Erythropoietin Causes Down Regulation of Colony-stimulating Factor (CSF-1) Receptors on Peritoneal Exudate Macrophages of the Mouse

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ABSTRACT We have shown that erythropoietin (epo), the primary regulator of erythrocyte formation, diminished the binding to peritoneal exudate macrophages (PEM) of the principal macrophage growth regulator, colony-stimulating factor (CSF-1). The effect of epo on ^{125}I-CSF-1 binding was dose-dependent; at a concentration of 1–2 U of epo/ml (10^{-10} M), CSF-1 binding was almost completely suppressed. Erythropoietin did not compete with CSF-1 for occupancy of the latter's receptors. The effect of epo on CSF-1 binding occurred at 37°C but not at 2°C, and during the continuous exposure of PEM to epo at 37°C we found that CSF-1 binding reached a nadir at 1 h and recovered to pre-exposure levels in 7 h.

Our novel results are consistent with the notion that specific receptors for epo exist on the cell surface of PEM and that binding of epo sets in motion a series of cellular events resulting in the internalization of CSF-1 receptors. Thus epo causes down regulation of CSF-1 receptors on PEM.

We have previously shown that epo causes suppression of CSF-induced granulocyte-macrophage colony formation by mouse bone marrow cells. The results we present here provide a possible mechanism for these results.

Erythropoietin (epo)[1] and colony-stimulating factor (CSF) are regulators of hemopoietic cell proliferation and differentiation. Epo is the primary, if not sole, effector of erythrocyte formation in vivo and in vitro, and CSF-1, at least in vitro, causes macrophage proliferation and differentiation (1–4).

One of us (G. Van Zant) has found that the two regulators compete for expression of their respective differentiation activities when added to bone marrow cells in vitro (5, 6). We therefore have determined whether or not epo affects the initial step in macrophage regulation by CSF-1, namely, binding of CSF-1 to specific cell surface receptors that are restricted to mononuclear phagocytes and their precursors and to established macrophage cell lines only (7, 8). It is believed that CSF-1 receptors mediate both proliferation and other biological functions of target cells (4, 7).

Other growth regulators have been shown to modulate the number of receptors with specificity for another effector binding to the same cell. For example, platelet-derived growth factor (PDGF) decreases the number of specific receptors for epidermal growth factor (EGF) on mouse 3T3 cells (9, 10) and EGF has been shown to reduce the number of receptors for human choriogonadotropin on Leydig tumor cells (11).

We report here that epo causes a reduction in the number of CSF-1 receptors on macrophages in a pattern dependent on dose, temperature, and time.

MATERIALS AND METHODS

Mice: We used male C3H/HeJ mice 8–12 wk old from our specific pathogen-free animal facilities for these studies.

Macrophage Harvest: We harvested peritoneal exudate cells (PEM) as described by Chen et al. (12) by peritoneal lavage with ice-cold alpha medium 3 d after a single intraperitoneal injection of 1.5 ml of thioglycollate medium (Difco Laboratories, Detroit, MI). Cells were washed once with alpha medium containing 10% fetal calf serum (alpha-10) (Hyclone, Logan, UT) by centrifugation (500 *g*, 10 min) in the cold. The lot of serum selected for these studies contained <1 ng of endotoxin/ml. Adherent macrophages were selected by plating exudate cells in 1 ml of alpha-10 in 35-mm polystyrene tissue culture dishes for 15 min at 37°C. We removed nonadherent cells by washing once with PBS at pH 7.2. Adherent cells consisting of more than 95% mononuclear phagocytes were cultured in alpha-10 at 37°C for 20 h in a humidified atmosphere of 10% CO_2 in air before they were used for study.

J774 Cells: This macrophage cell line was originally isolated by Ralph

[1] *Abbreviations used in this paper:* CSF-1, colony-stimulating factor-1; EGF, epidermal growth factor; epo, erythropoietin; LPS, lipopolysaccharide; PDGF, platelet-derived growth factor; PEM, peritoneal exudate macrophages.

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(13) and was obtained by us from the Cancer Cell and Virus Center at this university.

