

## Interleukin 4 regulates induction of sialoadhesin, the macrophage sialic acid-specific receptor

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**ABSTRACT** Sialoadhesin is a nonphagocytic lectin-like receptor found on a restricted population of tissue macrophages in lymphoid and hemopoietic tissues. In bone marrow, it is localized to areas of contact between the resident stromal macrophages and developing granulocytes, which together form myeloblastic clusters. Sialoadhesin is highly specific for sialylated glycoconjugates and may play a role in adhesion and trophic hemopoietic cell interactions, although its function is unknown. Resident peritoneal macrophages do not express high levels of sialoadhesin *in vitro* unless an inducing element found in normal mouse serum is present. The restricted *in vivo* location of this marker and its induction by mouse serum prompted us to examine the possible influence of various cytokines on its expression, measured by a sheep erythrocyte rosetting assay. None of the cytokines tested was able to induce sialoadhesin; however, interleukin 4 (IL-4) prevented the induction in the presence of serum. Expression of other macrophage markers was not influenced in parallel, and Western blotting showed that sialoadhesin antigen in cell lysates was selectively reduced by IL-4. Inhibition by IL-4 was dose dependent, could be blocked by antibodies to both IL-4 and the IL-4 receptor, and was overcome by increased serum concentrations. IL-4 is therefore a potent cytokine regulator of the sialic acid-specific receptor implicated in macrophage-hemopoietic cell interactions.

Resident macrophages are distributed throughout the organs and tissues of the body, where they are thought to play a central role in both innate and specific immune responses and in the maintenance of normal homeostatic mechanisms such as hemopoiesis. The precise function of macrophages within specific microenvironments such as the bone marrow or the brain is poorly understood, but it appears that these cells maintain a discrete set of surface receptors which are specific for their particular anatomical/functional location and which appear to be controlled in a spatially and temporally precise manner.

The way in which tissue macrophages interact with other cells and with components of the extracellular matrix is of particular interest. Our laboratory has described a macrophage-restricted receptor that has a lectin-like specificity for ligands containing terminal sialic acid residues (1). This receptor, known as SER or sialoadhesin, was characterized by means of its functional capacity to bind sheep erythrocytes in the absence of divalent cations. Following the development of a specific monoclonal antibody (mAb), SER-4 (2), the receptor was isolated by affinity chromatography and shown to be a glycoprotein of 185 kDa (reduced) or 175 kDa (nonreduced) (3). The isolated protein at nanomolar concentrations was capable of agglutinating sheep and human erythrocytes, and this agglutination could be inhibited with gangliosides such as GT1b and GD1a, suggesting that the receptor reacts preferentially with oligosaccharides containing terminal sialic acid as Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc.

Immunocytochemical analyses with SER-4 mAb demonstrated high levels of receptor expression within discrete locations, including resident macrophages of the bone marrow, subcapsular sinus macrophages of the lymph nodes, and marginal metallophil cells of the spleen (2). Immunoelectron microscopy on isolated hemopoietic clusters from adult bone marrow showed that sialoadhesin molecules appeared to concentrate at areas of contact between the central macrophages and developing granulocytes (4). This location is suggestive of a functional role for sialoadhesin in the development of granulocytes. While isolated resident or inflammatory elicited peritoneal macrophages express low levels of sialoadhesin, a striking upregulation in expression can be achieved by cultivating the cells in the presence of mouse serum (5). The inducing activity within the serum has not been fully characterized, but recent work suggests that it is a 60- to 70-kDa protein with a pI of 4.8 (A.S.M. and P.T., unpublished observations).

The possibility that a serum component is responsible for either induction or maintenance of sialoadhesin expression *in vivo* within very discrete cell populations suggested to us that there may be other factors responsible for regulating expression. In the present study we have examined a number of cytokines for their capacity to either induce sialoadhesin or regulate its expression in the presence of a positive serum induction signal. While none of the cytokines studied were able to induce expression of sialoadhesin, interleukin 4 (IL-4) specifically prevented its induction in the presence of mouse serum. This effect was selective for sialoadhesin, as F4/80, which was also upregulated by serum, was not affected.

