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Cross-Talk between ICAM-1 and Granulocyte-Macrophage Colony-Stimulating Factor Receptor Signaling Modulates Eosinophil Survival and Activation[1]

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Abstract

Reversal of eosinophilic inflammation has been an elusive therapeutic goal in the management of asthma pathogenesis. In this regard, GM-CSF is a primary candidate cytokine regulating eosinophil activation and survival in the lung; however, its molecular mechanism of propagation and maintenance of stimulated eosinophil activation is not well understood. In this study, we elucidate those late interactions occurring between the GM-CSF receptor and activated eosinophil signaling molecules. Using coimmunoprecipitation with GM-CSF-stimulated eosinophils, we have identified that the GM-CSF receptor β-chain (GMRβ) interacted with ICAM-1 and Shp2 phosphatase, as well as Slp76 and ADAP adaptor proteins. Separate experiments using affinity binding with a tyrosine-phosphorylated peptide containing an ITIM (ICAM-1 residues 480–488) showed binding to Shp2 phosphatase and GMRβ. However, the interaction of GMRβ with the phosphorylated ICAM-1-derived peptide was observed only with stimulated eosinophil lysates, suggesting that the interaction of GMRβ with ICAM-1 required phosphorylated Shp2 and/or phosphorylated GMRβ. Importantly, we found that inhibition of ICAM-1 in activated eosinophils blocked GM-CSF-induced expression of c-fos, c-myc, IL-8, and TNF-α. Moreover, inhibition of ICAM-1 expression with either antisense oligonucleotide or an ICAM-1-blocking Ab effectively inhibited ERK activation and eosinophil survival. We concluded that the interaction between ICAM-1 and the GM-CSF receptor was essential for GM-CSF-induced eosinophil activation and survival. Taken together, these results provide novel mechanistic insights defining the interaction between ICAM-1 and the GM-CSF receptor and highlight the importance of targeting ICAM-1

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and GM-CSF/IL-5/IL-3 receptor systems as a therapeutic strategy to counter eosinophilia in asthma.

Reversal of the chronic eosinophilic inflammatory response associated with asthma has been an elusive therapeutic goal in the management of this disease. To date, no therapeutic target has been identified that provides an effective strategy for successfully reducing tissue-activated eosinophils and ameliorating asthma symptoms in human trials. Tissue residence, activation, and survival of eosinophils involves, at least in part, the action of soluble factors, such as GM-CSF, IL-5, and IL-3, important cytokines that induce eosinophilic effector function and lung survival (1–3). Although several reports have suggested the importance of adhesion molecules, such as CD11b/CD18 and ICAM-1, in modulating the outcome of GM-CSF and IL-5 stimulation in human eosinophils (4–6), definitive molecular mechanisms are still lacking regarding the interaction between the IL-5/GM-CSF receptor and ICAM-1 signaling pathways.

Intercellular ICAM-1 belongs to the Ig receptor superfamily and is expressed on the surface of various cells such as endothelial cells, fibroblasts, macrophages, and activated T lymphocytes (7). ICAM-1 is not typically present on circulating eosinophils or monocytes; however, it is readily induced by activation with proinflammatory cytokines such as TNF-α, IFN-γ, IL-1, and GM-CSF (8–11). Significantly, however, lung tissue eosinophils do express ICAM-1 (12–14). ICAM-1 was indicated as a potential modulator of IL-5-induced activation of eosinophils because their adherence to fibronectin significantly up-regulated the release of cytotoxic mediators such as eosinophil-derived neurotoxin, eosinophil peroxidase (EPO),[3] and leukotriene C_4 (6, 15–17). Furthermore, blockade of ICAM-1 in GM-CSF-activated eosinophils inhibited the release of EPO (6). Although these results indicate that signaling pathways induced by cytokines interact with ICAM-1, the proximal interactions in this process still remain essentially unknown.

The receptor for GM-CSF is comprised of two glycoprotein subunits; a 60- to 80-kDa α-chain (GMRα), which by itself exhibits low-binding activity of the ligand, and a 120-to 140-kDa β-chain (GMRβ), which does not bind GM-CSF itself but is essential for binding with GMRα and the formation of a signaling complex that initiates cell activation (18). Although the GMRα subunit is specific for GM-CSF, the GMRβ subunit (β_c) is common to the

[3]Abbreviations

EPO	eosinophil peroxidase
GMRα	GM-CSF receptor α-chain
GMRβ	GM-CSF receptor β-chain
RT	room temperature
7-AAD	7-aminactinomycin D
DSP	dithio-bis(succinimydl)propionate
ODN	oligodeoxynucleotide

receptors of GM-CSF, IL-5, and IL-3, which explains some of the overlapping effects of these three cytokines on eosinophils (19). The role of GMRβ in GM-CSF signaling has been well described (18, 20, 21). Briefly, following the binding of GM-CSF to GMRα, subsequent GMRβ binding converts the interaction to one of high affinity. Upon receptor dimerization, multiple tyrosyl residues in GMRβ become rapidly phosphorylated; this process is mediated by members of the JAK family. Phosphorylated tyrosyl residues are reported target binding sites for proteins containing Src homology 2 domains. Several tyrosine kinases (Lyn, Btk, Tec, Fyn, and Hck), tyrosine phosphatases (Shp1 and Shp2), and adaptor proteins (Shc and Grb2) were reported to associate with GMRβ. Of the eight tyrosyl residues present in human GMRβ, two tyrosyl residues (Y^{577} and Y^{612}) were shown to bind with the Shc adaptor molecule and Shp2 phosphatase, which subsequently interact with Grb2/Sos and transduce activation of the Ras-ERK kinase pathway (22). Thus, there has been progress in our knowledge of the early events of GM-CSF receptor activation; however, a comprehensive understanding of the later signaling mechanisms governing the expression and activation of effector proteins in eosinophils is still needed. Early phosphorylation of GMRβ and interacting kinases is transient because it is followed by proteasome- and phosphatase- dependent signal termination, thereby rendering cells insensitive to further cytokine stimulation. However, despite feedback mechanisms regulating transient responses to GM-CSF or IL-5 stimulation, eosinophils are still able to maintain their GM-CSF-induced effector functions and survival even during diminished expression of surface receptors (23). Recent findings in this area have focused on the interaction of the GM-CSF receptor with signaling from receptors for stem cell factor, erythropoietin, G-CSF, and M-CSF and their role in transphosphorylation and transactivation of the GM-CSF signaling pathway (24). However, even demonstration of direct physical interaction involving the GMRβ, stem cell factor, or erythropoietin receptor systems did not extrapolate to physiological significance in primary hemopoietic cell studies (25).

