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Interactions Between Extracellular Matrix Components and Proteoglycans Released from Monocytes *In Vitro*

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^{35}S-labelled chondroitin sulfate proteoglycans isolated from conditioned media of cultured human monocytes (day 1 *in vitro*) and monocyte-derived macrophages (day 6 *in vitro*) were chromatographed on columns of immobilized fibronectin and collagen, respectively. The elution profiles prior to and after alkali treatment were compared with those of standards chondroitin 4-sulfate and chondroitin sulfate E and heparin. The day 6 ^{35}S-proteoglycans have a higher sulfate density than the day 1 species, but this difference did not affect the elution profiles after chromatography on collagen-Sepharose, whereas the day 6 proteoglycans bound more firmly than the day 1 fraction to fibronectin-Sepharose. The elution patterns obtained for these distinct proteoglycans closely resembled those of heparin and oversulfated chondroitin sulfate E standards, and clearly demonstrated the importance of sulfate density both for the affinity to fibronectin and collagen. Neither day 1 nor day 6 ^{35}S-proteoglycans were found to interact with hyaluronate.

Previous reports have established that monocytes differentiating *in vitro* into monocyte-derived macrophages (MDM) start to synthesize oversulfated chondroitin sulfate proteoglycan [1, 2]. Both in the monocyte and MDM cultures the chondroitin sulfate proteoglycan (CSPG) is synthesized and released into the medium [1]. The proteoglycans are not deposited into any matrix under *in vitro* conditions, but can be recovered from the conditioned media in both cell systems [3]. Cells belonging to the monocyte-macrophage lineage participate in antigen-presentation, phagocytosis of bacteria, virus and cellular debris, and congregate in inflammatory reactions such as exudates, granulomas and fatty streaks [4]. It has not been established whether endogenous synthesis and deposition of extracellular matrix is a major feature of such highly migratory and multifunctional cells *in vivo*. The question of biological basis for

Abbreviations used: CSPG - chondroitin sulfate proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulfate; CS-E, chondroitin 4,6 disulfate; MDM, monocyte-derived macrophages.

the synthesis and release of CSPG observed in cultured human monocytes therefore remains unclear, as does also the functional implications of the increased sulfation of CSPG during differentiation of moncytes into MDM. Another prominent component of extracellular matrix, fibronectin, has been shown to be synthesized by monocytes after differentiation into MDM and to be recovered from conditioned media as well [5]. Consequently, MDM synthesize and release two typical components of the extracellular matrix without incorporating them into a subcellular matrix *in vitro*. However, the cultivation of monocytes on coats of purified extracellular matrix proteins has been shown to modulate various cellular phenotypes. Monocytes cultured on fibronectin did not develop into typical MDM, nor did they synthesize oversulfated CSPG [2]. Furthermore, monocytes cultured on collagen were shown to develop into typical histiocytes and not MDM, and furthermore were demonstrated to have a lower capacity for tumor cell killing than MDM controls [6].

The concomitant expression of oversulfated CSPG [3] and fibronectin [5] in MDM and the demonstration of affinity of oversulfated CSPG for immobilized fibronectin [3] may suggest that such interactions have some biological relevance in the monocyte-MDM system. The interactions of monocyte and MDM CSPGs with fibronectin, collagen and hyaluronate was therefore investigated, and compared with the interactions of the glycosaminoglycan standards heparin, chondroitin sulfate and chondroitin sulfate E.

Materials and Methods

Materials

Blue dextran, DEAE-Sephacel, Sepharose 4B and Sepharose Cl-6B were bought from Pharmacia Fine Chemicals, Uppsala, Sweden. ^{35}S-sulfate (carrier-free) and ^{3}H-acetic anhydride were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. ^{3}H-labelled chondroitin 4-sulfate and ^{3}H-heparin were kind gifts from Dr. U. Lindahl, Swedish University of Agricultural Sciences, Uppsala, Sweden. Fibronectin isolated from human plasma and immobilized on cyanogen bromide-activated Sepharose 4B [7] was given by Dr. S. Johansson, University of Uppsala, Uppsala, Sweden. Rat skin collagen type I immobilized on Sepharose 4B was a gift from Dr. K. Rubin, University of Uppsala, Uppsala, Sweden. Heparin (stage 14) from pig intestinal mucosa was bought from Inolex Pharmaceutical Division, Park Forest South, IL, USA, and was purified as described [8]. Chondroitin 4-sulfate (CS) and DNP-alanine were obtained from Sigma Chemical Co., St. Louis, MO, USA. Chondroitin sulfate E (CS-E) isolated from squid cartilage was a kind of gift from Dr. N. Seno, Ochanomizu University, Tokyo, Japan, and was finally labelled with ^{3}H-acetic anhydride according to Höök *et al.* [9]. By high voltage paper electrophoresis [1] it was shown to contain approximately 50% monosulfated and 50% disulfated disaccharide units.