### MATERIALS AND METHODS

**Mice.** Female C57BL/6 mice were bred and housed at the Sir William Dunn School of Pathology, University of Oxford, and were 6-8 weeks old when used. Mice were bled by cardiac puncture and the serum was pooled and stored in aliquots at  $-70^{\circ}\text{C}$ . After thawing, serum was heated at  $56^{\circ}\text{C}$  for 30 min to inactivate complement.

**Macrophage Preparation and Culture.** Thioglycollate-elicited peritoneal macrophages (TPMs) were obtained 4 days following intraperitoneal injection of 1 ml of Brewer's complete thioglycollate broth. TPMs and resident peritoneal macrophages (RPMs) obtained after peritoneal lavage were washed in Opti-MEM, a defined serumless medium (GIBCO), prior to culture. All cell culture was performed in Opti-MEM plus antibiotics and 2 mM L-glutamine.

**Sheep Erythrocyte Binding Assay.** Expression of sialoadhesin was measured by the capacity of macrophages to bind washed sheep erythrocytes. Assays were performed in eight-chamber slides (ICN/Biomedicals), and each slide contained

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Abbreviations: IL-4, interleukin 4; mAb, monoclonal antibody; RPM, resident peritoneal macrophage; TPM, thioglycollate-elicited peritoneal macrophage.

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a positive control with 2% (vol/vol) mouse serum and a negative control with Opti-MEM alone. All cultures were performed on duplicate slides. For the assay,  $\approx 10^5$  RPMs or TPMs were added to each chamber of the slide and cultured in 300  $\mu$ l of medium for 48 hr. Washed erythrocytes were then added at 0.5% (vol/vol), and slides were incubated for 1 hr at 37°C. After removal of the chambers, the slides were immersed in phosphate-buffered saline (PBS) at room temperature, inverted, and agitated gently until all nonadherent erythrocytes had been removed. Slides were fixed in 0.1% (vol/vol) glutaraldehyde in PBS for 1 hr and stained with hematoxylin and eosin. The percentage of macrophages binding four or more erythrocytes was then counted. Binding was completely abolished by anti-sialoadhesin mAb.

**Reagents and Antibodies.** The mAbs SER-4 and 3D6, specific for sialoadhesin, were prepared as described (2, 3). F4/80 antibody was prepared and purified as previously (6). The IL-4-neutralizing antibody 11B11 (7) was purified from ascites fluid on a protein G-Sepharose column (Pharmacia). Both IL-4 and the M1 and M2 antibodies against the murine IL-4 receptor (8) were the gift of K. Grabstein (Immunex, Seattle). IL-5 was provided by C. Sanderson (Glycobiology, University of Oxford), and mouse interferon  $\gamma$  by F. Balkwill (Imperial Cancer Research Fund, London). All other cytokines or growth factors were purchased from Genzyme.

**Western Blotting.** After culture, cells were washed with ice-cold PBS and lysed in 250  $\mu$ l of 10 mM Tris Cl, pH 8.2/5 mM EDTA/1 mM phenylmethanesulfonyl fluoride/20  $\mu$ M leupeptin/5 mM iodoacetamide/1% (wt/vol) octyl glucoside (Sigma). Detergent-insoluble material was pelleted by centrifugation (10,000  $\times g$ , 5 min, 4°C). Solubilized proteins were separated in SDS/6.5% polyacrylamide gels at 20 mA, transferred to nitrocellulose (4 hr at 0.5 A), and probed with relevant antibodies. Blots were incubated with affinity-purified rabbit anti-rat IgG (Sigma) (3  $\mu$ Ci/ml; 1 Ci = 37 GBq) labeled with  $^{125}$ I by the Iodobead (Pierce) method (9). After autoradiography, bands were cut from nitrocellulose and assayed in a  $\gamma$  spectrometer.