In this study, we sought to elucidate the interactions between GMRβ and signaling molecules occurring in the late stages of eosinophil activation. Immunoprecipitation assays showed that a complex is formed with GMRβ and ICAM-1 12 h after stimulation. Moreover, we report for the first time the occurrence of Slp76 and adhesion and degranulation promoting ADAP in pull-down complexes of GMRβ and ICAM-1. Using the phosphorylated intracellular portion of ICAM-1 as bait, we have also identified Shp2 phosphatase as an interacting partner for both the GMRβ subunit and ICAM-1. Furthermore, we established the importance of this interaction in eosinophil survival studies. Thus, we provide strong evidence that ICAM-1 has an important role in the maintenance of GM-CSF receptor-mediated signaling in activated human eosinophils.

Materials and Methods

Eosinophil isolation from peripheral human blood

Peripheral blood (120 ml) was drawn from healthy nonallergic volunteers, with eosinophil counts in the 3–5% range. Eosinophils were obtained by sedimentation in 4–6% dextran for 50 min at room temperature (RT), followed by centrifugation in a Ficoll-Hypaque gradient

as described previously (26). Following centrifugation at 500 × *g*, upper layers of serum and mononuclear cells were removed and saved for further analysis. Erythrocytes were removed by hypotonic lysis and then eosinophils were negatively selected using anti-CD16 immunomagnetic beads to remove neutrophils using the MACS system 9 (Miltenyi Biotec). Final eosinophil purity was assessed by microscopic examination using a Wright-stained cytospin preparation. The purity of eosinophil preparations was ≥99%.

Human eosinophil cell culture

Eosinophils were stimulated for up to 48 h with 10 ng/ml GM-CSF, and their viability was determined as previously described (27). Briefly, eosinophils were suspended in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 2–5% FBS (HyClone), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen Life Technologies). Cells were cultured at a density of 1×10^6/ml in a humidified atmosphere containing 95% air and 5% CO_2. The cultures were maintained in 24-well sterile, flat-bottom plates (Costar) previously coated with 1% human serum albumin.

Eosinophil viability

An Annexin V^{PE} Apoptosis Detection kit (BD Biosciences) was used to quantitatively determine eosinophils undergoing apoptosis by virtue of their ability to bind annexin V and exclude 7-aminoactinomycin (7-AAD). This assay detected viable (annexin V-negative, 7-AAD-negative) cells undergoing early apoptosis (annexin V positive, 7-AAD negative) and dead cells (annexin V positive, 7-AAD positive). Eosinophils (2×10^5) were stained according to the manufacturer's instructions. Data were acquired on a FACScan instrument (BD Biosciences) and analyzed using CellQuest software (BD Biosciences); we acquired 10,000 events per sample.

Multiplex cytokine assay

The ability of eosinophils to produce and release inflammatory cytokines was tested using a fluorescent bead immunoassay kit (Luminex type) from BioSource International using a Bio-Rad Bio-Plex instrument. Ten cytokines were measured simultaneously in cell-free supernatants obtained from eosinophil cultures (10^6 cells/ml). A volume of 50 μl of culture medium, or of cytokine standards, was preincubated with 50 μl of blocking buffer (40% normal mouse serum (Sigma-Aldrich), 20% goat serum (DakoCytomation), and 20% rabbit serum (DakoCytomation)) for 30 min. A volume of 50 μl of diluted sample, or blocking buffer alone, was incubated with 25 μl of multiplex microspheres for 2 h. Microspheres were washed with PBS/0.05% Tween 20, incubated with 25 μl of biotinylated detection Ab, and diluted in 25 μl of blocking buffer and 50 μl of assay buffer (1% BSA (Sigma-Aldrich) in PBS/0.05% Tween 20) for 1 h. Microspheres were then washed, incubated with streptavidin-PE at RT for 30 min, and then washed again. Subsequently, the microspheres were resuspended in 100 μl of assay buffer and analyzed using a Bio-Rad Bio-Plex 200 multiplex system. Sample concentrations were determined relative to a standard curve.

Coimmunoprecipitation

Following cytokine treatment, cells were washed with three times PBS followed by incubation with 0.5 mM dithio-bis(succinimydyl)propionate (DSP; Pierce) for 20 min at RT. Subsequently, eosinophils were lysed in ice-cold immunoprecipitation buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 10 mM NaF, 1 mM EGTA, 1 mM sodium orthovanadate, protease inhibitors, and 50 mM Tris-HCl, pH 7.4). Lysates were cleared by centrifugation and frozen immediately at −80°C or subjected to incubation with the appropriate Ab. Ab preparations were bound to protein A-Sepharose at RT for 3 h. The Sepharose beads were washed twice with 10-bed volumes of 0.2 M sodium borate (pH 9) and then resuspended in 10-bed volumes of the same solution; dimethyl pimelimidate (Pierce) was added to a final concentration of 20 mM and mixed for 30 min at RT. The reaction was terminated by washing the beads with 0.2 M ethanolamine (pH 8) and resuspending in 10 volumes of ethanolamine for 2 h at RT. After discarding the ethanolamine, the beads were washed with 10 volumes of 0.2 M glycine-HCl (pH 3) and then stored at 4° in PBS containing 0.01% NaN_3.

