhibitor solution composed of 0.5% (vol/vol) Triton X-100, 0.25% (wt/vol) sodium deoxycholate, 2 mM PMSF, 10  $\mu$ g/ml leupeptin, and 10 mM EDTA dissolved in 10 mM phosphate-buffered saline (150 mM), pH 7.6 (27, 34). The incorporation of [ $^{35}$ S]methionine into total protein was determined by precipitation with trichloroacetic acid using the method described by Roberts and Paterson (35). Incorporation of [ $^{35}$ S]methionine into IGF-1 and Bf was quantified by serial immunoprecipitation of cell lysates with 5  $\mu$ l of a 1:20 dilution of polyclonal antiserum to IGF-1 followed by 5  $\mu$ l of anti-human Bf (properdin Bf) IgG as previously described (27). The lysates were precleared with "immunoprecipitin" between immunoprecipitations. Samples were separated by SDS-PAGE through a 7.5% (Bf) or 10% (IGF-1) polyacrylamide gel under reducing conditions. Localization of the radioactive bands was by fluorography and the specific incorporation of [ $^{35}$ S]methionine into Bf and IGF-1 was determined by excision of the radioactive bands, digestion in hydrogen peroxide (15% [vol/vol]), and counting in a liquid scintillation spectrometer. Disintegrations incorporated into Bf and IGF-1 were normalized to the level of total protein synthesis on the basis of the trichloroacetic acid-precipitable counts and are expressed as the dpm incorporated into Bf or IGF-1 per  $10^7$  dpm of trichloroacetic acid precipitable protein. Each experiment was conducted a minimum of three times.

**Purification of RNA and Northern blot analysis.** Total cellular RNA was extracted by lysis with 4 M guanidine isothiocyanate (36) and purified by centrifugation through 5.7 M cesium chloride at 100,000 g for 18 h. 15  $\mu$ g of total RNA was subjected to Northern blot analysis through 1.0% agarose-formaldehyde gels (37) and transferred to nitrocellulose or nylon filters. Blots were hybridized with IFN- $\beta$ -, TNF $\alpha$ -, GAPDH-, or IGF-1 exon III  $^{32}$ P-labeled cDNA probes (38, 39), washed to a final stringency of  $0.2 \times$  SSC, and autoradiographs prepared at  $-70^\circ\text{C}$  using Kodak XAR-1 film. The IGF-1 exon III cDNA probe was a generous gift from Dr. Peter Rotwein (Washington University, St. Louis, MO), the mouse TNF $\alpha$  probe was obtained from Dr. Arjun Singh (Genentech, San Francisco, CA), and the IFN- $\beta$  probes were provided by Drs. Tom Maniatis and Tamar Enoch (Harvard Medical School, Boston, MA).

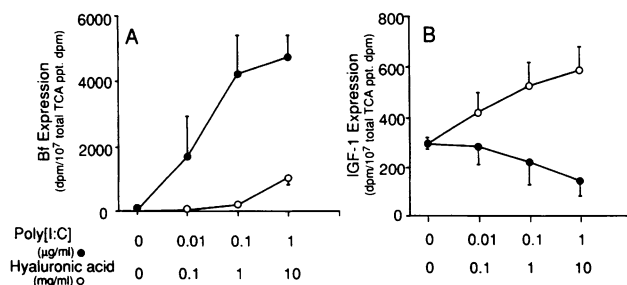
**TNF $\alpha$  cytotoxicity assay.** TNF $\alpha$  was assayed using the cell lytic assay as described by Ruff and Gifford (40). L929 cells in DME containing 2 mM glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, 0.37% (wt/vol) NaHCO $_3$  and 10% (vol/vol) heat-inactivated FBS, were plated in 96-well flat-bottomed microtiter plates at a density of  $5.5 \times 10^4$  cells in 100  $\mu$ l of culture medium and were incubated for 20 h at 37°C in a humidified 5% (vol/vol) CO $_2$  atmosphere. The medium was removed and 100  $\mu$ l of actinomycin-D (2  $\mu$ g/ml) dissolved in the above medium was added, followed by 100  $\mu$ l of sample or standard. After 20 h of incubation, the supernatants were removed, and 100  $\mu$ l of 0.1% (wt/vol) crystal violet in 1% (vol/vol) acetic acid was added. After 15 min incubation, the cells were washed three times in water and then solubilized in 1% (wt/vol) SDS. The concentration of the dye was determined by measuring the absorbance of the wells in an ELISA reader at 590 nm. The amount of TNF $\alpha$  in each sample was determined by comparison of the absorbances against a standard curve of mouse rTNF $\alpha$ . All samples were frozen immediately after collection and were assayed after a single thaw.

**Data analysis.** Results are presented as the mean  $\pm$  standard error of the mean for three or more independent experiments. Comparisons between groups were made using the Student's paired *t* test.