## RESULTS

We tested cytokines for their capacity to induce sialoadhesin or to prevent induction in the presence of a positive inducing signal from mouse serum. We used sheep erythrocyte rosette formation to detect functional surface expression of sialoadhesin. A serum-free culture medium (Opti-MEM) provided a suitably low background level of sialoadhesin on cultivated peritoneal macrophages.

**Effects of Cytokine Exposure on Sialoadhesin Expression.** None of the cytokines tested had any direct effect on sialoadhesin expression on RPMs (Table 1). Similar results were obtained using TPMs. We next determined whether cytokines were able to influence expression during induction by mouse serum. Only IL-4 was shown to have any effect and totally prevented induction by 2% (vol/vol) mouse serum (Table 1). The synthetic corticosteroid dexamethasone was also tested in both assays and had no effect either on direct expression or on induction by serum.

**IL-4 Selectively Influences Sialoadhesin Induction.** To determine selectivity, we examined the effects of IL-4 on total surface and intracellular levels of unrelated macrophage surface markers (Table 2). Cultivation in Opti-MEM plus 2% (vol/vol) mouse serum for 48 hr increased sialoadhesin antigen by 80% compared with levels found in cells cultivated in Opti-MEM alone. IL-4 alone at 20 ng/ml had no direct effect on basal sialoadhesin, whereas the increase in sialoadhesin content was reduced by >50% when IL-4 at this concentration was added with 2% mouse serum for 48 hr. In other experiments, not shown, sialoadhesin antigen was almost completely eliminated after 72 hr or longer incubation periods. Ia and Mac-2 were similarly examined as markers of

Table 1. Effects of cytokines on the *in vitro* expression of sialoadhesin

Treatment	% macrophages binding $\geq 4$ erythrocytes			
	Untreated	Treated	Untreated + MS	Treated + MS
M-CSF (100 units/ml)	4	3	97	97
G-CSF (100 units/ml)	7	5	90	92
GM-CSF (100 units/ml)	12	9	100	98
IL-1 $\alpha$ (100 units/ml)	15	9	99	98
IL-1 $\beta$ (100 units/ml)	15	14	99	95
IL-2 (100 units/ml)	15	15	99	99
IL-3 (100 units/ml)	0	2	89	88
IL-4 (20 ng/ml)	3	2	94	2
IL-5 (100 units/ml)	4	7	90	83
IL-6 (100 units/ml)	3	0	83	80
TNF- $\alpha$ (50 units/ml)	3	3	95	92
PDGF (50 units/ml)	5	3	92	90
IFN- $\gamma$ (100 units/ml)	8	5	88	89
Dex (1 $\mu$ M)	3	5	90	91

RPMs were cultured in Opti-MEM with or without cytokine or dexamethasone (Dex), for 48 hr in the presence or absence of 2% mouse serum (MS). Results are representative of three experiments. M-CSF, macrophage-colony-stimulating factor; G-CSF, granulocyte-CSF; GM-CSF, granulocyte/macrophage-CSF; TNF, tumor necrosis factor; PDGF, platelet-derived growth factor; IFN, interferon.

macrophage activation and F4/80 as another serum-inducible marker (A.S.M. and P.T., unpublished observations). Table 2 shows that exposure to IL-4 at 20 ng/ml for 72 hr increased expression of Ia by 50% compared with background levels in Opti-MEM alone. Macrophages cultivated in 2% mouse serum showed a 30% reduction in Ia, and in the presence of both mouse serum and IL-4 the increase seen with IL-4 alone was suppressed. This suggests that factors in mouse serum alter or prevent IL-4-mediated activation of macrophages. Levels of Mac-2 in macrophage lysates were not affected by exposure to either mouse serum or IL-4. F4/80 was upregulated by >90% in the presence of mouse serum, but this increase was not affected by IL-4.