The incubation of protein samples with appropriate Abs was conducted for 3 h at 4°C. For control experiments, protein A-Sepharose beads without Ab were incubated with the lysates under the same conditions. Next, Ab-Sepharose beads were washed once with 10-bed volumes of lysis buffer and 20- bed volumes of wash buffer (10 mM Tris-HCl, 25 mM NaCl, and 1 mM Na_3 VO_4, pH 7.5). Bound proteins were eluted three times from the beads, each time using 1-bed volume of wash buffer containing 150 mM phenylphosphate and incubated at 37°C for 10 min. The eluates were pooled, concentrated to 1 ml using a Centriprep centrifuge (Millipore) at 3000 × *g*, and concentrated to 20–50 *μ*l using a Microcon (Millipore) centrifuge at 12,500 × *g*. Concentrates were resolved by SDS-PAGE and stained with Sypro Ruby or subjected to Western blot analysis.

Protein composition of GMRβ immunoprecipitates

One-dimensional SDS-PAGE (4–12%) was used to separate proteins immunoprecipitated with anti-GMR*β*. After staining with Sypro Ruby fluorescent stain (Bio-Rad), UV-visible bands were excised and subjected to mass fingerprinting after trypsin digestion (28). Mass spectra of peptide digests were obtained using a model 4800, MALDI-TOF-TOF-MS (Applied Biosystems). Proteins were identified by peptide mass fingerprint analysis using the National Center for Biology Information protein database and Mascot algorithm. Positive protein identifications were accepted for proteins having expectation scores of 1×10^{-3} or smaller as we previously reported (28).

Inhibition of ICAM-1 expression and activation

ICAM-1 expression was inhibited with an antisense oligodeoxynucleotide applied to in vitro-cultured eosinophils. A phosphorothioate-modified antisense oligodeoxynucleotide directed against human ICAM-1 corresponding to the sequence of ISIS 2302 (29) (5′-CCCAAGCTGGCATCCGTCA-3′) and its sense control (5′-CCTGGAGTGATGCCTAATAAT-3′) were synthesized commercially (BioSource International). Cells were transfected with 50 nM oligonucleotide using the FuGENE 6 reagent as instructed by the manufacturer (Roche Molecular Biochemicals). All experiments

using sense and antisense oligonucleotides were monitored for the expression of ICAM-1. In a separate set of experiments, inhibition of ICAM-1 activation was achieved with a monoclonal anti-human ICAM-1-blocking Ab (clone BBIG-11C81; R&D Systems). The ability of ICAM-1 to interact with counter ligands was blocked by adding 100 μl of Ab solution (10 μg/ml) to 5×10^5 eosinophils suspended in 400 μl of culture medium.

Western blot analysis

For protein identification following gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore) and stained with Sypro Ruby to visualize proteins. After rinsing the Sypro stain from the blot, membranes were blocked with 5% milk in TBS containing 0.1% (v/v) Tween 20 for 1 h and then incubated (1/10,000 dilution) with the appropriate Ab (as annotated) overnight at 4°C. Membranes were washed four times with TBS containing 0.1% (v/v) Tween 20 and then incubated with HRP-conjugated secondary Ab. After washing, proteins in immune complexes were detected by reaction with an ECL assay (Amersham Biosciences) according to the manufacturer's protocol.

Analysis of phosphorylated eosinophil proteins

Phosphoproteins from eosinophils stimulated with GM-CSF were enriched on a Qiagen PhosphoProtein Purification column using the manufacturer's protocol. Briefly, eosinophil proteins were extracted by homogenization in lysis buffer containing 0.25% (w/v) CHAPS, protease/phosphatase inhibitors, and benzonase as described in the manufacturer's phosphoprotein purification protocol (PhosphoProtein Purification Kit; Qiagen) for 30 min at 4°C and centrifuged at 10,000 × *g* at 4°C for 30 min to remove insoluble material.

Total extracted eosinophil protein (200 μg from 4×10^6 eosinophils) was diluted to a concentration of 0.1 mg/ml in lysis buffer (described above) and was applied to a lysis buffer-equilibrated PhosphoProtein purification column at RT. After washing the column with 6.0 ml of lysis buffer, the phosphoproteins were eluted with 2 ml of PhosphoProtein Elution Buffer. The yield of phosphorylated protein was determined by the Bradford assay and constituted 6–8% of the initial total protein load. The flow-through samples were passed through two additional columns to ensure complete removal of phosphoproteins. The phosphoproteins were then concentrated by ultrafiltration using a 10-kDa cutoff Amicon Ultra column (Millipore) and resolved on SDS-PAGE followed by Western blotting.

Peptide affinity binding

Five micrograms of biotinylated ICAM-1 peptides, both phosphorylated (RKIKKpY^{485}RLQ) and nonphosphorylated (RKIKKYRLQ), was incubated with lysates of eosinophils or lysis buffer alone for 16 h. The biotinylated peptides with bound protein complexes were captured by addition of 20 μl of Ultralink-Immobilized-Streptavidin beads (Pierce) for 3 h at 4°C. The beads were washed four times with ice-cold lysis buffer and twice with ice-cold PBS. The bound proteins were then eluted for SDS-PAGE followed by Western blotting.

Data analysis for cell survival and cytokine release

The results of eosinophil viability and cytokine measurements are expressed as means ± SD. To determine significant differences between the two groups, a two-tailed Student *t* test was performed using a Sigma-Plot software program (SPSS); $p < 0.05$ was considered significant.