Determination of ^{125}I-CSF-1 Binding: We washed macrophages twice with cold PBS, and the cells were subsequently bathed in 0.9 ml of cold alpha-10 buffered to pH 7.2 with 20 mM morpholinopropane sulfonic acid (Sigma Chemical Co., St. Louis, MO) instead of the usual bicarbonate. Labeled CSF-1 was added in a volume of 100 μl, and the cells were incubated at 2°C for 16 h. We removed unbound ^{125}I-CSF-1 by washing the cells three times with cold PBS. We then lysed the cells with 0.75 ml of 50 mM Tris-HCl, 0.5% SDS at pH 7.4 (7, 8) and removed the lysate from the dish for counting in an automatic gamma counter. Nonspecific binding, determined as the amount of ^{125}I-CSF-1 bound in the presence of a 200-fold excess of unlabeled CSF-1, was always <2% of the total binding, and we subtracted this value from the total counts to obtain specific binding.

Purification and Iodination of CSF-1: Murine L-cell CSF-1 was purified according to the methods described previously (3, 8). To determine the purity, we used SDS PAGE at 9 and 12.5% acrylamide concentration. Comigration of biological activity and labeled protein was used as the index of purity (3, 8). Biological activity of CSF-1 was determined by the standardization method described by van den Engh (14) and the material we used had a specific activity of 5×10^7 U/mg of protein. Purified CSF-1 was iodinated by the method described by Stanley and Guilbert (15) with slight modification. We prepared high specific activity ^{125}I-CSF-1 (150–300 μCi/μg protein) by iodinating 1 μg of CSF-1 with 2 mCi of carrier-free NaI^{125} (Industrial Nuclear Co., St. Louis, MO). ^{125}I-CSF-1 prepared in this manner retained its biological activity and also reacted with either rabbit or rat anti–CSF-1 antiserum (15, 16). We used ^{125}I-CSF-1 within 4 wks of iodination since its biological activity decreases with time ($t_{1/2}$ = ~6 wks).

Erythropoietin (epo): We used partially purified human urinary epo (CAT-1) for these studies. It was obtained from the Division of Blood Diseases and Resources of The National Heart, Lung, and Blood Institute and had a specific activity of 1,140 U/mg of protein; pure human urinary epo has a specific activity of 70,400 U/mg of protein (17). The CAT-1 epo was prepared by Dr. Eugene Goldwasser of the University of Chicago and was essentially freed of any contaminating endotoxin by an ultracentrifugation step. Less than 20 pg of endotoxin per unit of epo was measured by *Limulus* lysate test of this preparation. In addition this preparation is virtually free of CSF activity, including CSF-1, and is free of burst-promoting activity.

Epo Bioassay: Epo in the medium over macrophages was assayed by measuring induced ^{59}Fe incorporation into hematin synthesized by mouse marrow cells, as we have previously described (18). Bone marrow cells (5×10^5/ml) were cultured for 48 h in the wells of Costar tissue culture trays (model 3524, Costar, Cambridge, MA); six replicate cultures were run for each experimental point. The medium consisted of α-minimal essential medium containing 0.8% methyl cellulose (4,000 cps, Dow Chemical Co., Midland, MI), 20% fetal calf serum, 1% BSA (Sigma Chemical Co.) and 10^{-4} M β-mercaptoethanol. At 24 h we added radioiron-labeled rat transferrin (0.2 μCi in 20 μl) to each well and 24 h later we harvested cells and extracted hematin into cyclohexanone. Results are expressed as mean ^{59}Fe cpm in hematin ± 1 SD.

RESULTS

Table I shows that preincubation of PEM with epo caused a dose-dependent inhibition of subsequent CSF-1 binding. In these two representative experiments, a 1-h exposure of cells at 37°C to 0.12 U of epo caused a 25% inhibition of subsequent CSF-1 binding. Higher epo concentrations caused progressive suppression and, at a concentration of 2 U of epo/ml, suppression was virtually complete. In three other experiments of this type we found that inhibition was always detectable at a concentration of 0.12 U/ml of epo and that 1–2 U/ml completely prevented CSF-1 binding. It appeared that a concentration of ~0.1 U of epo/ml was a threshold level since lower concentrations did not affect CSF-1 binding and higher concentrations reproducibly diminished binding.

We used partially purified human urinary epo for these experiments; in other experiments not reported here we found a qualitatively similar effect using a crude epo preparation from an entirely different source in another species (anemic sheep plasma). The sheep epo (4 U/mg of protein; Connaught, Swiftwater, PA) was about one-fourth as effective per unit as the human epo in causing inhibition of CSF-1 binding but was identical in potency to the human epo in stimulating hemoglobin synthesis in mouse marrow cells.