**IL-4 Prevents Expression of Functional Sialoadhesin.** The effect of IL-4 on the capacity of macrophages to form rosettes via sialoadhesin can be seen from Fig. 1. Control RPMs in the absence of mouse serum (Fig. 1A) showed no sialoadhesin expression after 48 hr in culture, whereas almost 100% formed rosettes in the presence of 2% mouse serum (Fig. 1B). mAbs SER-4 and 3D6 blocked rosette formation, and neuraminidase-treated erythrocytes were unable to rosette, confirming that the rosetting was entirely due to sialoadhesin. IL-4 by itself had no effect on sialoadhesin (Fig. 1C) but prevented its induction by mouse serum (Fig. 1D). The IL-4-neutralizing antibody 11B11 had no effect on sialoadhesin induction when tested by itself (Fig. 1E) or in the presence of mouse serum, but blocked the prevention of induction by IL-4 in the presence of mouse serum (Fig. 1F). Of two rat mAbs against the murine IL-4 receptor (8) tested, one (M1) blocked the inhibitory effects of IL-4 completely, and the second (M2) partially.

Table 2. Selectivity of IL-4 in regulating mouse serum (MS)-induced expression of sialoadhesin (Sn) in macrophages

Treatment	% control			
	Ia	Mac-2	F4/80	Sn
MS (2%)	71	80	191	180
IL-4 (20 ng/ml)	150	98	93	102
MS + IL-4	86	91	182	138

Data are from two separate experiments and represent counts obtained from bands located by autoradiography and excised from immunoblots of cell lysates. Results are presented relative to expression in Opti-MEM alone (control), which was taken as 100%.