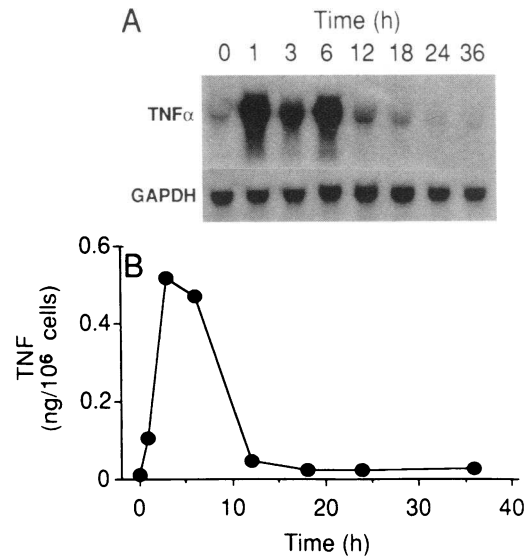
## Results

**Effect of poly(I:C) and hyaluronic acid on the synthesis of Bf and IGF-1.** The differential responses of macrophages to poly(I:C) and hyaluronic acid are illustrated in Fig. 1. Macrophage monolayers were incubated with either poly(I:C) (0.01–1  $\mu$ g/ml) or hyaluronic acid (0.1–10 mg/ml) for 18 h before biosynthetic labeling with [ $^{35}$ S]methionine and quantification of the level of synthesis of Bf and IGF-1. As shown in Fig. 1 A, exposure of macrophages to poly(I:C) resulted in a concentration-dependent stimulation of Bf synthesis but did not result in any stimulation of the synthesis of IGF-1 (Fig. 1 B). By contrast, when macrophages were incubated with increasing concentrations of hyaluronic acid, the synthesis of IGF-1 was significantly increased at concentrations  $>$  1 mg/ml ( $P = 0.04$ ) (Fig. 1 B), while the synthesis of Bf was only stimulated at the highest concentration of hyaluronic acid used ( $P = 0.02$ ) and only to  $\sim 25\%$  of the level seen with poly(I:C). Thus, poly(I:C) was a potent stimulant of Bf synthesis but not that of IGF-1, whereas hyaluronic acid stimulated IGF-1 expression but was a poor inducer of Bf.

**Both poly(I:C) and hyaluronic acid stimulate macrophages through a TNF $\alpha$ -dependent mechanism.** Previously reported findings have suggested that the expression and autocrine action of TNF $\alpha$  is required for the induction of macrophage cytotoxic activity by several stimuli (11, 41). Similarly, as will be shown subsequently in this paper, TNF $\alpha$  is also involved in the expression of Bf, a gene product whose expression accompanies cytotoxic activation after exposure to poly(I:C). In addition, other work from our laboratory has suggested that TNF $\alpha$  is required for the stimulation of IGF-1 synthesis in response to hyaluronic acid (4). Since the expression of TNF $\alpha$  is regulated at both pretranslational and translational levels, we quantified the effects of poly(I:C) and hyaluronic acid on the expression of both TNF $\alpha$  mRNA and secreted protein. Incubation of mouse bone marrow-derived macrophages with poly(I:C) (1  $\mu$ g/ml) (Fig. 2) was found to stimulate the expression of TNF $\alpha$  mRNA in a bimodal fashion with peaks at 1 and 6 h, before falling over the subsequent 6 h. However, only a single rise in protein was seen that peaked at 3 h (Fig. 2 B). The secondary rise in TNF $\alpha$  transcripts was a consistent finding that, we specu-

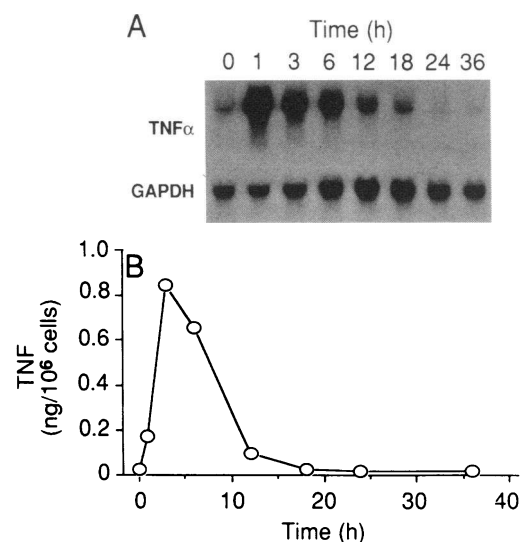


**Figure 1.** Concentration-dependent effect of poly(I:C) and hyaluronic acid on the synthesis of Bf (A) and IGF-1 (B). Bone marrow-derived macrophages were incubated with either poly(I:C) (10–1,000  $\mu$ g/ml) or hyaluronic acid (0.1–10 mg/ml) for 18 h before biosynthetic labeling.

