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# Inducible Expression of the $\alpha_2$-Macroglobulin Signaling Receptor in Response to Antigenic Stimulation: A Study of Second Messenger Generation

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**Abstract** Thioglycollate (TG)-elicited murine, peritoneal macrophages express two receptors for activated forms of the proteinase inhibitor $\alpha_2$-macroglobulin ($\alpha_2$M*)—namely, the low density lipoprotein receptor-related protein (LRP) and the $\alpha_2$M signaling receptor ($\alpha_2$MSR). We now report that resident peritoneal macrophages express only 400±50 $\alpha_2$MSR receptors/cell compared to 5000±500 receptor/TG-elicited macrophage. By contrast, LRP expression is only 2–2.5-fold greater on elicited cells. The low level of $\alpha_2$MSR expression by resident cells is insufficient to trigger signal transduction in contrast to TG-elicited cells which when exposed to $\alpha_2$M* demonstrate a rapid rise in inositol 1,4,5-trisphosphate and a concomitant increase in cytosolic free $Ca^{2+}$. We then studied a variety of preparations injected subcutaneously for their ability to upregulate $\alpha_2$MSR. Macroaggregated bovine serum albumin (macroBSA) injection upregulated $\alpha_2$MSR and triggered signaling responses by splenic macrophages. Nonaggregated BSA injection alone or in the presence of alum, by contrast, did not alter $\alpha_2$MSR expression. Recombivax (hepatitis B antigen adsorbed to alum) injection also upregulated $\alpha_2$MSR on splenic macrophages while the alum carrier had no effect. We conclude that macrophage $\alpha_2$M* receptors are inducible and their expression may be regulated, in part, by potential antigens. J. Cell. Biochem. 82: 260–270, 2001. 

**Key words:** monocytes/macrophages; cellular activation; antigens/peptides/epitopes; second messengers; cell surface molecules

$\alpha_2$-Macroglobulin ($\alpha_2$M) is part of a large superfamily which includes proteinase inhibitors and complement components [Sottrup-Jensen, 1987; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. $\alpha_2$M is a homotetramer and, like C3 and C4, each subunit contains an internal β-cysteinyl-γ-glutamyl thiolester [Sottrup-Jensen, 1987; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. Upon reaction of $\alpha_2$M with proteinases, the thiolesters rupture and the molecule undergoes a large conformational change [Sottrup-Jensen, 1987; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. This exposes cryptic determinants located in the carboxyl terminal domain which constitute receptor recognition sites for one of two receptors. These receptors are the low density lipoprotein receptor-related protein (LRP) and the $\alpha_2$M signaling receptor ($\alpha_2$MSR) [Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994; Misra et al., 1994a, 1999a]. Direct reaction of the thiolesters with small nucleophiles, such as $NH_3$ or $CH_3NH_2$, also triggers exposure of the receptor recognition sites [Barrett et al., 1979; Imber and Pizzo, 1981; Marynen et al., 1981; Gonias et al., 1982; Sottrup-Jensen et al., 1986; Van Leuven et al.,

Abbreviations used: $\alpha_2$M, $\alpha_2$-macroglobulin; $\alpha_2$M*, receptor recognized forms of $\alpha_2$M; $\alpha_2$MSR, the $\alpha_2$M* signaling receptor; RBF, the cloned and expressed receptor binding fragment; LRP, low density lipoprotein receptor-related protein; $PIP_2$, phosphatidyl inositol 4,5-bisphosphate; $IP_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$; PLC, phospholipase C; PI, phosphoinositide; PI 3-kinase, phosphatidyl inositol 3-kinase; RAP, receptor-associated protein; HHBSS, Hanks' balanced salt solution containing Hepes, pH 7.4 and 3.5 nM $NaHCO_3$; HBsAg, hepatitis B surface antigen; BSA, bovine serum albumin.

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1986; Enghild et al., 1989]. Collectively, $\alpha_2$M-proteinase complexes and $\alpha_2$M reacted with small nucleophiles are designated receptor recognized forms of $\alpha_2$M ($\alpha_2$M*) since both "activated" forms show identical receptor binding behavior [Barrett et al., 1979; Imber and Pizzo, 1981; Marynen et al., 1981; Gonias et al., 1982; Sottrup-Jensen et al., 1986; Van Leuven et al., 1986; Enghild et al., 1989]. The biological behavior of the cloned and expressed carboxyl terminal ~17 kDa receptor binding fragment (RBF) is indistinguishable from $\alpha_2$M* [Sottrup-Jensen et al., 1986; Van Leuven et al., 1986; Enghild et al., 1989; Salvesen and Pizzo, 1994]. Most of the data collected with respect to the expression of LRP and $\alpha_2$MSR have been obtained with thioglycollate (TG)-elicited murine, peritoneal macrophages. $\alpha_2$MSR is a high affinity ($K_d$~50 pM) low capacity (~4000 sites/cell) receptor while LRP has a lower affinity ($K_d$~1–5 nM) and higher capacity (~50,000 sites/cell) [Howard et al., 1996; Misra et al., 1997, 1999b; Odom et al., 1997].