Fig. 1 shows the results of an experiment in which we added graded concentrations of either epo or unlabeled CSF-1 to PEM at the same time as we added ^{125}I-CSF-1. The PEM were kept at 2°C during the incubation and after 16 h we determined the ^{125}I-CSF-1 bound. In contrast with the dramatic inhibition caused by unlabeled CSF-1, epo did not have a significant effect on the binding of CSF-1; only the 2 U/ml epo concentration caused any inhibition in CSF-1 binding, and that amounted to only 11%. These results show that epo caused a decline in the number of available CSF-1 receptors

TABLE I

Effect of Epo Concentration on Subsequent Binding of CSF-1 to Macrophages

	Experiment 1		Experiment 2	
Epo	^{125}I-CSF-1 bound	% of control	^{125}I-CSF-1 bound	% of control
U/ml	*cpm*		*cpm*	
0	42,290 ± 1,010	—	6,830	—
0.06	—	—	6,370	93
0.12	32,570 ± 3,030	77	4,890	72
0.25	9,940 ± 2,570	24	1,230	18
0.5	2,660 ± 65	6	410	6
1.0	760 ± 100	2	250	4
2.0	190 ± 2	0.4	—	—

Adherent PEM (1×10^6/ml in Experiment 1; 4×10^5/ml in Experiment 2) were incubated with the appropriate concentration of epo for 1 h at 37°C. The cells were subsequently washed twice with fresh medium and ^{125}I-CSF-1 was added (100,000 cpm in Experiment 1; 50,000 cpm in Experiment 2). The cells were then incubated for 16 h at 2°C and ^{125}I-CSF-1 (cpm) binding was determined. Cellular binding of CSF-1 in duplicate 35-mm dishes is reported in Experiment 1, and single dishes were used for each point in Experiment 2.

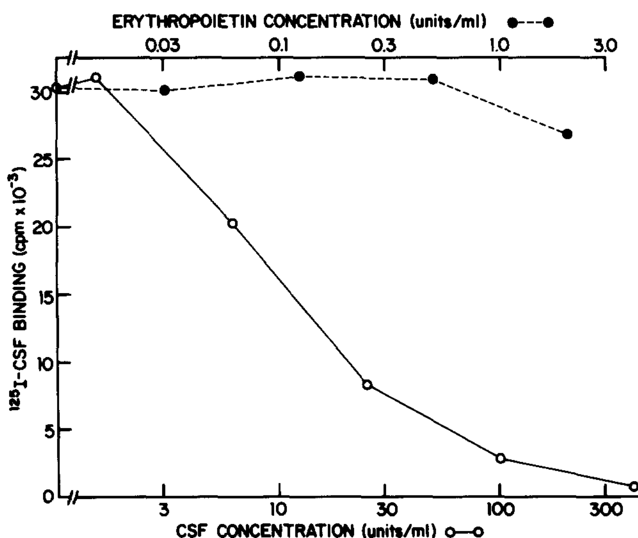

FIGURE 1 Effects on ^{125}I-CSF-1 binding of simultaneously incubating macrophages at 2°C with either epo (closed circles) or CSF-1 (open circles). Adherent exudate macrophages, prepared as described in Materials and Methods, were plated in duplicate 35-mm polystyrene dishes (1×10^6 cells/dish) and exposed to several concentrations of epo or CSF-1 at 2°C along with ^{125}I-CSF-1 (100,000 cpm). Binding was determined after overnight incubation (16 h); the data are reported as the means of specific cellular binding (cpm) in the duplicate dishes and they have been corrected for nonspecific binding.

(in Table I) through cellular mechanisms operable at 37°C, but not at 2°C, and, moreover, that epo does not compete for CSF-1 receptors.

Note that concentrations of epo and CSF-1 of 1 U/ml are equivalent to molar concentrations of 4×10^{-10} M and 3×10^{-13} M, respectively. The scales of the abscissas in Fig. 1 (and in Fig. 2, *a* and *b*) correspond to essentially equimolar concentrations of the two growth regulators, and the two curves can therefore be directly compared on a molar basis since one epo unit equals 1,000 CSF-1 units.