Methods

Monocytes were isolated from blood of healthy human volunteers and cultured *in vitro* as previously described [1]. The cells were exposed to ^{35}S-sulfate (50 μCi/ml and well) for 20 hours from day 0 or day 5 in culture, and ^{35}S-proteoglycans were isolated from conditioned media by DEAE-Sephacel ion exchange chromatography at 4°C. The column was

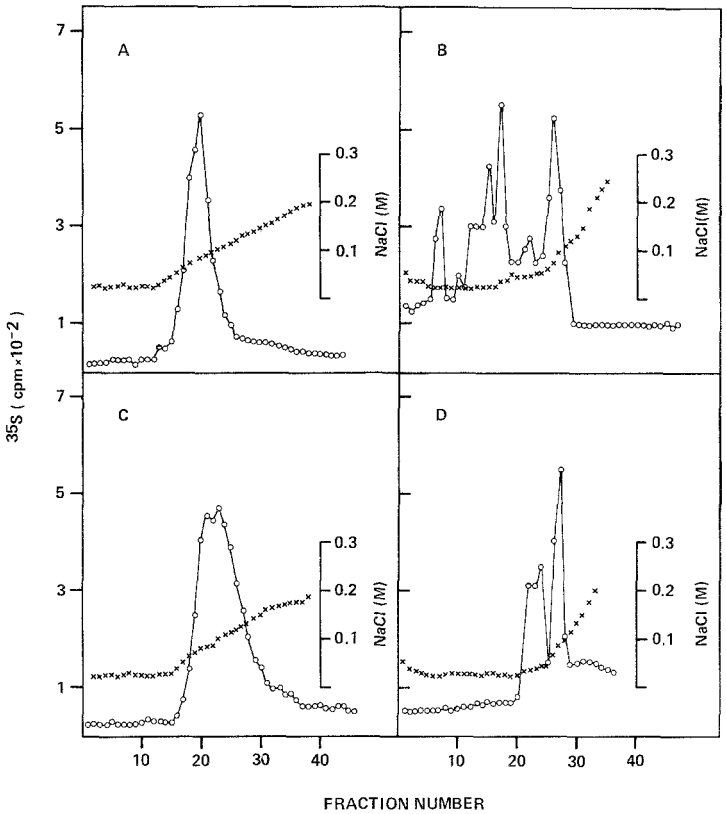

Figure 1. Affinity chromatography on fibronectin-Sepharose.

^{35}S-Proteoglycans from monocyte (panel A and B) and MDM cultures (panel C and D) were chromatographed on a column of fibronectin-Sepharose prior to (panel A and C) and after (panel B and D) alkali treatment. The column was run at 40°C in 0.05 M Tris/HCl-buffer, pH 7.4, in 0.05 M NaCl, at a rate of 6 ml/hour. The column was eluted with a gradient extending from 0.05 - 0.5 M NaCl in the same buffer as above. Fractions of 1 ml were collected and analyzed on a conductometer and for content of radioactivity.