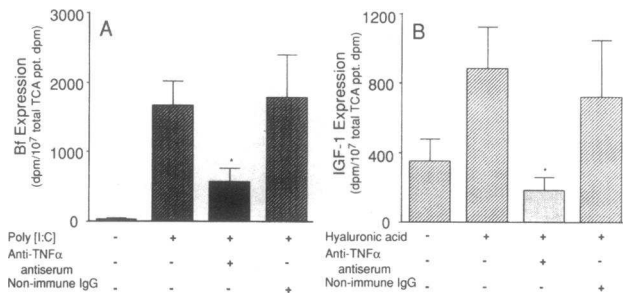


**Figure 2.** Time course of TNF $\alpha$  mRNA and protein expression after stimulation with poly(I:C). Bone marrow-derived macrophages were stimulated with poly(I:C) (1  $\mu$ g/ml) for times ranging from 0 to 36 h, after which the RNA was extracted and mRNA expression for TNF $\alpha$  assessed by Northern analysis (top). TNF protein levels were quantified in the culture supernatants using the L929 cytotoxicity bioassay (bottom). The top shows a representative experiment with GAPDH shown as a control.

late, may be mediated by IFN- $\beta$ , which we have previously shown to be induced by poly(I:C) over a similar time course (28). Exposure of macrophages to hyaluronic acid (1 mg/ml) also stimulated the expression of TNF $\alpha$  mRNA (Fig. 3 A), which peaked at 1 h, and secretion of TNF $\alpha$  protein (Fig. 3 B), which peaked at 3 h. Both poly(I:C) and hyaluronic acid led to the expression of comparable levels of TNF $\alpha$  mRNA and protein.



**Figure 3.** Time course for TNF $\alpha$  mRNA and protein expression after stimulation with hyaluronic acid. Macrophages were stimulated with hyaluronic acid (1 mg/ml) for times ranging from 0 to 36 h, after which the levels of TNF $\alpha$  mRNA and secreted protein were determined as detailed in the legend to Fig. 2. The top shows a representative experiment with GAPDH shown as a control.

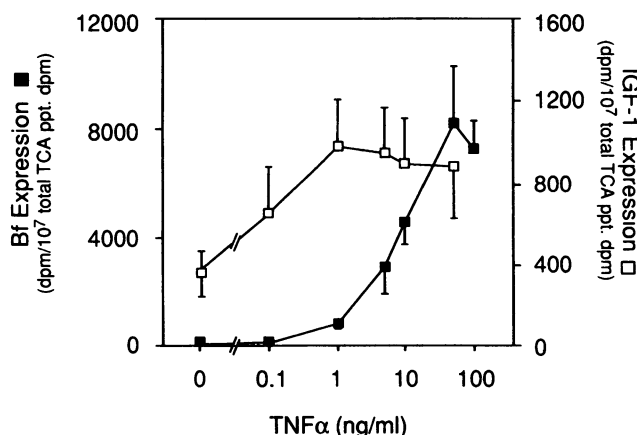


**Figure 4.** Effect of polyclonal TNF $\alpha$  antiserum on Bf expression (A) induced by poly(I:C) and IGF-1 expression (B) induced by hyaluronic acid. Bone marrow-derived macrophages were preincubated for 30 min with either anti-TNF $\alpha$  antiserum (20,000 neutralizing U/ml) or nonimmune IgG as a control, before the addition of the stimulating agents poly(I:C) (1  $\mu$ g/ml) or hyaluronic acid (1 mg/ml). Macrophages were labeled after 18 h of stimulation. \* $P < 0.03$  compared with stimulated macrophages.

The requirement for macrophage-derived TNF $\alpha$  in the expression of Bf and IGF-1 was investigated by stimulating macrophage monolayers with poly(I:C) or hyaluronic acid in the presence of a rabbit polyclonal anti-TNF $\alpha$  neutralizing antiserum. As will be seen in Fig. 4, the expression of Bf and IGF-1 was substantially blocked in the presence of the antiserum but was not affected by nonimmune rabbit IgG. Thus, TNF $\alpha$  was expressed in response to both poly(I:C) and hyaluronic acid and acted in an autocrine fashion to induce the expression of Bf and IGF-1, respectively.

#### Stimulation of Bf and IGF-1 expression by mouse rTNF $\alpha$ .

We next determined the effects of highly purified mouse rTNF $\alpha$  on the expression of Bf and IGF-1. Bone marrow-derived macrophages were incubated with mouse rTNF $\alpha$  (0.1–50 ng/ml) for 18 h before quantifying the level of synthesis of Bf and IGF-1. As shown in Fig. 5, Bf expression rose in a concentration-dependent fashion and was initially detected at a concentration of 1 ng/ml. A plateau was reached at between 50 and 100 ng/ml of TNF $\alpha$ . Synthesis of IGF-1 also increased in response to mouse rTNF $\alpha$  but the dose-response curve was shifted to the left in comparison with Bf and had reached a



**Figure 5.** Dose response of mouse rTNF $\alpha$  stimulation of Bf and IGF-1. Bone marrow-derived macrophages were stimulated with mouse rTNF $\alpha$  (0.1–50 ng/ml) for 18 h before biosynthetic labeling and quantification of Bf (■) and IGF-1 (□).

plateau at 1 ng/ml, i.e., at a 50–100-fold lower concentration of mouse rTNF $\alpha$ . Interestingly, the level of stimulation of IGF-1 synthesis was greater in response to TNF $\alpha$  than hyaluronic acid. It is possible that this fact may be related to the amount of TNF $\alpha$  produced after stimulation with hyaluronic acid. Thus, after stimulation with 1 mg/ml of hyaluronic acid, the concentration of TNF $\alpha$  in the culture supernatant was 5 pg/ml, a figure considerably lower than that applied exogenously.