LRP is a scavenger receptor located in clathrin-coated pits which binds many other ligands in addition to $\alpha_2$M* [Sottrup-Jensen, 1987; Strickland et al., 1990; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. The consequence of binding to LRP is ligand uptake and endosomal processing [Van Leuven et al., 1978; Sottrup-Jensen, 1987; Strickland et al., 1990; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. Binding of all known ligands to LRP is blocked by receptor-associated protein (RAP) [Williams et al., 1992]. $Ni^{2+}$ treatment of TG-elicited macrophages prior to $\alpha_2$M* exposure also blocks LRP binding [Hussain et al., 1995; Odom et al., 1997]. By virtue of the presence of the thiolesters, $\alpha_2$M can form covalent bonds with a number of non-proteolytic proteins during the course of proteinase attack on $\alpha_2$M [Sottrup-Jensen, 1987; Chu et al., 1991, 1994; Chu and Pizzo, 1993, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. Hormones, growth factors, cytokines, non-proteolytic enzymes, and antigens all may covalently co-incorporate when proteinases activate $\alpha_2$M [Borth, 1994; Chu and Pizzo, 1994; Gonias et al., 1994]. LRP-dependent uptake of $\alpha_2$M*-antigen complexes is linked to greatly increased efficiency of antigen presentation both in vitro and in vivo [Chu and Pizzo 1993; Chu et al., 1994]. Ligation of $\alpha_2$MSR, by contrast, triggers inositol 1,4,5-trisphosphate ($IP_3$) synthesis and increased levels of cytosolic free $Ca^{2+}$ ($[Ca^{2+}]_i$) followed by activation of both the $p21^{ras}$-dependent MAPK and phosphatidyl inositol 3-kinase (PI 3-kinase) downstream signaling pathways [Misra et al., 1993, 1994a,b, 1999a,b, 1995; Misra and Pizzo, 1998a,b]. Neither RAP nor $Ni^{2+}$ block binding to $\alpha_2$MSR or the subsequent activation of signaling cascades [Hussain et al., 1995; Odom et al., 1997].

In the present report we demonstrate that constitutive expression of $\alpha_2$MSR on murine peritoneal or splenic macrophages is 10–12-fold lower than observed on TG-elicited macrophages. $\alpha_2$MSR induction on splenic macrophages occurred to a similar extent in mice treated with hepatitis B surface antigen (HBsAg) or macroaggregated bovine serum albumin (macroBSA). Exposure of mice to alum, starch, or nonaggregated BSA did not alter $\alpha_2$MSR expression in macrophage populations. LRP is constituitively expressed at high levels on resident macrophages and this expression is also upregulated (2–2.5-fold) in response to TG, HBsAg, or macroBSA treatment of mice.

## MATERIALS AND METHODS

### Materials

Brewer's thioglycollate (TG) broth was purchased from Difco (Baltimore, MD). Culture media were purchased from Life Technologies, Inc. (Long Island, NY). Starch, bovine serum albumin (BSA), methylamine, neutral buffered formalin, and Hepes were purchased from Sigma Chemicals Co. (St. Louis, MO). A 1% solution of starch was prepared in sterile distilled water. Macroaggregated BSA (macroBSA) was prepared from BSA (Sigma Chemical Co.) according to Iio et al. [1963]. $\alpha_2$M*, RBF, and its mutant K1370A and RAP were prepared as reported previously [Salvesen et al., 1992; Misra and Pizzo, 1998a, b; Misra et al., 1993, 1994a, b, 1995, 1999a, b]. $^{125}$I-Bolton-Hunter reagent was from NEN (Boston, MA). M3/84 FITC-tagged Mac-3 antibody was from Pharmingen (San Diego, CA). Fura-2/AM was obtained from Molecular Probes, Inc. (Eugene, OR). Myo[2-$^3$H]inositol (specific activity 10–20 Ci mol) and was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other reagents used were of the highest grade available.

### Treatment of Mice and Isolation of Macrophages

Pathogen-free C57BI/6 mice (8–10 weeks old) were obtained from Charles River (Raleigh, NC). Animals were injected in groups of five peritoneally with thioglycollate (1 ml), starch (1 ml of a 1% suspension), or buffer (HHBSS). In experiments where the mice were exposed to hepatitis B virus surface antigen (HBsAg), groups of five mice were injected subcutaneously into the right thigh with Recombivax (recombinant, yeast-derived HBsAg equivalent to 0.5 μg antigen absorbed to alum in 0.2 ml in PBS). MacroBSA (100 μg) was, therefore, injected into the right thigh of a group of five mice. As a control, a group of five mice was injected subcutaneously as above with 100 μg of BSA (Sigma Chemical Co.). Mice were also injected with BSA absorbed on alum (alhydrogel "85", E.M. Sergeant Pulp and Chemical Co., Afton, NJ) as a control for the alum itself. MacroBSA is preferentially taken up, internalized, and degraded by cells of the reticuloendothelial system including macrophages [Iio et al., 1963]. Three days after intraperitoneal injections or seven days after subcutaneous injections, mice were euthanized. Peritoneal cells were obtained by lavage as previously described [Imber and Pizzo, 1981; Enghild et al., 1989; Misra et al., 1994a]. Spleens were gently removed with forceps and placed in Petri dishes in RPMI 1640 medium containing 5% fetal bovine serum 12.5 U/ml penicillin, 6 μg/ml streptomycin, and 2 mM glutamine. The spleen cells were obtained by injecting 10 ml of RPMI medium with the additions listed above into the spleens. The spleen cells were centrifuged at 1000*g* for 5 min and the pellet suspended in the modified RPMI medium. Spleen macrophages were separated from total splenocytes by allowing them to adhere either onto glass coverslips or 48 well plates for 30 min at 37°C in a humidified $CO_2$ (5%) incubator. The non-adherent cells were removed and monolayers washed three times with the modified RPMI medium. That the adherent cells were macrophages was also determined by washing adherent monolayers thrice with HHBSS containing 2% BSA and 0.1% sodium azide (staining buffer) followed by the addition of M3/84FITC-tagged Mac-3 antibody dissolved in staining buffer to each well. After incubation at 4°C overnight, the monolayers were washed as above, incubated in cell dissociation buffer for 30 min at 37°C, centrifuged at 1000*g* for 5 min and resuspended in 10% neutral buffered formalin. The mean relative fluorescence after excitation at $\lambda = 488$ nm was determined in a FACScan flow cytometer and analyzed with Cell QUEST software (Becton-Dickinson, San Diego, CA). Flow cytometry demonstrated that the adhered splenocytes were predominantly macrophages.