washed in phosphate-buffered saline, PBS (0.14 M NaCl/2 mM KCl/8mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) supplemented with 0.2 M NaCl, followed by a second wash in 0.05 M sodium acetate buffer pH 4.0 with 0.2 M NaCl. The proteoglycans from both monocyte and MDM cultures were eluted from the column as single peaks after applying a salt-gradient extending from 0.2 - 1.5 M NaCl [3]. Day 1 and day 6 ^{35}S-proteoglycans were finally dialyzed at 4°C against PBS containing 0.2 mM phenyl methylsulfonyl fluoride, 1.0 mM *N*-ethylmaleimide and 2 mM EDTA and finally stored at —20°C. Isolated ^{35}S-proteoglycans, ^{3}H-heparin, ^{3}H-chondroitin sulfate and ^{3}H-chondroitin sulfate E were chromatographed on columns of fibronectin-Sepharose (1 × 1 cm) and collagen-Sepharose (1 × 2 cm) at 4°C in 0.05 M Tris/HCl pH 7.4 containing 0.05 M NaCl. Material binding to the respective columns was eluted with salt gradients from 0.05 - 0.5 M NaCl in the same buffer as above. The ^{35}S-proteoglycans were chromatographed both before

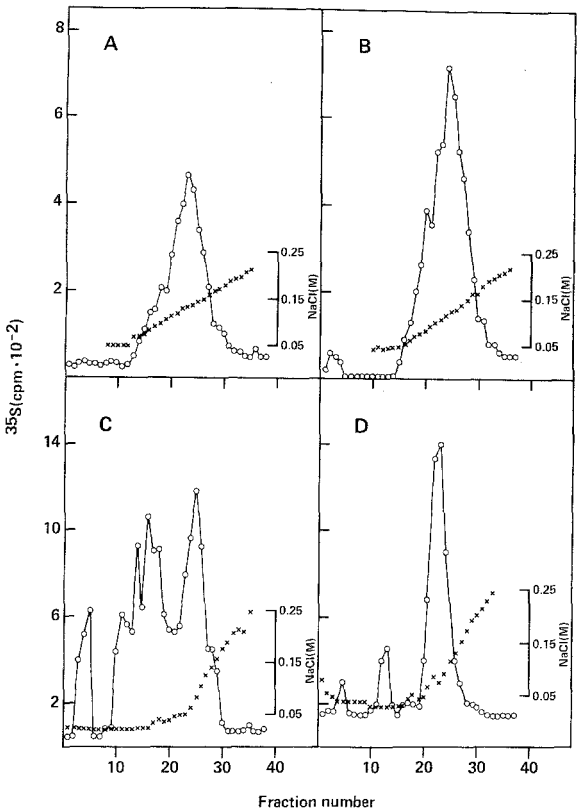

Figure 2. Affinity chromatography on collagen-Sepharose.

^{35}S-Proteoglycans from monocyte (panel A and C) and MDM cultures (panel B and D) were chromatographed on a column of collagen-Sepharose prior to (panel A and B) and after (panel C and D) alkali treatment. Elution conditions were as described in Fig. 1.

and after alkali treatment; 0.5 M NaOH at 20°C for 15 h followed by neutralization and dilution with distilled water to reduce the ionic strength under that of the starting elution buffer of the affinity chromatographies.

Interactions between hyaluronate and purified ^{35}S-CSPGs from monocyte and MDM cultures were studied by incubating 50 μg of Healon (Pharmacia Fine Chemicals, Uppsala, Sweden) with the respective proteoglycans for 30 min at 37°C in a Tris-HCl buffer, pH 8.0 with 2 mM EDTA, 1 mM *N*-ethylmaleimide and 0.2 mM phenyl methylsulfonyl fluoride. The incubation mixture (total volume of 0.5 ml) was thereafter applied directly to a column of Sepharose CI-6B and chromatographed in incubation buffer. Fractions of 1.5 ml were collected and counted for content of radioactivity. Blue dextran and DNP-alanine were used as markers for the void and total volumes, respectively.