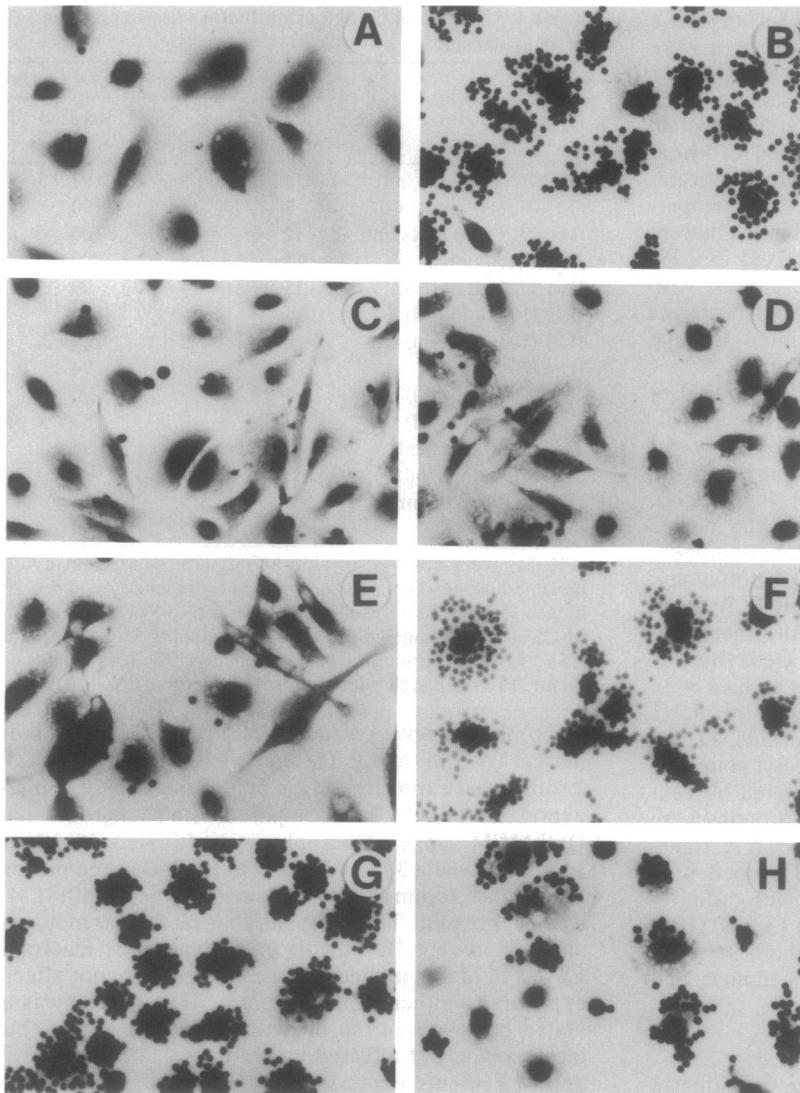


FIG. 1. Induction of sialoadhesin expression is prevented by IL-4. RPMs were cultured with Opti-MEM alone (A) or with Opti-MEM plus 2% (vol/vol) mouse serum (B), IL-4 (20 ng/ml) (C), 2% mouse serum plus IL-4 (D), affinity-purified 11B11 (E), 2% mouse serum plus IL-4 plus 11B11 (F), 2% mouse serum plus IL-4 plus M1 antibody (G), or 2% mouse serum plus IL-4 plus M2 antibody (H). The ability to form rosettes with sheep erythrocytes, induced by mouse serum (B), was prevented by IL-4 (D), and this effect was reversed by either anti-IL-4 (11B11) (F) or anti-IL-4 receptor (M1) (G) antibody. Similar results were obtained with TPMs.

Thus IL-4 was binding to its receptor on macrophages and modulated expression of sialoadhesin principally through the epitope recognized by the M1 antibody.

**IL-4 Prevents Sialoadhesin Expression by Blocking Induction of Protein.** To confirm the selective effect of IL-4 on sialoadhesin expression and to determine whether inhibition operated at the level of total cellular protein or surface function, RPMs were cultivated with mouse serum, IL-4, or both. Detergent lysates were analyzed for their content of F4/80 and sialoadhesin (3D6) antigen. Fig. 2 shows that cultivation of RPMs in 2% mouse serum resulted in an induction of sialoadhesin total antigen that was prevented by IL-4. This effect was again neutralized by antibody 11B11. In contrast, the upregulation of F4/80 by mouse serum was unaffected by IL-4. Interestingly, the combination of IL-4, 11B11, and mouse serum appeared to result in a greater expression of F4/80 than mouse serum and IL-4, suggesting that perhaps F4/80 levels are regulated by the presence of immune complexes. This observation has been confirmed by dot blotting (data not shown).

**Dose-Response and Kinetics of IL-4 Inhibition of Sialoadhesin Induction.** The effect of IL-4 is apparent at 2 ng/ml and fully expressed at 20 ng/ml (Fig. 3A). Sialoadhesin induction by 2% mouse serum can be detected by enhanced erythrocyte binding after 12 hr in culture (Fig. 3B). When IL-4 is present at the start of culture this induction is prevented, showing that IL-4 is able to suppress the induction signal at its inception and is therefore the overriding response. Incuba-

tion with as little as 0.5% mouse serum in Opti-MEM results in  $\approx 10\%$  of the RPMs expressing sheep erythrocyte rosetting after 48 hr. The percentage of positive cells increases with the amount of serum until 100% induction is achieved with 2% serum (Fig. 4B). At this level of mouse serum, IL-4 at 20 ng/ml will block induction (compare Fig. 3A); however,

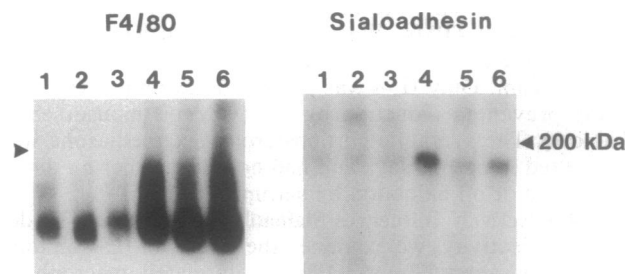


FIG. 2. IL-4 prevents synthesis of induced sialoadhesin. RPMs were cultivated in Opti-MEM alone (lanes 1) or with IL-4 (20 ng/ml) (lanes 2), IL-4 plus 11B11 (lanes 3), 2% mouse serum (lanes 4), 2% mouse serum plus IL-4 (lanes 5), or 2% mouse serum plus IL-4 plus 11B11 (lanes 6). Cell lysates were analyzed by SDS/PAGE and Western blotted with either F4/80 or anti-sialoadhesin (3D6) and  $^{125}\text{I}$ -labeled rabbit anti-rat IgG. Both F4/80 and sialoadhesin antigens were induced by mouse serum (lanes 4) but only in the case of sialoadhesin was IL-4 able to block induction (lanes 5). The broad band of F4/80 at high levels of antigen is characteristic.