### Measurement of $[Ca^{2+}]_i$ in Splenic and Peritoneal Macrophages

Changes in $[Ca^{2+}]_i$ levels in macrophages were determined as previously described [Misra et al., 1993, 1994a,b, 1995, 1999a,b]. Briefly, macrophages in RPMI 1640 medium containing the additions listed above were pipetted ($1 \times 10^6$ cells) onto a glass coverslip in a 35 mm petri dish and allowed to adhere for 30 min at 37°C in a humidified $CO_2$ (5%) incubator. The non-adherent cells were removed by washing with HHBSS thrice and adhered macrophages in the modified RPMI 1640 media incubated overnight as above. Macrophage monolayers were incubated with 5 μM Fura-2/AM for 30 min in dark at 25°C, washed with HHBSS and processed for the measurements of $Ca^{2+}$ after agonist stimulation as described previously [Misra et al., 1994a, 1999a,b]. The effect of $Ni^{2+}$ on $\alpha_2$M*-dependent regulation of $[Ca^{2+}]_i$ was tested by addition of 10 mM $NiCl_2$ (10 min/37°C) prior to $\alpha_2$M* exposure as previously described [Bhattacharjee et al., 1999]. RAP was also studied (100-fold excess) for its ability to block signaling induced by $\alpha_2$M* as previously described [Misra et al., 1999b].

### Measurement of $IP_3$ in Splenic and Peritoneal Macrophages

The formation of $IP_3$ in macrophages was determined as described previously [Misra et al., 1993, 1994a,b, 1995, 1999a,b]. Briefly, peritoneal and splenic macrophages ($2–2.5 \times 10^6$ cells/well) were adhered for 2 h or 30 min, respectively in six well plates in RPMI 1640 medium containing the additions listed above, myo[2-$^3$H]inositol (8 μCi/ml) was added and the cells incubated overnight at 37°C in a humidified $CO_2$ (5%) incubator. The labeled monolayers were washed several times with cold HHBSS containing 10 mM $Li^+$, 1 mM $Ca^{2+}$, and 1 mM $Mg^{2+}$ and a volume of the wash buffer added to each well. After a 3 min temperature equilibration, the monolayers were stimulated

with agonist and cells processed for the isolation and separation of $IP_3$ as previously described [Misra et al., 1994a, b, 1999b]. To demonstrate the role of PI-specific phospholipase C (PLC) in $IP_3$ synthesis, macrophages were treated with a specific inhibitor of this enzyme, U73122 (4 μM/10 min) [Yule and Williams, 1992] prior to treatment with $\alpha_2$M*. Previous studies demonstrate that the G protein coupled to $\alpha_2$MSR on TG-elicited peritoneal macrophages is pertussis toxin insensitive [Misra et al., 1993, 1994a, b, 1995, 1999a, b]. The effect of this agent in the current study was assessed by adding pertussis toxin (1 μg/2 h at 37°C) prior to treating macrophages with $\alpha_2$M*. The effect of $Ni^{2+}$ or RAP on $IP_3$ synthesis was tested as above.

### Binding of $^{125}$I-$\alpha_2$M* to Splenic and Peritoneal Macrophages

Direct binding of $^{125}$I-$\alpha_2$M* was determined as described earlier [Howard et al., 1996; Odom et al., 1997; Bhattacharjee et al., 1999]. Briefly $^{125}$I-$\alpha_2$M* was added to macrophages in wells ($1 \times 10^6$ cells/well) at varying concentrations and cells incubated overnight at 4°C. Free ligand was separated from bound ligand by aspirating the medium and monolayers carefully washed five times with HHBSS containing 2% BSA. The cells were lysed with 0.5 M NaOH/0.05% SDS at 4°C and bound radioactivity was determined in a γ-counter. The specific binding of $^{125}$I-$\alpha_2$M* was calculated by subtracting nonspecific binding determined in the presence of a 100-fold excess of $\alpha_2$M*. The dissociation constant ($K_d$) for binding to the lower affinity LRP was calculated by direct fit to the one site binding equation using the non-linear data program Sigma plot from Jandel Scientific (San Raphel, CA). The $K_d$ for binding to the high affinity $\alpha_2$MSR was determined from Scatchard analysis.

## RESULTS

### Induction of Macrophage Responsiveness to $\alpha_2$M* by Thioglycollate Treatment of Mice

$\alpha_2$MSR and its signaling pathway has been well characterized in TG-elicited macrophages [Misra et al., 1993, 1994a,b, 1995, 1999a,b]. In this study, we compared TG-elicited macrophages to resident cells which were obtained from both the peritoneal cavity and the spleen. Surprisingly, neither resident peritoneal nor splenic macrophages exposed to $\alpha_2$M* demonstrated the characteristic increase in $[Ca^{2+}]_i$ seen with TG-elicited peritoneal macrophages [Misra et al., 1993, 1994a,b, 1995, 1999a,b]. Macrophages prepared from spleens as well as the peritoneal cavity of mice exposed to TG showed a significant increase in $[Ca^{2+}]_i$ when treated with $\alpha_2$M* (Fig. 1). The studies presented in Figure 1 are typical single cell responses in $[Ca^{2+}]_i$ after macrophages are exposed to $\alpha_2$M*. Figure 2 shows a representative field of splenic macrophages exposed to $\alpha_2$M*. Table I summarizes results from these various macrophage study groups. Overall 70 to 80% of TG-elicited macrophages responded by an increase in $[Ca^{2+}]_i$ when exposed to $\alpha_2$M*. Peritoneal injection of starch did not alter the subsequent responsiveness to $\alpha_2$M* of macrophages obtained from the peritoneal cavity or spleen (data not shown).