Cells of the macrophage line J774 have previously been shown to possess CSF-1 receptors (7, 19, and Chen and Hsu, unpublished observation), and, as is true with PEM, prior or simultaneous exposure of these cells to unlabeled CSF-1 diminishes binding of the labeled derivative. We therefore asked whether or not epo had similar effects on J774 cells and PEM. We found that whereas CSF-1 inhibited its subsequent binding to both cell types, epo suppressed CSF-1 binding to only the PEM (Fig. 2). This result demonstrates that epo inhibits CSF-1 binding by a cell-specific mechanism(s) rather than by having nonspecific, perhaps toxic, effects on macrophages. It is readily apparent in Fig. 2 that the dose-dependent inhibition caused by epo and CSF-1 follows different patterns. In the region of 50% inhibition on the two curves, the rate of decline with increasing epo concentrations is steeper than with increasing CSF-1 concentrations and, as we mentioned before, inhibition occurred only at epo levels above a threshold concentration. Inhibition caused by CSF-1 appeared to be strictly dose-dependent and did not have a threshold since the curve extrapolated through the 100% value.

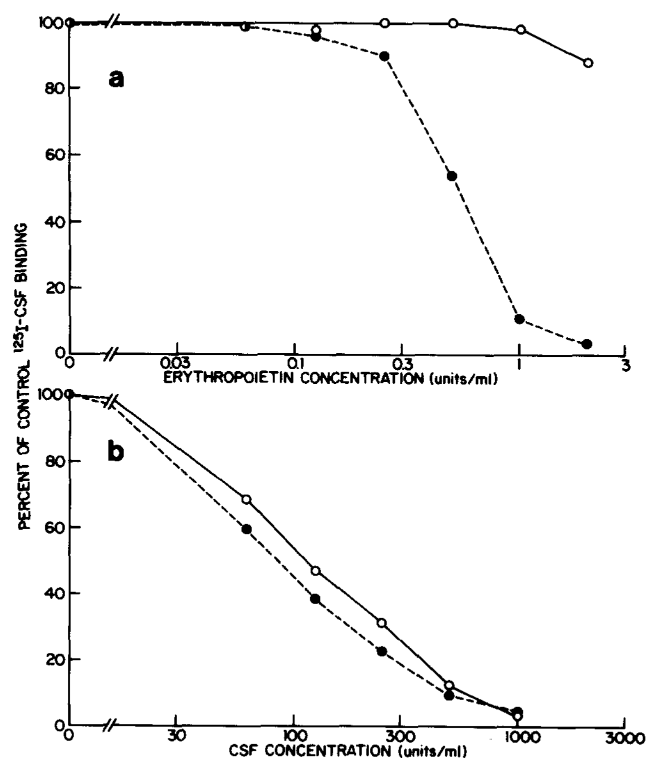

FIGURE 2 The effects of epo and CSF-1 on ^{125}I-CSF-1 binding to J774 cells (open circles) and to PEM (closed circles). ^{125}I-CSF-1 binding to macrophages (1×10^6/ml) or J774 cells (5×10^5/ml) was measured as described in Materials and Methods after the cells were first exposed for 1 h at 37°C to either epo (*a*) or CSF-1 (*b*). The results are expressed as means of duplicate dishes at each point. 50,000 cpm of ^{125}I-CSF-1 was added to each dish, and the control binding to macrophages and J774 cells was 24,700 and 27,908 cpm, respectively, in *a*; in *b*, it was 30,750 and 26,580 cpm, respectively. Nonspecific binding to dishes containing 200-fold excess of unlabeled CSF-1 averaged 2% in these experiments and was subtracted from the experimental results to give specific cellular binding.

We next studied ^{125}I-CSF-1 binding to macrophages after first exposing them (at 37°C) to 1 U/ml of epo for times ranging from 30 min to 7 h. These results in Table II show, first, a rapid eclipse in CSF-1 binding that reached a nadir of ~1% of control at 1 h and, secondly, a recovery in binding commencing at 2 h that reached 87% of control levels by 7 h.