Results

Affinity of ^{35}S-Proteoglycans for Immobilized Fibronectin

Medium-associated ^{35}S-proteoglycans isolated from day 1 (monocytes) and day 6 MDM cell cultures were subjected to affinity chromatography on immobilized fibronectin. Both cell types synthesize CSPG [3] and in Fig. 1 it is demonstrated that both the monocyte species (Fig. 1A) and the MDM species (Fig. 1C) displayed affinity for fibronectin. However, the oversulfated CSPG from MDM cultures was eluted at a higher ionic strength than the corresponding monocyte macromolecules, as has previously been demonstrated [3]. The difference in elution positions varied somewhat, but the MDM-CSPG was invariably eluted off the column at 0.15 M NaCl in 0.05 M Tris/HCl-buffer, whereas the peak of the monocyte ^{35}S-proteoglycan was eluted from the fibronectin column at 0.1 M NaCl in 0.05 M Tris/HCl, and in some cases 30 - 40% of this latter fraction was recovered in the wash, prior to the application of the salt gradient. After the release of the galactosaminoglycan chains from the respective proteoglycans by alkali treatment the binding of both fractions to fibronectin changed considerably. Only a small fraction of the ^{35}S-glycosaminoglycan (GAG) chains from the monocyte CSPG was found to bind to the immobilized fibronectin and with a low affinity (see Fig. 1B). In contrast, ^{35}S-GAG chains from MDM-CSPG was found to bind to a somewhat larger extent (Fig. 1D). It is, however, debatable whether these differences may be considered significant when taking into account the ionic strength at which these ^{35}S-macromolecules were eluted off the column. The difference noted may, however, reflect the higher sulfate density in the individual GAG-chains from MDM-proteoglycans (see below and Table 1).

Affinity of ^{35}S-Proteoglycans for Immobilized Collagen

The monocyte and MDM ^{35}S-CSPGs were found to bind to immobilized collagen with the same affinity (Fig. 2A and B). In contrast, after alkali treatment the ^{35}S-GAG-chains from the MDM-proteoglycans exhibited a slightly higher affinity than the corresponding monocyte chains (Fig. 2D and C, respectively). Accordingly, the binding of free ^{35}S-GAG-chains to both collagen and fibronectin may, at least in part, be dependent upon the sulfate density of the chains. The interactions of the proteoglycans with collagen and fibronectin do, however, seem to differ to a certain extent. Binding of the two distinct ^{35}S-CSPGs to fibronectin does seem to depend to a certain extent on the sulfate density of the proteoglycans (see Table 1), whereas the affinity for collagen is mostly dependent upon intact proteoglycan molecules (Fig. 2A-D), suggesting the possible importance of the protein core for such interactions to occur.

The binding to hyaluronate is a prominent feature of CSPG from cartilage [10]. The possible binding of the secretory CSPGs from monocytes and MDM to hyaluronate was investigated by co-incubation of the latter with isolated medium ^{35}S-proteoglycans followed by Sepharose CI-6B gel chromatography. Incubation of the two different ^{35}S-CSPGs with hyaluronate did not induce any shift in the elution pattern. Both the monocyte and MDM ^{35}S-proteoglycans were eluted with a peak K_{av} of approximately 0.2 both prior to and after incubation with hyaluronate, suggesting no binding between the CSPGs and hyaluronate.

Table 1. DEAE-Sephacel ion exchange chromatography elution positions for monocyte and MDM ^{35}S-CSPGs and ^{3}H-GAG standards.

Monocyte and MDM ^{35}S-PGs and ^{3}H-labelled GAGs were chromatographed on DEAE-Sephacel ion exchange columns. Fractions were collected and counted for radioactivity. The salt concentration of each fraction was measured on a conductometer (Konduktometer E 527 Metrohm Herisan, Switzerland) and the concentration of the fraction containing the radioactivity peak is displayed.

Sample	Peak elution position (salt concentration, M)
Monocyte ^{35}S-CSPG	0.70
MDM ^{35}S-CSPG	0.88
^{3}H-Chondroitin 4-sulfate	0.65
^{3}H-Heparin	1.02
^{3}H-Chondroitin sulfate E	1.08

Affinity of Glycosaminoglycan Standards for Fibronectin and Collagen

The affinities of monocyte and MDM ^{35}S-CSPGs for collagen and fibronectin were further compared with those of GAG standards. In advance, the elution patterns of both the ^{35}S-proteoglycans and the GAG standards following DEAE-ion exchange chromatography had been compared. The previously reported difference in sulfate density between monocyte and MDM ^{35}S-proteoglycans was evident from the differences in the peak elution positions as shown in Table 1. It was further shown that heparin and CS-E were the most negatively charged macromolecules used in this study, eluting at considerably higher ionic strengths than the monocyte and MDM CSPGs. The approximate molecular sizes of CS, heparin and CS-E were determined by Sepharose Cl-6B gel chromatography and found to be 12 000, 15 000 and 60 000 respectively (not shown).