$\alpha_2$M* binds to both LRP and $\alpha_2$MSR on TG-elicited peritoneal macrophages, but only the latter binding results in activation of signaling cascades and increases in $[Ca^{2+}]_i$ [Misra and Pizzo, 1998a,b; Misra et al., 1993, 1994a,b, 1995, 1999a,b]. As evidence that the responses of TG-elicited splenic macrophages occurred via $\alpha_2$MSR, we exposed TG-elicited splenic macrophages to the highly specific $\alpha_2$M* receptor binding site mutant K1370A which binds only to $\alpha_2$MSR and not to LRP or any other known receptor [Howard et al., 1996]. RBF mutant K1370A triggered a response in splenic macrophages, similar to the response of TG-elicited macrophages (Fig. 1). RAP or $Ni^{2+}$ treatment of macrophages blocks binding of $\alpha_2$M* to LRP, but not to $\alpha_2$MSR [Misra and Pizzo, 1998a,b; Misra et al., 1993, 1994a,b, 1995, 1999a,b]. Neither RAP nor $Ni^{2+}$ prevent $\alpha_2$M*-induced increases in $[Ca^{2+}]_i$ in splenic macrophages, behavior identical to TG-elicited peritoneal macrophages (data not shown).

### Induction of $IP_3$ Synthesis by $\alpha_2$M* in Macrophages From TG-Elicited Mice

Previous studies of TG-elicited peritoneal macrophages demonstrate that increases in cellular $[Ca^{2+}]_i$ are dependent on increased $IP_3$ synthesis mediated by PLC which catalyzes its synthesis from phosphatidyl inositol 4,5-bisphosphate ($PIP_2$) [Misra and Pizzo, 1998a,b; Misra et al., 1993, 1994a,b, 1995, 1999a,b]. The increase in $IP_3$ synthesis in TG-elicited macrophages treated with $\alpha_2$M* activates specific $IP_3$

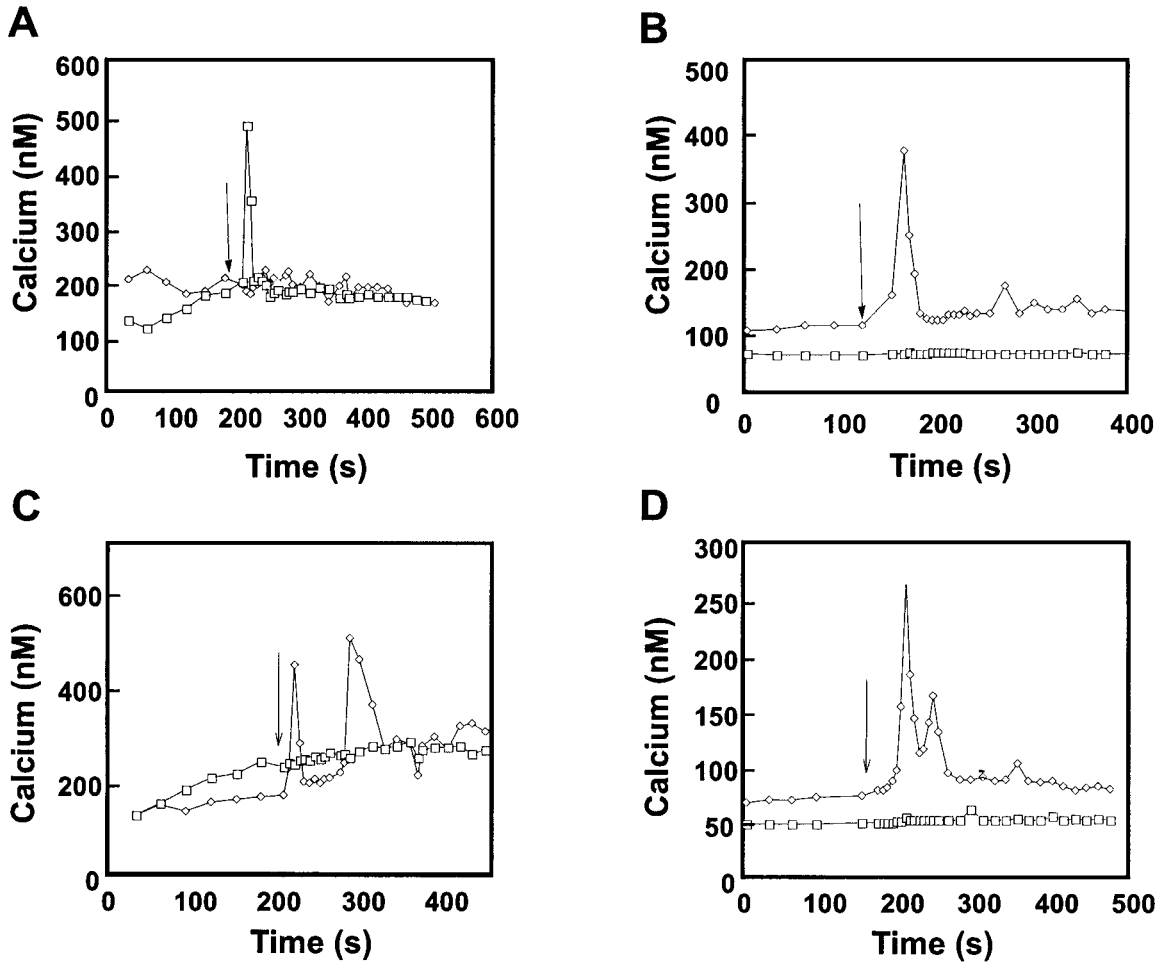


**Fig. 1.** Representative changes in $[Ca^{2+}]_i$ levels in single murine peritoneal or splenic murine macrophages isolated from buffer- or TG-injected mice stimulated with $\alpha_2$M*. **Panel A:** Peritoneal macrophages from TG (□)- or buffer (◇)-injected mice stimulated with $\alpha_2$M* (100 pM). **Panel B:** Splenic macrophages from TG (◇)- or buffer (□)-injected mice stimulated with $\alpha_2$M* (100 pM). **Panel C:** Peritoneal macrophages from TG (◇)- or buffer (□)-injected mice stimulated with binding site mutant K1370A (100 pM). Panel D. Splenic macrophages from TG (◇)- or buffer-injected mice (□) stimulated with binding site mutant K1370A (100 pM). The arrows indicate the time of ligand addition.