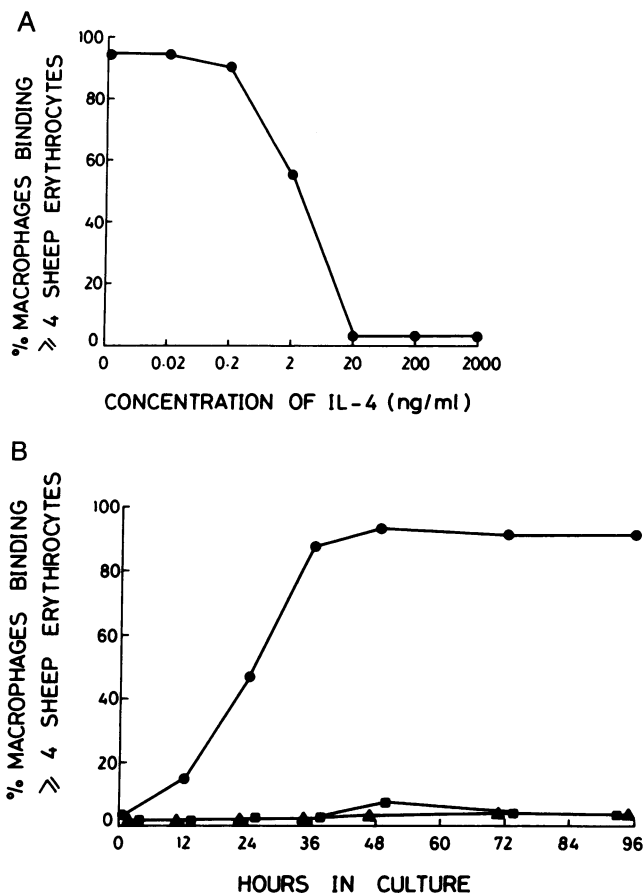


FIG. 3. Dose-response and kinetics of IL-4 inhibition of sialoadhesin induction. (A) RPMs were cultivated for 48 hr in Opti-MEM plus 2% mouse serum and various concentrations of IL-4. (B) Macrophages were cultured in Opti-MEM alone (■) or with 2% mouse serum (●) or 2% mouse serum and IL-4 (20 ng/ml) (▲). Cells were assayed for rosette formation at the times shown. IL-4 inhibition of sialoadhesin induction was complete at 20 ng/ml and observable within 12 hr of incubation. IL-4 had no effect on the rosetting assay itself.

increasing the concentration of serum to 30% will overcome the blocking effect of IL-4 (Fig. 4A).

**IL-4 Actively Downregulates Expressed Sialoadhesin.** For macrophages to continue expressing sialoadhesin, the inducing signal provided by mouse serum must be present continuously. If the serum is removed from the culture, levels of expression begin to fall, and there is no expression within a few days. To investigate whether IL-4 could modulate expression of preexisting sialoadhesin, RPMs that had been cultured with mouse serum for 3 days were exposed to IL-4

for a further 3 days. The results (Fig. 5) show that in the absence of serum there is no induction of sialoadhesin and that when serum is withdrawn after 3 days the level of sialoadhesin falls to  $\approx 25\%$  expression within a further 3 days. However, if IL-4 is added when the serum is removed, the levels fall to 5% expression within the same period. If after 3 days incubation the serum is replaced with fresh serum and IL-4, then levels of expression fall to 15% by 6 days compared with  $\approx 100\%$  in fresh serum without IL-4. Again, these effects of IL-4 are neutralized with 11B11. We conclude that IL-4 is able to switch off sialoadhesin activity even in the face of a preexisting and continuing induction signal.