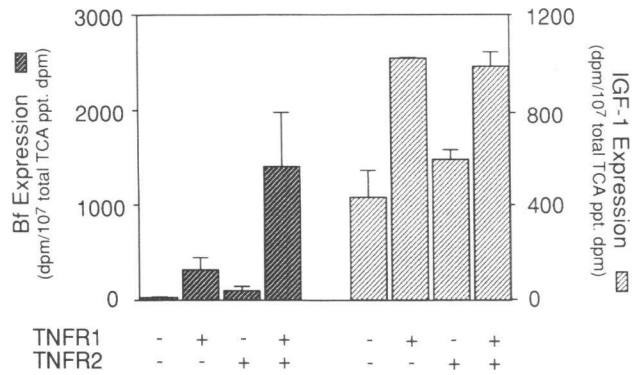
*Involvement of TNF receptor classes in the expression of Bf and IGF-1.* A potential explanation for the diversity in the expression of Bf and IGF-1 after stimulation with poly(I:C), hyaluronic acid (and TNF $\alpha$ ) is that each response is mediated by a different TNF receptor class. Two strategies have been applied to address this question. First, mouse TNFR1 (p55) ligates both human and mouse TNF $\alpha$ , whereas TNFR2 (p75) recognizes mouse TNF $\alpha$  alone (21). Thus, human TNF $\alpha$  can be exploited as a TNFR1-specific agonist. As will be seen in Table I, exposure of mouse macrophages to human rTNF $\alpha$  and mouse rTNF $\alpha$  (10–20 ng/ml) for 18 h resulted in an indistinguishable level of stimulation of IGF-1 synthesis, suggesting that ligation of TNFR1 is sufficient to fully induce this response. However, quantification of the level of Bf synthesis revealed that human rTNF $\alpha$  was significantly less effective than mouse rTNF $\alpha$  at inducing this response. These findings thus implicate TNFR1 in the stimulation of Bf synthesis by TNF $\alpha$  but also suggest that ligation of TNFR2 may contribute to this response.

To further address this question, we investigated the effects of rabbit polyclonal antibodies reactive against each TNF receptor on the synthesis of Bf and IGF-1. These antibodies have previously been shown to act as specific and independent agonists of TNFR1- and TNFR2-dependent responses (18, 21). Macrophage monolayers were incubated with saturating concentrations (2  $\mu$ g/ml) of anti-TNFR1 or anti-TNFR2 antibodies, or the combination of both antibodies, for 18 h before quantifying the level of synthesis of Bf and IGF-1. As shown in Fig. 6, ligation of TNFR1 alone resulted in a stimulation of both Bf ( $P = 0.05$ ) and IGF-1 ( $P = 0.05$ ) synthesis, whereas ligation of TNFR2 had only a marginal effect on the synthesis of both proteins. Of significance, and in confirmation of the findings described above using human and mouse TNF $\alpha$  to discriminate between the two receptors, simultaneous exposure of macrophages to both antibodies led to a synergistic increase in the synthesis of Bf, but failed to further increase the synthesis of IGF-1. Thus, in summary, ligation of TNFR1 ap-

**Table I.** Induction of Bf and IGF-1 by Mouse rTNF $\alpha$  and Human rTNF $\alpha$

	Bf*	IGF-1*
	<i>dpm/10<sup>7</sup> total TCA ppt. dpm</i>	
Control	39±11	559±40
Mouse rTNF $\alpha$	2,816±914	1,901±960
Human rTNF $\alpha$	1,103±393	2,040±757

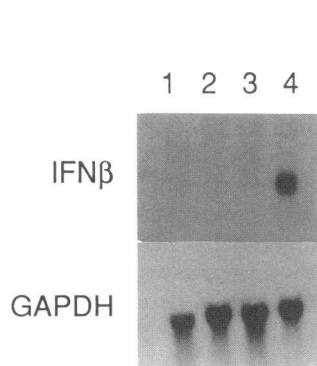
\* Bone marrow-derived macrophages were incubated with either mouse rTNF $\alpha$  (10 ng/ml) or human rTNF $\alpha$  (20 ng/ml) for 18 h before immunoprecipitation and quantification of Bf and IGF-1. Results show the mean  $\pm$  SE for three separate experiments.



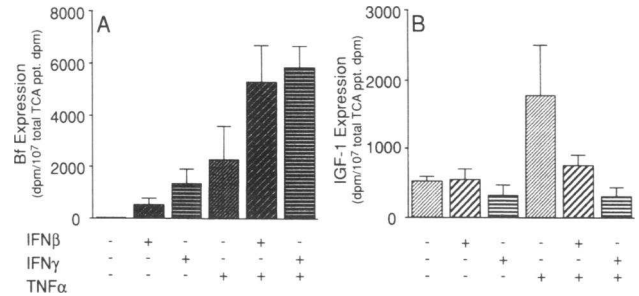
**Figure 6.** Stimulation of Bf and IGF-1 expression by agonist antibodies to the TNFR1 and TNFR2. Agonist TNF receptor antibodies (2  $\mu$ g/ml), either alone or together, were used to stimulate bone marrow-derived macrophages for 18 h before quantifying the level of synthesis of Bf and IGF-1.

peared qualitatively sufficient to stimulate the expression of both Bf and IGF-1, while ligation of TNFR2 appeared to amplify the expression of Bf. These findings therefore suggest that factors in addition to TNF $\alpha$  receptor usage underlie the differences in the ability of poly(I:C) and hyaluronic acid to differentially stimulate the synthesis of Bf and IGF-1.