receptors on the endoplasmic reticulum which are also $Ca^{2+}$ channels [Putney and Bird, 1993; Berridge, 1995] thereby raising the levels of $[Ca^{2+}]_i$. Both TG-elicited peritoneal and splenic macrophages synthesized increased amounts of $IP_3$ after $\alpha_2$M* treatment (Fig. 3). These results are consistent with our earlier reports employing TG-elicited peritoneal macrophages stimulated with $\alpha_2$M* [Misra et al., 1993, 1994b]. Neither $Ni^{2+}$ nor pertussis toxin pretreatment prior to $\alpha_2$M* stimulation of splenic macrophages prevented the increase in $IP_3$ (data not shown). These results with splenic macrophages are identical to the behavior of TG-elicited peritoneal macrophages [Iio et al., 1963; Misra and Pizzo, 1998a, b; Misra et al., 1993, 1994a,b, 1995, 1999a,b].

### $\alpha_2$MSR and LRP Expression in Resident and TG-Elicited Macrophages

In order to directly demonstrate the increase of $\alpha_2$MSR in TG-elicited peritoneal or splenic macrophages relative to resident populations, we performed direct ligand binding assays and Scatchard analysis (Table II). These studies demonstrate that LRP increased only 2–2.5-fold on TG-elicited as compared to nonelicited macrophages. By contrast, there is a 10–12-fold increase in $\alpha_2$MSR expression in both splenic and peritoneal macrophages derived from TG-

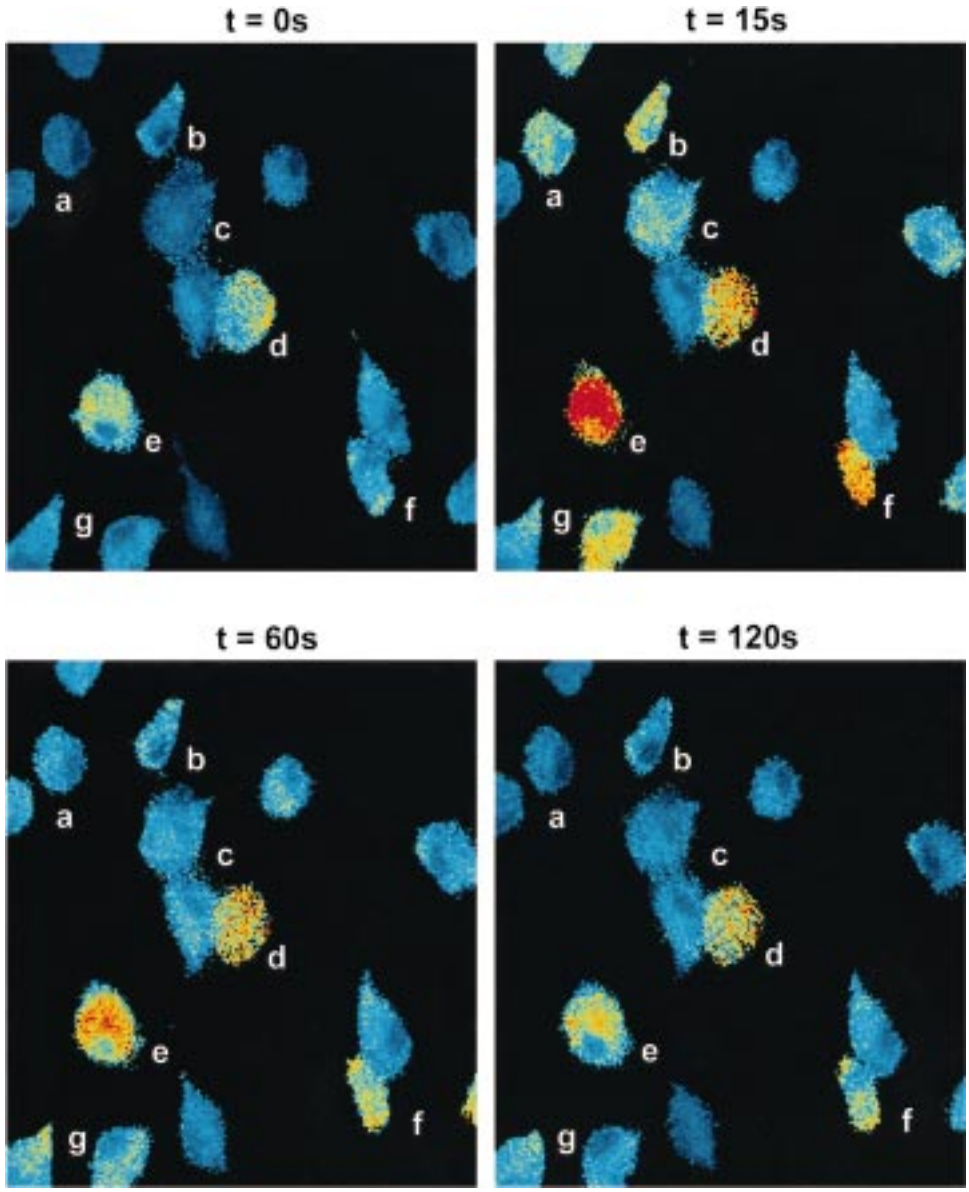


**Fig. 2.** Changes in $Ca^{2+}$-dependent fluorescence in Fura-2/AM loaded TG-elicited splenic macrophages stimulated with $\alpha_2$M* at different time intervals as indicated on the figure. Cells labeled ''a''–''g'' show changes in fluorescence in $\alpha_2$M* stimulated splenic macrophages.