^{3}H-Heparin was found to bind almost quantitatively to fibronectin (Fig. 3A), whereas ^{3}H-CS displayed no binding activity (Fig. 3B). The oversulfated and high molecular weight ^{3}H-CS-E bound to fibronectin to a 60-70% extent (Fig. 3C). The material binding, however, was eluted at a lower ionic strength than was heparin (Fig. 3C and A, respectively). In contrast, no significant difference could be observed between the elution pattern of MDM ^{35}S-CSPG and heparin (Fig. 1C and 3A).

The binding of heparin to collagen was also almost quantitative, only a small portion was eluted in the wash fractions (Fig. 4A), whereas CS did not bind to the immobilized collagen (Fig. 4B). Approximately 80% of the CS-E bound to the same column (Fig. 4C), and the peak fraction was eluted at the same ionic strength as the heparin peak (Fig. 4C and A, respectively). Both the monocyte and MDM ^{35}S-CSPGs were eluted at a slightly higher ionic strength than heparin and CS-E (see Fig. 2A, B and Fig. 4A, C). Taking the large difference in molecular size, and the somewhat smaller difference in polyanionic properties (Table 1) between CS-E and heparin into account, results might suggest that the sulfate density is more important for the binding of both proteoglycans and GAG to fibronectin than to collagen.

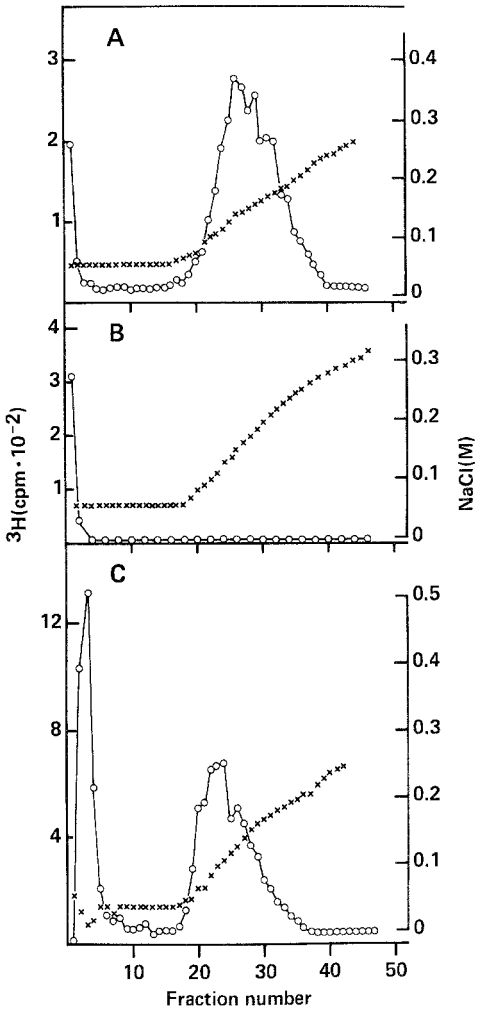

Figure 3. Affinity chromatography on fibronectin-Sepharose.

^{3}H-Heparin (panel A), ^{3}H-chondroitin sulfate (panel B) and ^{3}H-chondroitin sulfate E (panel C) were chromatographed on fibronectin-Sepharose under conditions described in Fig. 1.

Discussion

A large number of interactions between proteoglycans and other macromolecules are electrostatic in nature, whilst some, for instance the binding of heparin to the protease inhibitor antithrombin, depend upon unique and well-defined sequences within the glycosaminoglycan chains [11]. It is generally accepted that interactions not dependent upon such specific structural requirements are promoted by increasing M_r, increased sulfation and content of iduronic acid in the GAG chains [12]. The results presented