It should be stressed that the recovery of CSF-1 binding, presumably through the reappearance of CSF-1 receptors, occurred while epo was still present in the incubation medium. To verify that epo was not degraded during incubation, we assayed the incubation medium after various periods and, as shown in Table III, we found no loss of epo after as much

TABLE II

Time-Course of Inhibition of CSF-1 Binding Caused by Epo

Incubation period	Epo	^{125}I-CSF-1 bound	Effect of epo as % of control binding
h	*1 U/ml*	*cpm*	
0.5	−	46,470	—
	+	5,230	11
1	−	43,310	—
	+	600	1
2	−	44,830	—
	+	14,850	33
3	−	47,590	—
	+	31,120	65
5	−	44,640	—
	+	30,380	68
7	−	40,990	—
	+	35,810	87

Two sets of 35-mm dishes containing 1×10^6 PEM were set up: one set with 1 U/ml of human urinary epo and the other set without epo. The plates were cultured at 37°C and at selected times the media were collected for epo assay (Table III), the cells were washed twice, and ^{125}I-CSF-1 binding was determined as described in Materials and Methods. 100,000 cpm of ^{125}I-CSF-1 was added to each dish.

TABLE III

Epo Bioassay of Macrophage Culture Medium

Group	Addition	^{59}Fe in hematin	Δ
		cpm	
1	None	12 ± 2	—
2	20 mU/ml of epo	183 ± 22	171
3	7 h macrophage medium, without epo	14 ± 5	2
4	1 h macrophage medium, with epo	161 ± 18	149*
5	7 h macrophage medium, with epo	194 ± 14	182*

PEM culture medium either with or without 1 U/ml of added epo was assayed after 1 or 7 h of incubation at 37°C. A volume of 20 µl of the medium was added per milliliter to the suspension of bone marrow cells in the assay. If there was no loss of epo during incubation, 20 µl should have contained 20 mU of epo, and we therefore compared the culture medium with a 20 mU/ml concentration of epo added to the cells in the assay. Epo-induced ^{59}Fe incorporation into hematin extracted from the marrow cells was determined as described in Materials and Methods.

* Not significantly different ($P > 0.05$) than the response to 20 mU/ml of epo.

as 7 h of incubation with the macrophages, a time at which CSF-1 binding had recovered to near control levels (Table II).

DISCUSSION

This is the first direct demonstration that one hemopoietic growth regulator can affect the interaction of a second regulator with its target cells. Our results clearly show that epo inhibits CSF-1 binding to mouse peritoneal exudate macrophages. Inhibition was dose-dependent above a concentration of 0.1 U/ml of epo, and, at a concentration of 1–2 U/ml, inhibition was complete. Human urinary epo has a molecular weight of ~34,000 and purified material has a specific activity of 70,400 U/mg of protein (17). We therefore found that epo suppressed CSF-1 binding at 10^{-11}–10^{-10} M, i.e., in the concentration range of physiologic regulators.

Secondly, we found that inhibition was temperature-dependent; incubation of macrophages with epo at 37°C, but not at 2°C, caused a diminution in subsequent CSF-1 binding. We (Fig. 1) as well as others (7) have shown that simultaneous addition of epo and ^{125}I-CSF-1 to PEM in the cold does not affect binding of the latter. These data taken together show that epo does not inhibit CSF-1 binding by competing for the latter's receptors. In contrast, our data are consistent with the notion that epo caused down regulation of CSF-1 receptors by binding to macrophages, perhaps via specific receptors for epo, and subsequently causing a disappearance of CSF-1 receptors from the cell surface. Cells of the macrophage line J774, like PEM, possess CSF-1 receptors but, in contrast with what we found with PEM, epo did not cause down regulation of CSF-1 receptors on J774 cells. On the basis of our working hypothesis we would conclude that J774 cells lack epo receptors. Therefore, in the absence of specific epo binding, CSF-1 receptor down regulation does not occur on J774 cells as a consequence of epo exposure. In support of this view that J774 cells lack epo receptors, we find no change in J774 cell proliferation in the presence of epo (unpublished observations). Since at present there is no epo tracer, either radiolabeled or fluorescent, that retains biological activity, it is not possible to identify epo receptors on macrophages or, for that matter, on erythroid cells. Until a biologically active epo derivative is available, the identity of cells with receptors will remain speculative. From a teleological viewpoint, it is difficult to understand the occurrence of putative epo receptors on PEM since epo plays no known role in macrophage growth and function.