treated animals. Consistent with previous studies of TG-elicited peritoneal macrophages, $\alpha_2$MSR demonstrated an extremely low $K_d$ (~50 pM) in contrast to LRP (~1 nM) [Howard et al., 1996; Odom et al., 1997; Bhattacharjee et al., 1999; Misra et al., 1999b].

### The Effect of HBsAg or MacroBSA Exposure on the Expression of $\alpha_2$MSR by Splenic Macrophages

In order to better understand the nature of upregulation of $\alpha_2$MSR on macrophages, we sought to determine whether elicitation could be evoked by injecting mice with a well characterized antigen. We, therefore, subcutaneously administered to mice HBsAg in the form of Recombivax$^{TM}$. The antigen in these preparations is large, virus-like particles formed from HBsAg and absorbed onto alum. This treatment also resulted in upregulation of $\alpha_2$MSR on splenic macrophages as demonstrated by $[Ca^{2+}]_i$ and $IP_3$ responses (Fig. 4 and Table I). Overall 80–90% of the splenic macrophages responded with an increase in $[Ca^{2+}]_i$ when treated with $\alpha_2$M*. Direct binding studies confirmed the upregula-

**TABLE I. Changes in Intracellular Calcium in Macrophages Isolated From Mice Injected With TG, MacroBSA, or HBsAG and Stimulated With $\alpha_2$M*[a]**

| Treatment | $[Ca^{2+}]_i$ (nM) | |
|---|---|---|
| | Basal | $\alpha_2$M* peak response |
| | Splenic macrophages | |
| Buffer | 48.56±2.89 | 51.77±2.39 |
| TG | 60.64±3.50 | 147.05±8.65 |
| HBsAG | 56.89±2.13 | 375.01±89.12 |
| BSA | 80.38±8.99 | 88.33±11.47 |
| MacroBSA | 89.97±7.37 | 318.0±4.20 |
| | Peritoneal macrophages | |
| Buffer | 70.52±3.19 | 77.87±3.83 |
| TG | 79.52±7.11 | 275.09±31.53 |

The results shown are the mean±SEM. The cell populations analyzed are those described in legends of Figures 1, 3, and 4.

**TABLE II. Changes in Receptor Number in Macrophages Isolated From Mice Injected With TG, MacroBSA, or HBsAG[a]**

| Treatment | Receptor number | |
|---|---|---|
| | $\alpha_2$MSR ($K_d$~50 pm) | LRP ($K_d$~1 nM) |
| | Splenic macrophages | |
| Buffer/BSA | 400±50 | 60,000±5,000 |
| TG | 5000±500 | 1,40,000±10,000 |
| HBsAG | 4500±500 | 1,30,000±15,000 |
| BSA | 400±50 | 60,000±5,000 |
| MacroBSA | 6000±600 | 1,20,000±14,000 |
| | Peritoneal macrophages | |
| Buffer | 300±50 | 70,000±7,500 |
| TG | 4000±750 | 1,40,000±15,000 |

[a]Values reported are the mean±SEM from three separate studies.

tion of $\alpha_2$MSR in mice which were treated with HBsAg (Table II). In order to control for any possible effects of the alum administered with this particulate agent, we also subcutaneously injected mice with a soluble, non-particulate antigen, BSA, absorbed onto alum. Macrophages derived from the spleens of these animals behaved like resident cells and did not demonstrate significant changes in $[Ca^{2+}]_i$ or $IP_3$ when exposed to $\alpha_2$M* (data not shown). Pretreatment of splenic HBsAg-injected mice with U73122 abolished the induction of $IP_3$ synthesis seen when these macrophages were treated with $\alpha_2$M* (Fig. 4). This agent is a specific inhibitor of PI-dependent PLC activity [Yule and Williams, 1992]. MacroBSA is a particulate agent well known for its ability to be taken up and degraded by macrophages [Iio et al., 1963]. We, therefore, also studied the response of mice subcutaneously injected with macroBSA. This treatment upregulated $\alpha_2$MSR as evidenced both by increases in $[Ca^{2+}]_i$ (Table I) and $\alpha_2$MSR receptor number (Table II). Overall 80–90% of the splenic macrophages responded with an increase in $[Ca^{2+}]_i$ when treated with $\alpha_2$M*. By contrast, subcutaneous injection of nonaggregated BSA did not upregulate $\alpha_2$MSR (Tables I and II).

Injection of both HBsAg and macroBSA also resulted in an increase of LRP expression, but only at a level 2–2.5-fold greater than seen with resident splenic cells (Table II). Injection of nonaggregated BSA had no effect on the expression of LRP (Table II).