**DISCUSSION**

We have shown that IL-4 at concentrations as low as 20 ng/ml will selectively prevent induction of sialoadhesin in the presence of a strong inductive signal. This effect was restricted to IL-4 and could be prevented by an IL-4-neutralizing antibody (11B11) and by a mAb (M1) against a specific epitope of the murine IL-4 receptor, suggesting that the M1 epitope is critical for this activity of IL-4 (8). By immobilizing IL-4 to the bottom of the culture vessel, we have established that IL-4 could act by interacting with its receptor and that internalization is not necessary (data not shown). To investigate the possibility that IL-4 was influencing the general expression of macrophage surface receptors, we examined the effect of IL-4 on several surface markers unrelated to sialoadhesin. IL-4 produced an expected 50% increase in surface expression of Ia but had no effect on expression of Mac-2. In the presence of mouse serum the levels of Ia were not increased by IL-4, which suggests that factors present in mouse serum may be able to influence the activation of macrophages by this cytokine. Since the levels of the macrophage marker F4/80 are also upregulated by serum, we looked for possible effects of IL-4 on F4/80 induction and found that in this case the induction of expression was refractory to IL-4. These results point to a selective effect of IL-4 in downmodulating induction and expression of sialoadhesin.

We assessed sialoadhesin expression with an erythrocyte binding assay in order to measure the functional capacity of macrophages to attach to other cells via sialoadhesin. It was hoped that this assay system would mimic the *in vivo* phenomenon of cluster formation seen in the bone marrow and spleen. Thus, a lack of functional sialoadhesin may have reflected production of a nonfunctional form of the receptor, a block in protein synthesis and surface expression, or enhanced degradation. Western blotting demonstrated that IL-4 was preventing the increase in total cellular sialoadhesin protein induced by serum. At 20 ng/ml, IL-4 was able to block the inductive effects of 2% mouse serum totally; however, when the serum was increased to 30% this effect could be overcome. Two explanations are possible: (i) the

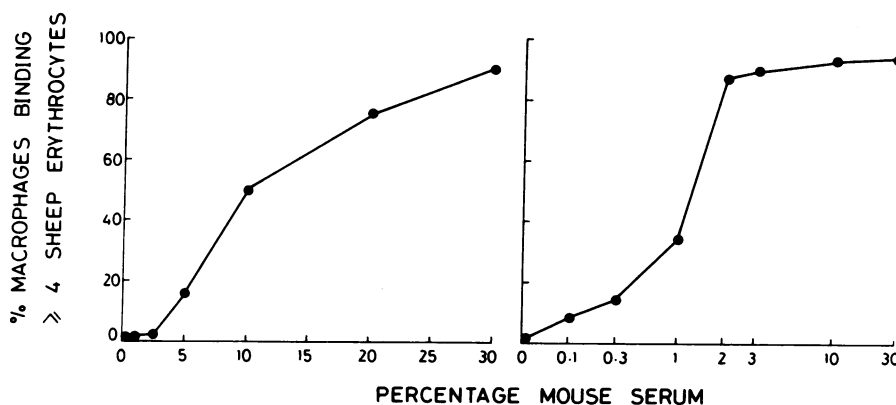


FIG. 4. Effect of increasing percentage of mouse serum on IL-4 inhibition of induced sialoadhesin. RPMs were cultivated in the different concentration of mouse serum for 48 hr in Opti-MEM with (Left) or without (Right) IL-4 (20 ng/ml) before assay. Maximum induction of sialoadhesin was obtained with 2% serum in the absence of IL-4, and the inhibition of induction due to IL-4 could be overcome by increasing concentrations of serum.