Results

Signaling proteins interacting with the GMRβ receptor

A systematic analysis of proteins coprecipitating with the GMRβ receptor after 6 h of GM-CSF stimulation was performed. To increase the recovery of receptor-associated proteins, we used the amino-reactive cross-linking reagent DSP, which is sufficiently hydrophobic to permeate the lipid bilayer. As shown in Fig. 1, the Sypro Ruby fluorescent staining of GMRβ immunoprecipitates revealed >20 bands corresponding to proteins associated with the GMRβ protein complex. In coimmunoprecipitates from eosinophils stimulated with GM-CSF for 6 h, using MALDI-TOF-MS mass fingerprint analysis, we positively identified ICAM, Slp76, and ADAP. Western blot analysis additionally identified Shp2 phosphatase and GMRα in immunoprecipitates of GMRβ obtained from stimulated eosinophils (Fig. 1).

Additional coprecipitation experiments evaluated the time course of the GMRβ-ICAM-1 interaction (Fig. 2*A*). Eosinophil lysates from nonstimulated cells and cells stimulated for 3 and 12 h were immunoprecipitated with anti-GMRβ followed by Western blotting with anti-ICAM-1. An ~80-kDa protein corresponding to ICAM-1 was detected in the GMRβ coprecipitates obtained from eosinophils stimulated for 12 h but not in eosinophil lysates stimulated for 3 h. In addition, we investigated the expression and phosphorylation of proteins coprecipitating with GMRβ. To examine the time course of ICAM-1 induction and GM-CSF receptor expression in eosinophils, we performed Western blotting on total cell lysates obtained from peripheral blood eosinophils stimulated in vitro with GM-CSF. Fig. 2*B* shows that incubation of eosinophils with GM-CSF induced ICAM-1 expression within the first 6 h of stimulation, reaching maximal expression by 24 h. Furthermore, we found that GM-CSF stimulation led to a rapid reduction of the high-affinity subunit of the GM-CSF receptor (GMRα) detectable as early as 3 h and extending up to 24 h after stimulation. The expression of GMRβ responsible for signal transduction from GM-CSF, IL-5, and IL-3 showed a transient reduction with the most pronounced decrease detectable after 3 h of GM-CSF stimulation and increasing after 6 h of stimulation. Our results were consistent with previous flow cytometric studies showing reduced GMRα cell surface expression in response to chronic GM-CSF stimulation, suggesting a limited role on eosinophil survival (30, 31).

To examine phosphorylation of the proteins identified in coprecipitation studies, the eosinophil lysates were subjected to phosphoprotein enrichment using phosphoaffinity columns followed by Western blotting. The phosphoprotein enrichment using immobilized metal affinity columns separated the serine-, tyrosine-, and threonine-phosphorylated protein fraction and constituted ~6% of the total eosinophil proteins. Western blots detected an increase of GMRβ, ICAM-1, Slp76, and ERK1 and ERK2 in the phosphoprotein fraction

obtained from GM-CSF-stimulated eosinophils albeit with different kinetics (Fig. 3). Phosphorylated ICAM-1 and Slp76 were detectable in eosinophil lysates obtained after 24 h of GM-CSF stimulation, whereas the highest phosphorylation of ERK kinases was detectable after 15 min of GM-CSF stimulation. Interestingly, phosphorylation of the ADAP protein was detected in nonstimulated cells and GM-CSF further increased ADAP phosphorylation after 15 min of stimulation. Furthermore, comparison of the level of GMRβ in the phosphoprotein fraction with the level of GMRβ in total cell lysates strongly indicated both early and prolonged phosphorylation of the GMRβ receptor in activated eosinophils. Taken together, our results showed the presence of phosphorylated GMRβ, Slp76, ADAP and ICAM-1 in GM-CSF-activated eosinophils.

ICAM-1 peptide binding to Shp2 and GMRβ receptor

A short intracellular peptide portion of ICAM-1 contains the ITIM between aa 480 and 488 that mediates interaction with signaling proteins (32). Earlier work has shown that a synthetic peptide containing the ICAM-1 ITIM was able to bind to Shp2 phosphatase and this interaction was phosphorylation dependent (32). Since Shp2 interacted with the GMRβ receptor upon GM-CSF stimulation (33), we tested whether GMRβ associated with ICAM-1 through the Shp2 adaptor molecule. We studied the affinity of a peptide containing the ICAM-1 ITIM (RKIKKpY^{485}RLQ) as a potential GMRβ-associating molecule in eosinophils by coprecipitation. Biotin-tagged peptides were incubated with eosinophil lysates and complexed molecules were pulled down using streptavidin immobilized on agarose beads. Affinity-bound complexes were then analyzed by Western immunoblotting. Both phosphorylated and nonphosphorylated versions of the peptide were used. Using this peptide affinity-binding method, we found that Shp-2 bound only to the phosphorylated ITIM-containing peptide (Fig. 4*A*); no binding was detected when the nonphosphorylated peptide was used. In contrast, the interaction of Shp2 with the ICAM-1 peptide did not require Shp2 phosphorylation because incubation of lysates from both GM-CSF-stimulated (with phosphorylated Shp2) and nonstimulated cells (containing nonphosphorylated Shp2) provided similar binding to the phosphorylated ICAM-1 peptide. However, interaction of GMRβ and ADAP with phosphorylated ICAM-1-derived peptide was detected only when lysates from stimulated eosinophils were used, suggesting that the interaction of the GMRβ and ADAP with ICAM-1 required phosphorylated Shp2 and/or phosphorylated GMRβ (Fig. 4, *B* and *C*). Taken together, these results supported the view that the tyrosine-phosphorylated fragment of ICAM-1 can transduce the interaction with GMRβ via phosphorylated Shp2 phosphatase and/or phosphorylated GMRβ.