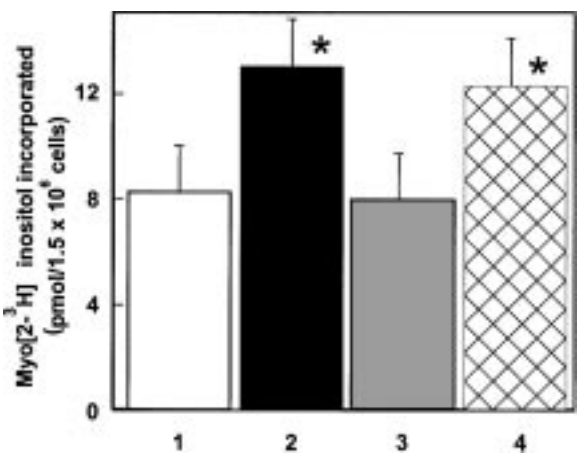


**Fig. 3.** $IP_3$ synthesis in resident and elicited macrophages treated with $\alpha_2$M*. The bars represent maximal $IP_3$ synthesis by: (1) resident peritoneal macrophages, (2) TG-elicited peritoneal macrophages, (3) resident splenic macrophages, and (4) splenic macrophages from TG-elicited mice. Values of $IP_3$ synthesis are expressed in terms of myo[2-$^3$H]inositol incorporated into $IP_3$ (mean±SEM) from three separate experiments performed in duplicate. $P < 0.05$ compared to controls.

## DISCUSSION

The proteinase inhibitor $\alpha_2$M when activated is removed from the circulation by the scavenger receptor LRP [Borth, 1994; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. This receptor also binds a number of other ligands which then undergo endosomal degradation [Borth, 1994; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. LRP is widely distributed, being found on macrophages, fibroblasts, adipocytes, hepatocytes, and other epithelial cells [Borth, 1994; Chu and Pizzo, 1994; Krieger and Herz, 1994;

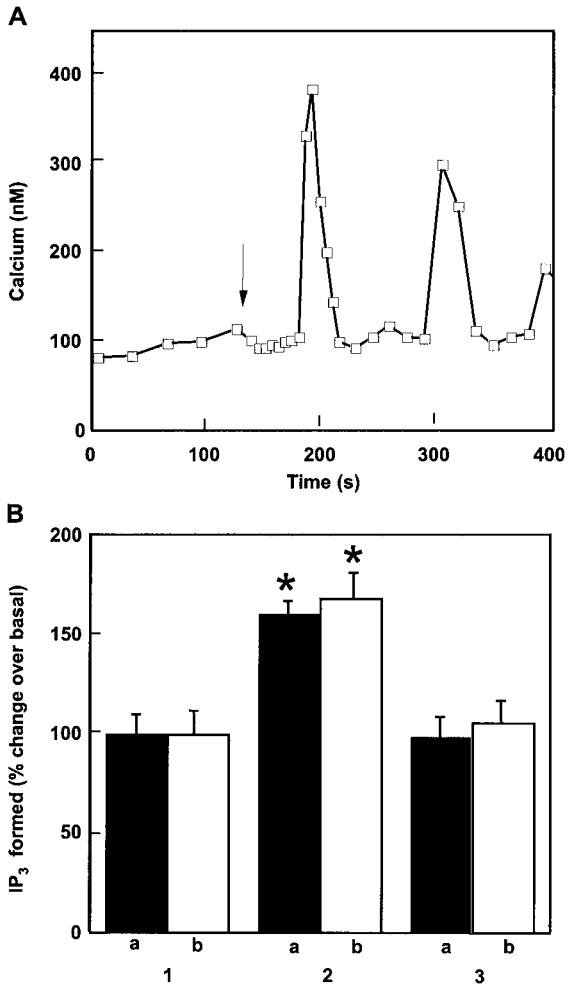


**Fig. 4.** Changes in the levels of two second messengers, $Ca^{2+}$ and $IP_3$, in peritoneal and splenic macrophages from mice injected subcutaneously with HBsAg. **Panel A:** Single cell changes in $[Ca^{2+}]_i$ by splenic macrophages stimulated with $\alpha_2$M*. Data presented are representative of 80–100 splenic macrophages performed in three separate experiments. **Panel B:** Synthesis of $IP_3$ and its sensitivity to U73122 in splenic macrophages from HBsAg injected mice and peritoneal macrophages from TG-injected mice stimulated with $\alpha_2$M*. Synthesis of $IP_3$ was quantified at the peak response (60 sec) after $\alpha_2$M* stimulation and is expressed as % $IP_3$ formation compared to basal values expressed as 100%. The values are mean±SEM from three individual experiments performed in duplicate. The studies shown are: (1) buffer-treated, (2) $\alpha_2$M*-treated, and (3) U73122 (4 μM/10 min) before $\alpha_2$M* treatment. The bar ''a'' is peritoneal macrophages from TG-injected mice, and the bar ''b'' is splenic macrophages from HbsAg-injected mice, respectively. $P < 0.05$ compared to controls. For analysis of the $[Ca^{2+}]_i$ responses of these populations see Table I. Table I also contains the data obtained for macroBSA and nonaggregated BSA.

Salvesen and Pizzo, 1994]. When $\alpha_2$M is activated by proteinase attack, the inhibitor exists in an intermediate state, during which the thiolesters become highly labile and reactive with almost any nucleophile [Sottrup-Jensen et al., 1981; Sottrup-Jensen, 1987; Chu et al., 1991; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994; Berridge, 1995]. This so-called "nascent" state is short-lived, but while $\alpha_2$M is in this intermediate state, it can covalently incorporate numerous "bystander" proteins including hormones, cytokines, and growth factors [Sottrup-Jensen, 1987; Chu et al., 1991, 1994; Chu and Pizzo, 1993, 1994; Borth, 1994; Gonias et al., 1994; Krieger and Herz 1994; Salvesen and Pizzo, 1994]. Activation of $\alpha_2$M also allows the molecule to bind covalently to proteins which are antigens [Chu and Pizzo, 1993; Chu et al., 1994]. In a murine in vitro system, complexes of $\alpha_2$M-elastase-lysozyme are presented 100 to 1000-fold better than free lysozyme [Chu and Pizzo, 1993; Chu et al., 1994]. In an in vivo rabbit model, this complex is more effective than the mixture of lysozyme and complete Freund's adjuvant with respect to immunizing these animals to produce antigen-specific IgG's [Chu and Pizzo, 1993; Chu et al., 1994]. We have demonstrated comparable results in murine models with various peptides and proteins, including a monomeric form of HBsAg, which we covalently incorporated into $\alpha_2$M*. This preparation results in several orders of magnitude greater response than subcutaneous injection of monomeric HBsAg with complete Freund's adjuvant.