**Role of IFN in the TNF $\alpha$ -dependent regulation of Bf and IGF-1 expression.** A significant feature of the response of most cell types, including macrophages, to poly(I:C), is the secretion of IFN- $\beta$ . Furthermore, as we and others have previously shown, IFNs, either exogenously applied ( $\alpha$ ,  $\beta$ , or  $\gamma$ ) or endogenously derived (IFN- $\beta$ ), markedly enhance the expression of Bf by macrophages by increasing the sensitivity of these cells to other agonists (14, 28, 31, 42, 43). Hyaluronic acid, on the other hand, has not, to our knowledge, been reported to induce the expression of IFN. Thus, it seemed plausible that the inability of hyaluronic acid to stimulate the synthesis of Bf was related to its inability to induce IFN- $\beta$  expression by macrophages. If this were so, it would also imply that IFNs would modify the response of the macrophages to TNF $\alpha$  and to TNF $\alpha$ -dependent stimuli. We addressed these hypotheses in two ways. First, we determined the effects of poly(I:C), hyaluronic acid, and TNF $\alpha$  on the expression of IFN- $\beta$  transcripts by mouse bone marrow-derived macrophages. Second, we investigated the effects of IFNs on the expression of Bf and IGF-1 by TNF $\alpha$  and hyaluronic acid.



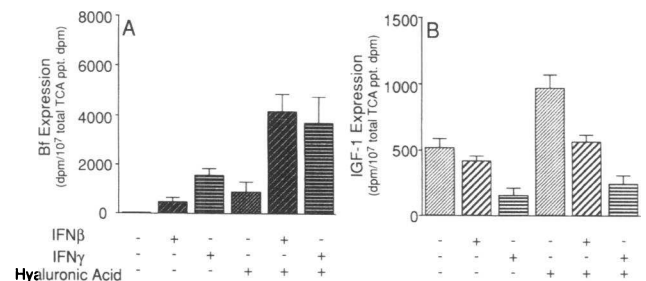
**Figure 7.** Effect of TNF $\alpha$ , hyaluronic acid, and poly(I:C) on the expression of IFN- $\beta$  mRNA by mouse macrophages. Cells were incubated in either medium alone (lane 1), TNF $\alpha$  (5 ng/ml, lane 2), hyaluronic acid (1 mg/ml, lane 3), or poly(I:C) (1  $\mu$ g/ml, lane 4) for 3 h before determination of the level of IFN- $\beta$  mRNA expression by Northern blot analysis. GAPDH mRNA is shown as a control.



**Figure 8.** Effect of IFN ( $\beta$  and  $\gamma$ ) on Bf and IGF-1 expression induced by TNF $\alpha$ . Bone marrow-derived macrophages were incubated with medium alone, IFN- $\beta$  (200 U/ml), or IFN- $\gamma$  (20 U/ml) for 3–4 h. They were then stimulated with TNF $\alpha$  (5 ng/ml) in the continued presence of IFN for 18 h before quantification of the level of synthesis of Bf (A) and IGF-1 (B).

The effect of poly(I:C), hyaluronic acid, and TNF $\alpha$  on the expression of IFN- $\beta$  mRNA was quantified by Northern analysis. As shown in Fig. 7, exposure of macrophages to poly(I:C) (1  $\mu$ g/ml for 3 h) led to the expression of IFN- $\beta$  mRNA. By contrast, hyaluronic acid (1 mg/ml for 3 h) and TNF $\alpha$  (5 ng/ml for 3 h) failed to induce the expression of IFN- $\beta$  mRNA. Additionally, mouse rTNF $\alpha$  failed to stimulate an increase in the steady state level of IFN $\beta$  mRNA in mouse bone marrow-derived macrophages at any time point studied (30 min to 24 h). The level of glyceraldehyde-3-phosphate dehydrogenase mRNA was found to be relatively constant, indicating that similar amounts of RNA were loaded in each lane (Fig. 7). Thus, while both poly(I:C) and hyaluronic acid stimulate the expression of TNF $\alpha$ , only poly(I:C) was able to induce the expression of IFN- $\beta$ .