Blockade of ICAM-1 expression inhibits GM-CSF-induced intracellular signaling and cytokine release and prolongation of eosinophil survival

The observation that ICAM-1 expression correlated with the GM-CSF-induced inhibition of eosinophil apoptosis and the previously reported requirement of ICAM-1 for eosinophil degranulation (6) led us to investigate whether ICAM-1 played a role in GMRβ-induced eosinophil activation. To address this question, we inhibited expression of ICAM-1 using a specific antisense oligonucleotide and investigated the ability of eosinophils to express c-myc and c-fos, transcription factors involved in the inhibition of apoptosis (34, 35). Pretreatment of eosinophils with the phosphorothioated antisense oligonucleotide ISIS 2302

at 50 nM for 1 h before GM-CSF stimulation efficiently prevented the expression of ICAM-1 24 h later, whereas control sense oligonucleotide had no effect on ICAM-1 up-regulation (Fig. 5*A*). Reprobing the blots with anti-c-fos revealed significant inhibition of c-fos expression in ISIS 2302-treated cells, suggesting the requirement of ICAM-1 for c-fos induction by GM-CSF. A similar effect of ICAM-1 inhibition was observed with c-myc induction, whereas there was no effect of ICAM-1 inhibition on several other signaling molecules investigated, notably ERK1 and ERK2.

Because phosphorylation and activation of MAPKs were proposed to transduce "outside-in" signaling from adhesion molecules (9), we tested the time course of ERK phosphorylation and its modulation by ICAM-1 inhibition. Western blotting with an Ab that recognized the phosphorylated forms of MAPKs revealed prolonged phosphorylation of ERK1 and ERK2 kinases that correlated with the emergence of ICAM-1 expression (Fig. 5*B*). Furthermore, inhibition of ICAM-1 expression with antisense oligonucleotides reduced ERK1 and ERK2 phosphorylation to baseline levels, suggesting a dependence of late ERK phosphorylation on signaling from ICAM-1. Stripping the same blot and subsequently reprobing with Ab against GMRβ showed that ICAM-1 inhibition had no significant effect on the decrease of GMRβ expression during the first 3 h of GM-CSF stimulation; however, GMRβ expression was slightly decreased after 24 h when compared with control GM-CSF-stimulated cells. In contrast, ICAM-1 antisense oligodeoxynucelotide (ODN) prevented the decrease of GMRα due to GM-CSF stimulation. The mechanism of the differential effect of ICAM-1 on GMRα and GMRβ expression is currently unknown and perhaps is linked to the effect of ICAM-1 on c-fos and c-myc transcription factors.

We subsequently tested whether GM-CSF-inducible expression of ICAM-1 played a role in the production of cytokines by activated eosinophils (Fig. 6). Analysis of several cytokines in cultured eosinophil supernatants revealed a consistent release of IL-8 and TNF-α from GM-CSF- stimulated cells. Inhibition of ICAM-1 expression significantly abrogated IL-8 and TNF-α from GM-CSF-activated eosinophils, indicating a critical role for ICAM-1 in mediating GM-CSF-induced cytokine release. Analysis of eight cytokines showed no increase in response to GM-CSF stimulation under the conditions measured; these included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and GM-CSF.

Blockade of ICAM-1 expression inhibits prolongation of eosinophil survival

Flow cytometric analysis with annexin V and 7-AAD staining performed 48 h after cytokine stimulation showed a significant decrease in eosinophil viability (42% of viable cells) upon inhibition of ICAM-1 when compared with control GM-CSF-treated (78%) and GM-CSF/sense oligonucleotide-treated cells (73%; Fig. 7*A*). Because reports of down-regulation of high-affinity receptors (α-chains) and proteolytic degradation of β-chain GM-CSF/IL-5/IL-3 receptor (30, 36, 37) suggested relative independence of GM-CSF-induced eosinophil activation with constant GM-CSF stimulation, we tested the effect of GM-CSF inhibition on eosinophil survival and compared it with the effect of ICAM-1 blockade. First, we added GM-CSF-neutralizing Ab before and 3 h after GM-CSF stimulation. We preincubated several concentrations of anti-GM-CSF-neutralizing Ab (VB22 Ab) with GM-CSF for 30 min followed by the addition of 10^6 eosinophils suspended in culture medium. We found

that at 20 μg/ml the VB22 Ab completely neutralized the stimulatory effect of GM-CSF on eosinophils since there was no difference in survival rate between nonstimulated eosinophils and cells pretreated with GM-CSF-bound VB22 Ab. However, GM-CSF VB22 Ab was ineffective in inhibiting eosinophil survival when added 3 h after GM-CSF stimulation because there was only a slight reduction in the percentage of viable eosinophils (58%) when compared with VB22 pretreatment (32%) and treatment with GM-CSF alone (72%; Fig. 7*B*). These results indicated that there was partial independence of eosinophil survival provided by constant stimulation with GM-CSF and suggested the involvement of other stimuli in the maintenance of antiapoptotic signaling occurring 3 h after GM-CSF stimulation. Furthermore, neutralization of ICAM-1 with the BBA3 ICAM-1-blocking Ab added 3 h after GM-CSF stimulation resulted in a significant reduction of eosinophil survival (43%), exceeding the inhibition elicited by anti-GM-CSF treatment and supported results obtained with the use of anti-ICAM-1 antisense oligonucleotide. A combination of anti-GM-CSF and anti-ICAM-1 treatment resulted in the complete blockade of antiapoptotic activity of GM-CSF because eosinophil survival did not differ from that of nontreated cells (Fig. 7*C*). These results, along with those obtained from blocking ICAM-1 with antisense oligonucleotide, indicated ICAM-1's critical role in supporting GM-CSF-induced eosinophil survival and activation.

Discussion

In this study, we have demonstrated the occurrence of biochemical and functional cross-talk between GMRβ and ICAM-1 in GM-CSF-activated eosinophils. Using coimmunoprecipitation and peptide affinity-binding techniques, we have shown that GMRβ can form signaling complexes with ICAM-1, Shp2, Slp76, and ADAP. Furthermore, we showed that ICAM-1 was required for GM-CSF-induced prolongation of ERK kinase phosphorylation and expression of the transcription factors c-myc and c-fos. Moreover, we found that inducible ICAM-1 was required for prolongation of eosinophil survival because blockade of ICAM-1 resulted in increased eosinophil apoptosis, independent of the presence of prosurvival cytokines such as GM-CSF. Thus, we have demonstrated for the first time the role of ICAM-1 as a critical signaling molecule for propagating and supporting antiapoptotic signals for the GM-CSF receptor.