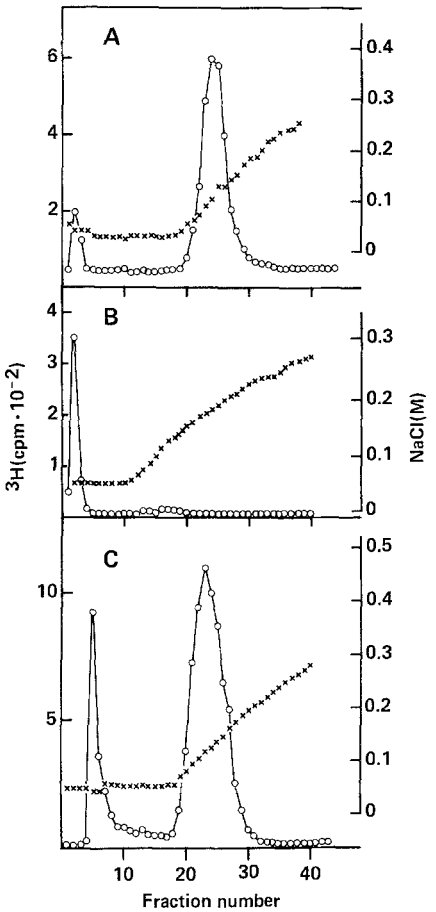

Figure 4. Affinity chromatography on collagen-Sepharose.

^{3}H-Heparin (panel A), chondroitin sulfate (panel B) and chondroitin sulfate E (panel C) were chromatographed on collagen-Sepharose. Experimental conditions were as described in Fig. 1.

here clearly indicate the ability of secretory CSPG from both monocyte and MDM to interact with both collagen and fibronectin, but not with hyaluronate. The difference in sulfation between the two distinct proteoglycans did not affect the binding to collagen, whereas the oversulfated MDM-CSPG bound more strongly to fibronectin than the monocyte counterpart. Heparin was found to bind fibronectin and collagen with affinities equal to those of MDM-CSPG. The binding to DEAE-ion exchange columns was, however, higher for heparin than the MDM-proteoglycan (Table 1). This result might suggest that intact proteoglycans, the presence of a peptide core or cooperative effects of GAG chains may promote interactions of proteoglycans with both fibronectin and collagen.

Various *in vitro* systems have been employed to investigate possible interactions between proteoglycans, collagen and fibronectin. Precipitation studies with collagen revealed the formation of complexes of collagen, fibronectin, and CSPG from a rat yolk sac tumor [13], and affinities of glycosaminoglycans and proteoglycans to immobilized collagen and fibronectin have been shown in a series of studies [14, 15, 16]. The pericellular matrix of fibroblasts was shown to contain a codistribute of proteoglycans, fibronectin and procollagen fibers [7]. In addition, the small dermatan sulfate proteoglycan from bovine tendon has been demonstrated to inhibit type I and II collagen fibrillogenesis; a property lost after alkali treatment [17]. Fibronectin has been demonstrated to bind heparin efficiently, whereas chondroitin sulfate was almost devoid of binding activity [18]. Using proteolytic fragments of fibronectin in binding studies confirmed the presence of heparin-binding domains, but also revealed the existence of cryptic fragments being capable of binding to heparan, dermatan and chondroitin sulphate as well [19]. It may be argued that the affinities of the monocyte and MDM proteoglycans for fibronectin and collagen presented here are relatively low, and therefore of minor biological importance. However, the affinities of these proteoglycans are almost equal to those of heparin. Binding of the latter GAG to fibronectin has been demonstrated to increase the affinity to this glycoprotein to collagen [20]. Whether such a property is harboured within the monocyte and MDM proteoglycans is not known, although chondroitin sulfate binding fragments within the fibronectin molecule [19], and complexes of CSPG, collagen and fibronectin have been identified [13]. Also, cartilage proteoglycans have been shown to inhibit fibronectin-mediated adhesion of fibroblasts to collagenous surfaces [21].

Both oversulfated CSPG and fibronectin are secretory products of cultured MDM [3, 5]. The association of these two macromolecules under *in vitro* conditions is only indicative as to such interactions *in vivo*. These findings do, however, bring into focus the biological basis for the synthesis of extracellular matrix components in monocytes and macrophages. Whether they are secreted in order to associate with collagen and other components when monocytes/macrophages migrate in various tissues under normal and pathological conditions is not known. These cells have a high secretory potential, at least 100 different substances with a large variety of functions and effects have been identified [22]. Fibronectin and proteoglycans may alone, or in association with other macromolecules provide a protective coat for the macrophages against some of their own damaging or lethal products, or be modulators of the functions of some of these. A further understanding of the functions of monocyte/macrophage synthesis of extracellular matrix components, and the possible effect of these molecules on the macrophages themselves may provide new insight into basic macrophage biology.

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