We next investigated the effect of IFNs on the synthesis of Bf and IGF-1. Bone marrow-derived macrophages were incubated with either IFN- $\beta$  (200 U/ml) or IFN- $\gamma$  (20 U/ml) for 3–4 h before adding either TNF $\alpha$  (5 ng/ml) or hyaluronic acid (1 mg/ml). The cells were then incubated for a further 18 h in the continued presence of IFN before quantifying the level of synthesis of Bf and IGF-1. As shown in Fig. 8 A, both IFN- $\beta$  and IFN- $\gamma$  enhanced the expression of Bf in response to TNF $\alpha$ . Importantly, as can be seen in Fig. 9 A, the expression of Bf by hyaluronic acid was also significantly enhanced in the presence of IFN- $\beta$  or IFN- $\gamma$  (Fig. 9 A). By contrast, both IFN- $\beta$  and IFN- $\gamma$  substantially inhibited the increase in IGF-1 synthesis



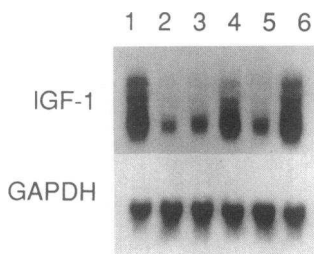
**Figure 9.** Effect of IFN ( $\beta$  and  $\gamma$ ) on Bf (A) and IGF-1 (B) expression induced by hyaluronic acid. Bone marrow-derived macrophages were incubated with medium alone, IFN- $\beta$  (200 U/ml), or IFN- $\gamma$  (20 U/ml) for 3–4 h before being stimulated with hyaluronic acid (1 mg/ml) in the continued presence of IFN for 18 h.

seen in response to  $TNF\alpha$  (Fig. 8 B) or hyaluronic acid (Fig. 9 B). Moreover, exposure of macrophages to  $IFN\gamma$  also decreased the baseline level of expression of IGF-1 (Fig. 9 B). These findings therefore suggest that IFNs serve an important function in determining the pattern of gene expression by macrophages in response to  $TNF\alpha$  itself or  $TNF\alpha$ -dependent stimuli.

**Effect of IFN on IGF-1 mRNA expression.** Previously reported studies (30, 44) have shown that the expression of IGF-1 is regulated at both pretranslational and translational levels, and thus it was important to determine the level at which IFN blocked the expression of IGF-1. Macrophage monolayers were incubated with  $IFN\gamma$  for 3–4 h before stimulation with either  $TNF\alpha$  (5 ng/ml) or hyaluronic acid (1 mg/ml) for 12 h. The levels of IGF-1 transcripts were then quantified by Northern analysis. As shown in Fig. 10, unstimulated macrophages contained detectable levels of IGF-1 mRNA, which essentially remained unchanged upon stimulation with  $TNF\alpha$  or hyaluronic acid. Thus, the increase in IGF-1 protein synthesis appears to be mediated at a translational level. However, exposure of macrophages to  $IFN\gamma$  was found to substantially reduce the level of IGF-1 mRNA both in the absence of further stimulation and after stimulation with  $TNF\alpha$  or hyaluronic acid.  $IFN-\beta$  similarly inhibited IGF-1 mRNA expression, but, like the pattern seen with respect to IGF-1 protein synthesis, was less effective by comparison with  $IFN-\gamma$ . These findings suggest that IFNs act at least in part to control the level of IGF-1 mRNA, although an additional effect at the level of translation cannot be excluded.

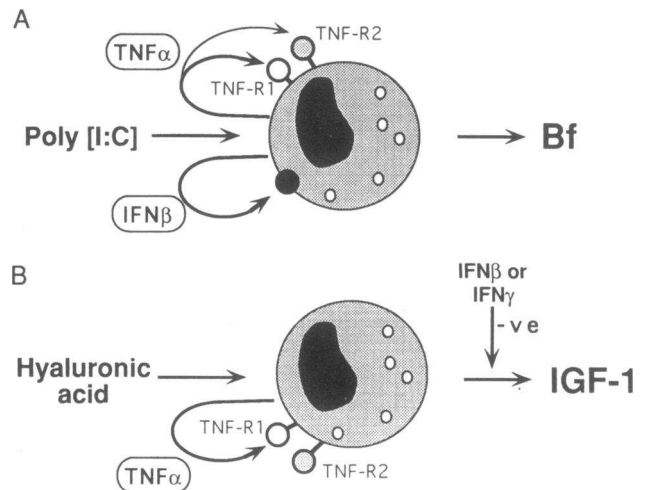
## Discussion

The question of how  $TNF\alpha$  is able to induce such broad diversity in cellular responses in the face of a binary system of cell surface receptors has become a major focus of research into the biology of this cytokine. The findings of this study show that diversity in macrophage gene expression can be induced when macrophages are exposed to  $TNF\alpha$  in combination with IFNs. We have shown that in the absence of IFNs, macrophages responded to picogram to nanogram quantities of  $TNF\alpha$  with a stimulation in the expression of the cell cycle progression factor, IGF-1, while the synthesis of the complement component, Bf, was stimulated at ~ 50–100-fold higher concentrations of  $TNF\alpha$ . However, when macrophages were exposed to  $TNF\alpha$  in the presence of  $IFN-\beta$  or  $IFN-\gamma$ , there was an abrupt cessation



**Figure 10.** Effect of and  $IFN-\gamma$  on the expression of IGF-1 mRNA by hyaluronic acid and  $TNF\alpha$ . Bone marrow-derived macrophages were incubated with medium alone or  $IFN-\gamma$  (20U/ml) for 3–4 h, and were then treated with either medium alone or with hyaluronic acid (1 mg/ml) or  $TNF\alpha$  (5 ng/ml) in the continued presence

of IFN for 12 h before the RNA was extracted and IGF-1 mRNA levels were assessed by Northern blotting. GAPDH mRNA is shown to indicate that equal amounts of RNA were loaded in each lane. Lane 1, unstimulated cells; lane 2,  $IFN-\gamma$ ; lane 3,  $IFN-\gamma$  + hyaluronic acid; lane 4, hyaluronic acid; lane 5,  $IFN-\gamma$  +  $TNF\alpha$ ; lane 6,  $TNF\alpha$ .