Although hematopoietins such as IL-3, IL-5, and GM-CSF are considered to be major activating and prosurvival factors for eosinophils, there is a significant gap in our knowledge of the critical factors controlling eosinophil function once they enter tissues. Studies by Bates et al. (38) using eosinophils obtained from bronchoalveolar lavage fluid documented a decreased responsiveness of cells to IL-5 stimulation that was paralleled by a decreased expression of the α-chain of the IL-5 receptor. These findings were independently confirmed by Liu et al. (36, 37) who reported distinct regulation of the receptors for IL-3, GM-CSF, and IL-5. In vitro studies showed that 24 h of stimulation of eosinophils with IL-5 resulted in the down-regulation of expression of the α-chains of the receptors for IL-5 and GM-CSF and in up-regulation of the α-chain of the IL-3 receptor (30). Although these findings suggested a role for IL-3-mediated activated eosinophils in lung eosinophils, further studies showed down-regulation of GMRβ upon IL-5 stimulation in both in vitro and in vivo models (36, 39). Taken together, down-regulation of the GMRβ receptor that extended beyond 18 h

after in vitro stimulation and decreased GMRβ expression observed in bronchoalveolar lavage eosinophils after segmental allergen challenge suggested the relative independence of late signaling events from cytokine stimulation with GM-CSF, as well as IL-5 and IL-3. Results presented herein, using a GM-CSF-blocking Ab, complemented these findings with regard to the late independence of eosinophil survival on a constant presence of GM-CSF.

Our results show for the first time a critical role for ICAM-1 in antiapoptotic pathways elicited from the GM-CSF receptor. The precise mechanism for the role of ICAM-1 in supporting GMRβ signaling is currently not known, but may be through outside-in signaling from ICAM-1. The outside-in signaling could be mediated by the engagement of ICAM-1 with ligands expressed on other cells and/or expressed on the extracellular matrix. Ligands for ICAM-1 include LFA-1, Mac-1, rhinovirus, influenza virus, and extracellular matrix components, such as fibronectin, which are present either within lung tissue or on eosinophils themselves (11). The importance of ICAM-1 for eosinophil functions other than locomotion was suggested in several reports. First, in GM-CSF-activated eosinophils, a blockade of ICAM-1 inhibited release of eosinophil-derived neurotoxin and superoxide production (17, 40). Second, adherence of eosinophils to fibronectin, an ICAM-1-ligand, significantly up-regulated the release of cytotoxic mediators such as EDN, EPO, and leukotriene C_4 (4–6, 15, 16), suggesting that cytokine-induced signaling and signaling from ICAM-1 do interact. Our results showing coprecipitation of GMRβ and ICAM-1 provide compelling evidence of interaction between these two receptors. Furthermore, coprecipitation and affinity pull-down experiments suggested an important role for the Shp2 adaptor molecule in mediating this interaction. This is in agreement with a previous report for the role of Shp2 in mediating prosurvival signaling from ICAM-1 in endothelial cells stimulated with TNF-α (32). In this study, the ICAM-1-Shp2 interaction was proposed as a limiting factor for the TNF-α antiapoptotic effect (32), analogous to the cross-talk between GMRβ and ICAM-1 demonstrated here. Tyrosine- phosphorylated Shp2 functions as an adapter protein and positively effects downstream signaling from IL-5 (33). In our studies, we demonstrated by coimmunoprecipitation and affinity pull-down experiments that Shp2 associated with both GMRβ and ICAM-1 upon stimulation of eosinophils with GM-CSF. These results demonstrated the formation of a signaling complex, which included GMRβ, ICAM-1, and the adapter proteins Slp76 and ADAP. These adapter proteins form a macromolecular complex bridging signaling pathways from both ICAM-1 and GMRβ. We reported previously that upon IL-5 stimulation, Shp2 becomes phosphorylated and associates with GMRβ and Grb2, thus leading to phosphorylation and activation of ERK kinases (33). In this study, we show that Shp2 becomes associated with ICAM-1; however, we did not observe dependence of the Shp2-ICAM-1 interaction on phosphorylation of Shp2. In contrast, phosphorylation of ITIM-related residues present on receptors has been shown to be critical for binding Shp2 (41, 42). This is in agreement with the proposed positive or negative mechanism of action of Shp2 depending on the receptor that recruits it (43, 44). Thus, interference of the Shp2 interaction by GMRβ or ICAM-1 may provide receptor-specific modulation of downstream signaling pathways. For example, specific inhibition of the Shp2 interaction with GMRβ or ICAM-1 may specifically prevent linking Shp2 to the Grb2/Sos/Ras/ MAPK pathway which transduces prosurvival signals.

We report herein for the first time the presence of the adapter protein Slp76 in complexes containing GMRβ and ICAM-1. Slp76 is known to interact with the adapter protein ADAP and serves as a substrate for Fyn and ZAP70 kinases (45). Slp76 is also essential for FcεRI-mediated signaling, degranulation, and IL-6 production in mast cells; however, Slp76 does not appear to be necessary for differentiation and maturation of mast cells or other granulocytes (42). Our results showed that, although peripheral blood eosinophils contained little or no Slp76, Slp76 was significantly up-regulated by GM-CSF stimulation. Slp76 expression occurred within the first 4 h of eosinophil stimulation. Slp76-deficient bone marrow-derived mast cells have been reported to retain the ability to phosphorylate a number of substrates, including Vav, Btk, and ERK, suggesting that several components of intracellular signaling and perhaps prosurvival signaling remain intact upon the absence of Slp76 (46). However, interactions of leukocyte-specific molecules with the GM-CSF receptor and ICAM-1 that contribute to cell degranulation may represent cross-talk between GMRβ and ICAM-1. It has been reported that the blockade of ICAM-1 abrogates degranulation of activated eosinophils (6, 15). Future ongoing studies will continue to address the relevance of Slp76 in eosinophil effector functions.