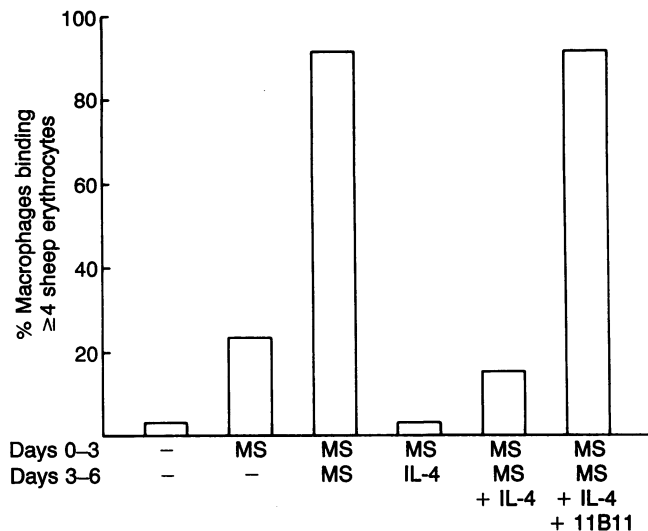


FIG. 5. Effect of IL-4 on preinduced sialoadhesin. RPMs were cultivated for 72 hr in Opti-MEM alone (-) or with 1% mouse serum (MS). After 72 hr the medium was replaced by either Opti-MEM or Opti-MEM with fresh serum, IL-4, or a combination of these and 11B11. Sheep erythrocyte rosette formation was then determined. IL-4 reduced the expression of sialoadhesin even in the presence of a continuing induction signal. This effect was neutralized by 11B11.

increased amount of serum may provide a more powerful induction signal, so that any effect of IL-4 is minimized; (ii) soluble, high-affinity IL-4-binding protein, present in mouse serum (10, 11), can bind the IL-4 and render it inactive.

The role of the stromal resident macrophages in hemopoiesis is unclear, but it is well known that they are able to form close associations with immature myelomonocytic cells (12-14) and with erythroblastic precursors (15-19). This association can be demonstrated both *in vivo*, following staining of bone marrow with mAbs such as F4/80, and in long-term *in vitro* bone marrow cultures (20, 21). Although these associations are well documented, there is little direct evidence defining the role of macrophages in the developmental regulation of the clustered cells.

Distinct hemagglutinins appear to be important in the attachment of myeloid and erythroid precursors to bone marrow macrophages via sialoadhesin and a divalent cation-dependent adhesion receptor (22). Our present results show that IL-4 is highly selective in its downmodulation of sialoadhesin and other, unpublished observations show that IL-4 does not influence divalent cation-dependent erythroblast-binding activity. While IL-4 is assuming the position of a dominant immunoregulatory molecule (23), its role in influencing normal hemopoiesis is unclear. IL-4 can act as a stimulant of mast cell growth, in that it will enhance IL-3-mediated effects *in vitro* (24), and can also inhibit the capacity of bone marrow stromal layers to support the formation of granulocyte/macrophage colonies (25). In these studies the nature of the stromal element involved was not confirmed, although the macrophages appeared to be implicated. IL-4 can also reduce macrophage colony formation after addition to bone marrow progenitors stimulated with either GM-CSF or M-CSF (26). It is therefore possible that IL-4 may influence the development or growth of these cells by downregulating the expression of sialoadhesin receptors on stromal macrophages.

IL-4 has other effects on macrophage function and/or receptor expression. For instance, treatment of murine macrophages with IL-4 has been found to enhance antigen-presenting ability (27), to increase expression of Ia antigen

(28, 29), to enhance tumoricidal (28) and microbicidal activities (30), and to prime cells for a respiratory burst (31). In contrast, in human monocytes, IL-4 can inhibit H<sub>2</sub>O<sub>2</sub> production and anti-leishmanial capacity due to interferon  $\gamma$  (32) and can suppress tumor necrosis factor  $\alpha$ , IL-1, and prostaglandin E<sub>2</sub> production (33). IL-4 may be suppressive or stimulatory to the macrophages, possibly depending on the degree of differentiation, the local tissue microenvironment, and the influence of other cytokines. The present data indicate that IL-4 may influence the nature of the macrophage response to inflammation and the trophic functions of resident stromal macrophages by selectively regulating specific surface receptors involved in interactions with other cells.

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