**Figure 11.** Cartoon illustrating the proposed ability of  $IFN-\beta$  and  $IFN-\gamma$  to influence the synthesis of IGF-1 and Bf by stimuli that induce the expression of  $TNF\alpha$ . (A) Exposure to poly(I:C) initiates the expression of  $IFN-\beta$  and  $TNF\alpha$ , which act in combination in an autocrine fashion to stimulate the expression Bf. Ligation of  $TNF\alpha$  by TNF receptors TNF-R1 and TNF-R2 is required for optimal expression of Bf. (B) Exposure to hyaluronic acid initiates the expression of  $TNF\alpha$  alone. In the absence of IFNs, macrophages respond in an autocrine fashion to  $TNF\alpha$  by expressing IGF-1. In contrast, in the presence of exogenously applied  $IFN-\beta$  or  $IFN-\gamma$ , synthesis of IGF-1 ceases. The increase in synthesis of IGF-1 by  $TNF\alpha$  is fully supported by TNF receptor TNF-R1.

of the synthesis of IGF-1 and a concomitant enhancement of the synthesis of the complement component, Bf. Thus, since  $TNF\alpha$  is secreted by macrophages in response to a multitude of structurally and functionally diverse stimuli, the presence and/or secretion of IFNs would appear to be an important determinant of the eventual responses of the cells to  $TNF\alpha$ . This conclusion, illustrated schematically in Fig. 11, is supported in the present study by the finding that exposure of macrophages to poly(I:C), a polyribonucleotide that induces the expression of both  $IFN-\beta$  and  $TNF-\alpha$ , led to a protein synthetic phenotype characterized by increased synthesis of Bf, whereas hyaluronic acid, a connective tissue matrix component that stimulates a similar degree of  $TNF\alpha$  expression but in the absence of  $IFN-\beta$ , led to a phenotype characterized by increased synthesis of IGF-1.

The dose-response curves for the stimulation of IGF-1 and Bf synthesis by  $TNF\alpha$  differed quite strikingly. Although we did not quantify the number or affinity of  $TNF\alpha$  receptors in this study, previously reported findings have shown that unstimulated macrophages express ~ 4,000 receptors per cell (45, 46) with a  $K_d$  for TNFR1 (p55) of ~ 500 pM, while TNFR2 (p75) displays a  $K_d$  of ~ 100 pM (17, 20, 22, 47). In this study, half-maximal stimulation of IGF-1 synthesis was achieved at a concentration of  $TNF\alpha$  of ~ 6 pM, suggesting first, that this response is stimulated at low receptor occupancy, and second, in view of the  $K_d$ 's of the two receptors, the likelihood that at such a low concentration,  $TNF\alpha$  preferentially binds to TNFR2. This latter conclusion, however, is clearly at variance with the finding that IGF-1 expression was stimulated by exposure of macrophages to TNFR1-specific agonists such as human  $TNF\alpha$  or agonistic antibodies directed against



TNFR1 and, moreover, that agonist antibodies reactive against TNFR2, applied either alone or combination with anti-TNFR1 antibodies, were inactive. These data could be reconciled in several ways: (a) TNFR2 may play an adjunct role to TNFR1, thereby allowing transmembrane signaling to be initiated by TNFR1 in a fashion similar to that proposed by Tartaglia and Goeddel (48) involving presentation of TNF $\alpha$  to the lower affinity TNFR1 by TNFR2; (b) a proportion of the TNFR1 pool may exist in a high affinity state; or (c) the  $K_d$  for the constitutive wild type TNFR1 expressed by macrophages may be lower than the  $K_d$  obtained for cell lines expressing transfected receptors and from which the  $K_d$  data discussed above were determined.