Several animal models of lung inflammation have shown a dramatic decrease in granulocyte infiltrations upon inhibition of ICAM-1 (47), and the immunosuppressive effect observed in these studies was thought to be mediated by inhibition of the interaction between circulating inflammatory cells and the vascular endothelium, thereby preventing migration of leukocytes to sites of inflammation (48). However, these approaches seriously compromised the host defense. More effective strategies may be better served by targeting ICAM-1-mediated interactions that are more specific to particular inflammatory conditions and cellular compartments. In this regard, an attractive therapeutic strategy may be to target eosinophils activated in tissues, since significant up-regulation of ICAM-1 has been reported in eosinophils in lung tissue, bronchoalveolar lavage, and skin during allergic inflammation (8, 49). Peripheral blood eosinophils express little or no ICAM-1 but most eosinophil prosurvival and proinflammatory cytokines, such as GM-CSF, IL-5, TNF-α, IFN-γ, and PGE, are known to induce expression of ICAM-1. Our results show that blockade of inducible-ICAM-1 prevents prolongation of eosinophil survival upon GM-CSF stimulation. Thus, understanding how signaling from ICAM-1 supports GM-CSF-driven eosinophilic function and identification of eosinophil-specific molecules critical for this process should make it possible to modulate the activation of eosinophils.

In summary, we have shown that ICAM-1 expressed on eosinophils interacts with the GMRβ receptor and its adaptor molecules Shp2 and Slp76, perhaps via the phosphorylation of specific tyrosine residues in its cytoplasmic domain. Shp2, in turn, may act as both a positive effector to downstream GM-CSF and ICAM-1-dependent ERK1/2 activation and as an adapter protein to bridge between ICAM-1 and GMRβ-associated signaling molecules comprised of Shc, Grb2, Sos, and ADAP. These interactions thus outline a possible molecular mechanism by which expression and cross-linking of ICAM-1 on the activated eosinophil surface can initiate a transmembrane signaling cascade, resulting in transactivation of GMRβ signaling. Fig. 8 is a proposed schematic representation of these molecular events. The downstream consequences of these signaling events, including

support and maintenance of eosinophil survival during diminished cytokine stimulation in later stages of eosinophil activation, may have implications for the maintenance and regulation of eosinophil function in lung tissue. Overall, these findings suggest that signaling from ICAM-1 may be critical in supporting effector function of eosinophils in later stages of activation and make this molecule and components of its signaling pathways a potential target for the development of novel therapies for the treatment of asthma and allergic inflammation.

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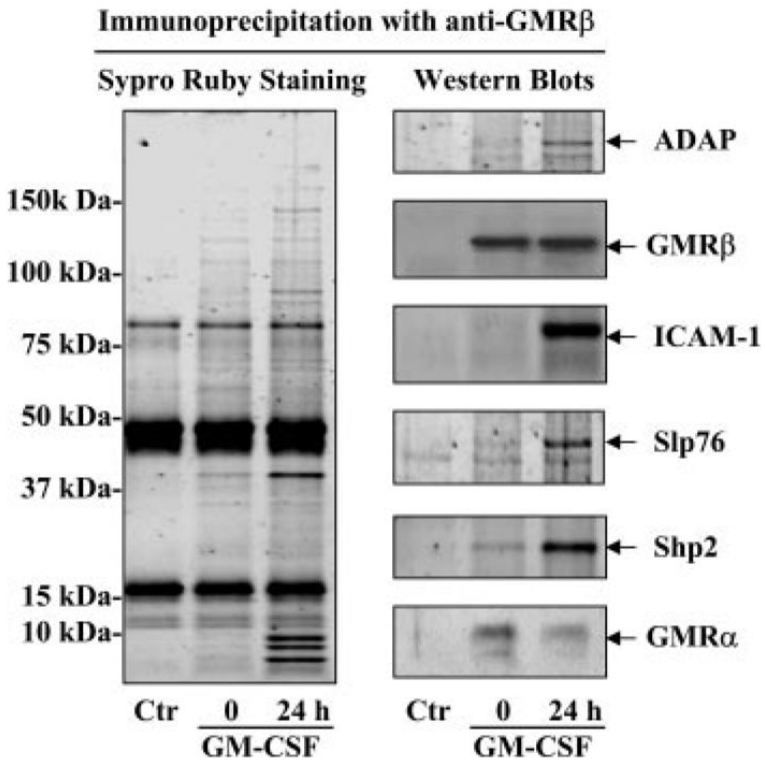

FIGURE 1.

Detection and identification of GMR*β*-associated proteins in eosinophils stimulated with GM-CSF (10 ng/ml for 24 h). After GM-CSF stimulation, eosinophils were treated with a membrane- permeable cross-linker (DSP) followed by cell lysis. Cell lysates were then immunoprecipitated with an anti-GMR*β* polyclonal Ab and separated by SDS-PAGE. Sypro Ruby fluorescent staining of proteins (*left panel*) coimmunoprecipitated with anti-GMR*β* revealed patterns of protein bands differentially expressed after GM-CSF treatment. Several proteins from the *left panel* one-dimensional gel were positively identified by mass fingerprint analysis including ADAP, Slp76, and ICAM. *Right panel*, Western blotting with annotated Abs validating the presence of Slp76, ICAM-1, Shp2, ADAP, and GMR*α* in the coimmunoprecipitates from GM-CSF-stimulated cells.