$\alpha_2$MSR is highly restricted in its distribution as well as its selectivity for ligands [Misra and Pizzo, 1998a,b; Misra et al., 1993, 1994a,b, 1995, 1999a,b]. Ligation of $\alpha_2$MSR triggers typical signaling cascades which are comparable to those activated by hormones and growth factors [Misra et al., 1993, 1994a,b, 1995, 1999a,b; Misra and Pizzo, 1998a,b]. The present study represents the first examination of resident, nonelicited macrophages for the presence of $\alpha_2$MSR. We now find that both resident peritoneal and splenic macrophages express very little $\alpha_2$MSR and there is a marked upregulation in $\alpha_2$MSR expression (10–12-fold) by either peritoneal or splenic macrophages studied three days after intraperitoneal administration of TG. By contrast to the eliciting effects of TG on macrophage $\alpha_2$MSR, LRP is induced to a much lower extent on these cells (2–2.5-fold). Ligation of $\alpha_2$MSR expressed on these elicted cells triggers signal transduction producing elevated levels of $IP_3$ which increases $[Ca^{2+}]_i$. We have

studied the signaling pathways activated in splenic macrophages in some detail. These data are not shown since we found no significant differences in these pathways as compared to the published data with TG-elicited peritoneal macrophages [Misra and Pizzo, 1998a,b; Misra et al., 1993, 1994a,b, 1995, 1999a,b].

TG is a complex mixture of various salts, small molecules, and killed yeast cells. Thus the preparation contains a number of foreign and potentially antigenic proteins. This led us to ask the question of whether macroBSA or a defined particulate antigen, such as HBsAg, might also trigger upregulation of $\alpha_2$MSR and LRP expression. Both macroBSA and HBsAg subcutaneous treatment upregulated $\alpha_2$MSR and LRP in splenic macrophages at a level comparable to the effect seen when mice are treated intraperitoneally with TG. The present study considerably extends our original observation that $\alpha_2$MSR is an inducible receptor. Normal synovial fibroblasts express LRP, but not $\alpha_2$MSR, by contrast, rheumatoid synovial fibroblasts express $\alpha_2$MSR at levels comparable to those present on TG-elicited macrophages [Misra et al., 1997]. In rheumatoid synovial fibroblasts, ligation of $\alpha_2$MSR triggers increased synthesis of protein and DNA and the cells have a higher proliferative rate than normal synovial fibroblasts [Misra et al., 1997]. Ligation of $\alpha_2$MSR on TG-elicited macrophages results in similar biological effects to those observed with rheumatoid synovial cells [Misra et al., 1997]. The present study suggests that upregulation of $\alpha_2$MSR is part of the phenotype of macrophages activated by stimuli which may render these cells more responsive during inflammatory states, or as a consequence of their response to malignant cells. With respect to the upregulation of LRP, the change seen while less dramatic than the effect on $\alpha_2$MSR, could have significant consequences with respect to the uptake of $\alpha_2$M*-antigen complexes and, therefore, antigen presentation.

Our previous studies have suggested that $\alpha_2$M will form complexes with non-proteolytic proteins in vivo under pathophysiologic conditions [Chu et al., 1991]. The concentration of $\alpha_2$M in extracellular fluids approaches that of the plasma compartment which is 2–5 μM [Sottrup-Jensen, 1987; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. Many types of cells, including macrophages, are also capable of local production of $\alpha_2$M [Sottrup-Jensen, 1987; Chu and Pizzo, 1994; Krieger and Herz, 1994; Salvesen and Pizzo, 1994]. Tissue injury inevitably results in the release of proteinases which will convert large amounts of $\alpha_2$M–$\alpha_2$M*. Where foreign antigens are present, such as in bacterial infection sites, our previous studies strongly suggest that $\alpha_2$M*-antigen complexes will form [Chu et al., 1991, 1994; Chu and Pizzo, 1993]. The uptake rate of ligands bound to LRP is very high and it is very difficult to saturate this process at 37°C. These complexes will, therefore, be taken up by macrophages at a markedly greater rate than possible by bulk phase uptake of free antigens. Once the complexes are endocytosed, processing occurs and the antigen presented at far greater efficiency than possible for free antigen taken up by pinocytosis [Chu et al., 1991, 1994; Chu and Pizzo, 1993].

As noted above, $\alpha_2$M*-antigen complexes are taken up about 10-fold more efficiently than free antigens, but presentation efficiency is 100–1,000-fold greater [Chu et al., 1991, 1994; Chu and Pizzo, 1993]. It is unclear whether the increased uptake rate can account for the entire effect seen in vitro or in vivo. The in vivo rabbit studies suggested that subcutaneous injection of unbound lysozyme with $\alpha_2$M* improved response to lysozyme, despite the fact that it cannot incorporate into preformed $\alpha_2$M* [Chu et al., 1991, 1994; Chu and Pizzo, 1993]. This raises the possibility that the signaling receptor plays a role in the process of antigen presentation by its effects on signal transduction in macrophages. We suggest, therefore, that upregulation of both $\alpha_2$MSR and LRP by antigen exposure may be important with respect to the ability of the host to mount an immune response to such antigens.

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