In contrast to the effect of TNF $\alpha$  on IGF-1 synthesis, half-maximal stimulation of Bf expression was achieved at a TNF $\alpha$  concentration of  $\sim 600$  pM, indicating that the expression of this gene product is only achieved at relatively high receptor occupancy. Furthermore, unlike the situation leading to the stimulation of IGF-1 synthesis, ligation of TNFR1 led to a suboptimal stimulation of Bf synthesis, while the combined ligation of both TNFR1 and TNFR2 led to a synergistic increase in Bf expression, suggesting cooperation between the two classes of TNF receptor in this situation. We have considered three potential explanations of this novel finding. First, it is conceivable that different signals may be generated by each receptor. Ligation of TNFR1 has been shown to result in the activation of a phosphatidylcholine-specific phospholipase C (PLC), leading to the formation of diacylglycerol (49) and the subsequent activation of protein kinase C (PKC). In other work, we have shown PKC to be involved in the induction of Bf expression (50). The mechanism of signaling through TNFR2, however, is largely unknown, although given the differences in specific cellular responses induced by ligation of this receptor and the marked differences in the primary structure of the intracellular domains of TNFR1 and TNFR2, it is likely that the signals transduced by TNFR2 will be distinct from those emanating from TNFR1. Conceivably, the signals generated by ligation of TNFR2 may amplify those signals generated by TNFR1. Second, and speculatively, incubation with high concentrations of TNF $\alpha$  may result in the coaggregation of both TNFR1 and TNFR2 in the plane of the plasma membrane leading to a different signaling pattern from that seen during aggregation of a single receptor class. Such aggregation may also result in the recruitment of additional signaling molecules as seen with the IFN- $\gamma$  receptor (51). Third, dual ligation of TNFR1 and TNFR2 may stimulate the expression of additional cytokines that may enhance the synthesis of Bf. It has recently been reported that IL-1 $\beta$  enhances the expression of GM-CSF after ligation of TNFR2 in transfected PC60 cells (26). However, we were unable to detect any increased expression of IFN $\beta$  or IL-1 $\beta$  when macrophages were stimulated with the TNFR1 and TNFR2 antibodies (F. R. Lake and D. W. H. Riches, unpublished observations).

Pretreatment or coinubation of macrophages with IFN- $\beta$  or IFN- $\gamma$  was found to be a major determinant of the direction of gene expression induced by TNF $\alpha$  or stimuli that induce TNF $\alpha$  expression. Thus, from a functional standpoint, exposure to IFN- $\beta$  or IFN- $\gamma$  led to a "switch" in the protein synthetic phenotype from one characterized by IGF-1 to one characterized by Bf. Interestingly, using a different system, Kirstein et al. (52) recently noted that both IFN $\gamma$  and LPS (which induces IFN- $\beta$  expression in macrophages) were able to inhibit

the induction of IGF-1 synthesis after stimulation of peripheral blood monocytes with advanced glycosylation end-products-BSA. In their system, however, IL-1 $\beta$  and PDGF, but not TNF $\alpha$ , act in an autocrine fashion to induce IGF-1 (52). It would seem unlikely that the cessation of IGF-1 synthesis was mediated by a decline in the number of TNF receptors since work reported by others has shown that, if anything, IFNs lead to an increase in the level of expression of TNF receptors (53, 54), particularly TNFR2 (55). Moreover, as we have shown, TNF receptors are also required for the stimulation of Bf expression. These findings therefore support the notion that the negative effect of IFNs on the induction of IGF-1 by TNF $\alpha$  is expressed at a postreceptor level, and efforts are now being directed to address the step(s) at which this effect is manifest. This conclusion is also consistent with previous observations made in this laboratory with respect to the enhancing effect of IFN- $\beta$  on Bf expression (28, 56), where the effect of IFN was distal to the early signaling events initiated by the calcium ionophore, ionomycin. Thus, we speculate that IFNs may allow the TNF $\alpha$  signal transduction pathways to be used for different cellular responses by redirecting the signals at a relatively distal point in the pathway.

The findings of this study have significant implications for the regulation of macrophage functional activity in disease. For example, TNF $\alpha$  has been implicated in the pathogenesis of both pulmonary fibrosis in humans (57, 58) and in a variety of inflammatory systems in animals (6, 7, 59, 60), and we have previously suggested that this may be mediated in part through its ability to increase the expression of IGF-1. Studies in animal models have also shown that both poly(I:C), through its ability to induce IFN- $\alpha/\beta$  expression, and IFN $\gamma$  can substantially reduce the pulmonary fibrosis in response to intratracheal instillation of bleomycin (61, 62). Similarly, the elevated level of serum IFN- $\gamma$  seen in patients with the granulomatous lung disease sarcoidosis has been shown to be associated with a lower grade of disease activity and a lower incidence of lung fibrosis (63). Although IFNs can affect multiple cell types and can directly suppress collagen synthesis by fibroblasts, it is tempting to speculate that part of their effect may be mediated by their ability to restrict macrophage expression of IGF-1 and thereby decrease fibroblast proliferation. Support for this possibility comes from the observation that fibroblast numbers are reduced in the lungs of mice instilled with bleomycin in the presence of IFN $\gamma$  (61). Interestingly, the ability of IFNs to block fibroblast growth factor expression is not restricted to IGF-1, since we have also observed that IFN- $\beta$  also blocks the induction of PDGF-B mRNA expression by macrophages (unpublished observations). Thus, in this respect, IFNs, either exogenously applied or endogenously derived, may serve to negatively regulate fibrogenic gene expression by macrophages.

## Acknowledgments

We thank Linda Remigio for her outstanding technical assistance and Barry Silverstein, Leigh Landskroner, and Nadi deStackleberg for preparing the photographs.

This work was supported by Public Health Service grant HL-27353. Fiona Lake was supported in part by a W.A. and M.G. Saw Fellowship from the University of Western Australia.

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