An alternative explanation for our results, namely that epo caused a change in CSF-1 receptor affinity for its ligand, cannot be ruled out in the present study. However, 12-O-tetradecanoyl-phorbol-13-acetate, a tumor-promoting phorbol ester, and bacterial lipopolysaccharide (LPS), the only other known inhibitors of CSF-1 binding to PEM, cause a decline in the number of CSF-1 receptors on the PEM cell surface rather than altering CSF-1 receptor binding affinity (12, 27).

Internalization of ligand-receptor complexes is a frequently reported consequence of the binding of polypeptide hormones to cells (20–23), and recently this response has been characterized by two helpful descriptive terms (11). The term homologous down regulation has been used to describe the loss of receptors, caused by a prior exposure of cells to the specific ligand of the receptor in question. CSF-1 down regulates its receptors in this way (24) as do other hormones and growth factors (9–11, 25, 26). The term heterologous down regulation has been used to describe the disappearance from the cell surface of specific receptors for one ligand as a consequence of the binding of another ligand to its unique receptors on the same cell. We believe that the results reported in this paper are an example of heterologous down regulation.

Other examples of heterologous down regulation include (*a*) the loss of EGF receptors on mouse 3T3 cells or human fibroblasts caused by PDGF (9, 10) and (*b*) the loss of receptors for human choriogonadotropin on mouse Leydig tumor cells caused by mouse EGF (11). Bowen-Pope et al. (10) found that the PDGF-induced loss of EGF receptors on 3T3 cells occurred at either 4° or 37°C. This is in contrast with our results showing that epo caused a loss in CSF-1 receptors only at 37°C and suggests that the mechanisms involved may be different.

Our results in Table II suggest that after the initial eclipse of CSF-1 receptors at 1 h, receptors reappeared in a time-dependent manner even in the continuous presence of epo. It is significant that the rate of reappearance of CSF-1 receptors after their initial exposure to epo is approximately the same as the rate of their reappearance after they were removed by trypsinization or the rate of appearance of unoccupied receptors after they were first saturated with unlabeled CSF-1 (24). In each of the above cases, full ^{125}I-CSF-1 binding was attained in 6–8 h. These results are similar to those of Wrann et al. (9) in which they found that EGF receptors reappeared on 3T3 cells after their loss was triggered by incubation with PDGF. These workers found that EGF receptors reappeared despite the continued presence of PDGF, and the rate of reappearance was similar to what we found for CSF-1 receptors.

One of us (B. D.-M. Chen) has previously reported that tumor-promoting phorbol esters (27) and bacterial LPS (12) cause down regulation of CSF-1 receptors on PEM. Several points rule out the possibility that LPS contamination of our epo preparations accounts for the effects on CSF-1 binding that we see. First, we used C3H/HeJ mice, a strain resistant to the effects of LPS. Chen et al. (12) found that under conditions identical to those used in the present studies, as much as 100 ng/ml of LPS caused no diminution of CSF-1 binding whereas in the LPS-susceptible strain C3H/Anf, this concentration of LPS reduced binding to macrophages >95%. Secondly, the human urinary epo preparation used in most of our experiments, distributed by The National Institutes of Health, contains <20 pg of LPS per epo unit according to the *Limulus* lysate test. Lastly, we have found that polymyxin B does not affect the inhibition of CSF-1 binding caused by our epo preparation (data not shown). Chen et al. (12) have shown that polymyxin B completely abrogates the effect of LPS on CSF-1 binding to macrophages of C3H/Anf mice.

We have tested a number of other growth regulators for their ability to modulate CSF-1 binding to macrophages (Chen, unpublished data). Pure PDGF (a gift of Jung San Huang and Thomas Deuel, Jewish Hospital and Washington University School of Medicine) had no effect at concentrations up to 300 ng/ml (10^{-8} M). Pure porcine insulin had no effect at concentrations as high as 600 ng/ml. Lastly, a purified preparation of mouse submaxillary gland EGF had no effect at the highest concentration tested (600 ng/ml).

Van Zant and Goldwasser (5, 6) have shown that epo causes suppression of CSF-induced granulocyte and macrophage colony formation by mouse bone marrow cells in vitro; conversely, CSF causes suppression of epo-induced erythroid burst formation by marrow cells. The mechanisms by which

these competitive influences are manifested on target cells is not clear; however, the results we report here provide a possible mechanism for our previous results.

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