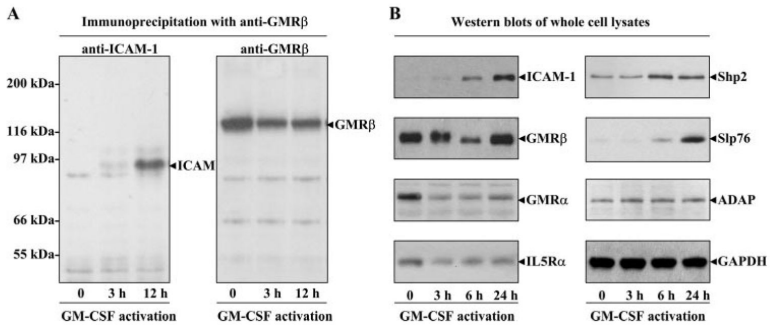

FIGURE 2.

Western blots of a time course of proteins interacting with the GMRβ receptor from activated eosinophils. *A*, Untreated and GM-CSF-treated eosinophils were lysed and immunoprecipitated with anti-GMRβ followed by Western blotting with anti-ICAM-1 (*left panel*). A band of ~80 kDa corresponding to ICAM-1 was detected in GMRβ coprecipitates obtained from eosinophils stimulated for 12 h. Reblotting of the membrane with anti-GMRβ showed diminishing amounts of coimmunoprecipitated GMRβ from GM-CSF-stimulated cells. *B*, Western blot analysis of GMRβ-coimmunoprecipitated proteins from whole cell lysates using commercial Abs as shown. Eosinophils were stimulated with GM-CSF for indicated times and whole cell lysates were resolved by SDS-PAGE. Western blot analysis showed a significant reduction of GMRβ and GMRα expression occurring between 3 and 6 h of GM-CSF stimulation and that the α-chain of the IL-5 receptor (IL-5Rα) was also diminished within 24 h of GM-CSF stimulation. Conversely, there was increased expression of Slp76, Shp2, and ICAM-1 within 6 h of eosinophils stimulation, whereas there were no significant changes in expression of ADAP in GM-CSF-stimulated eosinophils. Reblotting with anti-GAPDH as control confirmed equal protein loading.

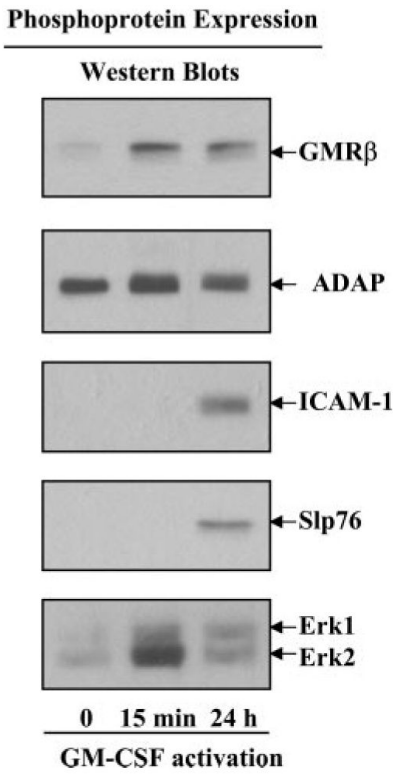

FIGURE 3.

Western blots of phosphorylated proteins present in eosinophil lysates fractionated on phosphoprotein affinity columns. Eosinophils were stimulated with GM-CSF for 15 min and 24 h and whole cell lysates were loaded on the phosphoprotein affinity columns. Column eluates containing phosphorylated proteins were concentrated, electrophoresed on 8–16% SDS-PAGE gels, and transferred to ECL membranes for Western blot analyses. Increased detection of ICAM-1, Slp76, and ADAP in column eluates indicated phosphorylation of GMRβ-interacting molecules as observed 24 h after GM-CSF stimulation. Control Western blot with anti-ERK1 and anti-ERK2 validated phosphoprotein enrichment showing highest ERK kinase phosphorylation in early time points of eosinophil stimulation.

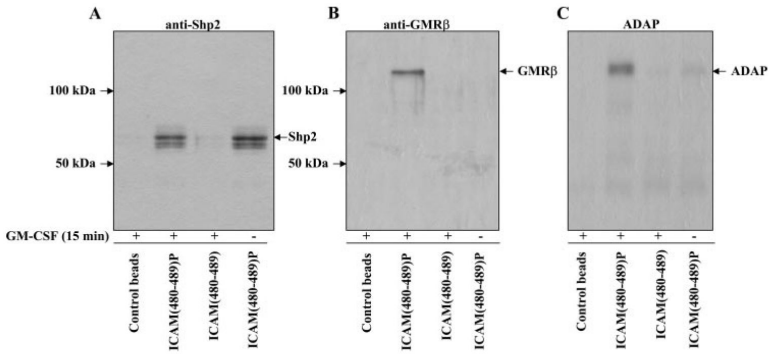

FIGURE 4.

Western blots showing the ability of Shp2 and GMRβ to complex with an ICAM-1 phosphopeptide containing an ITIM (residues 480–489). Biotinylated ICAM-1 peptides (5 μg), both phosphorylated and nonphosphorylated, were incubated with lysates of GM-CSF-stimulated eosinophils or control, nonstimulated cells for 16 h. The biotinylated peptides with bound protein complexes were captured by addition of streptavidin beads and eluted for SDS-PAGE analysis followed by Western blotting. *A*, Shp2 from both stimulated and nonstimulated cells bound to phosphorylated ICAM-1 but did not interact with nonphosphorylated ICAM-1 peptide. *B*, Phosphorylated ICAM-1 peptide interacted with GMRβ from GM-CSF-stimulated eosinophils; however, there was no binding to GMRβ when protein lysates from nonstimulated cells were used. *C*, Similar to GMRβ, the ICAM-1 peptide did bind ADAP protein extracted from GM-CSF-stimulated cells, indicating requirement of protein phosphorylation for interaction between ADAP and ICAM-1.

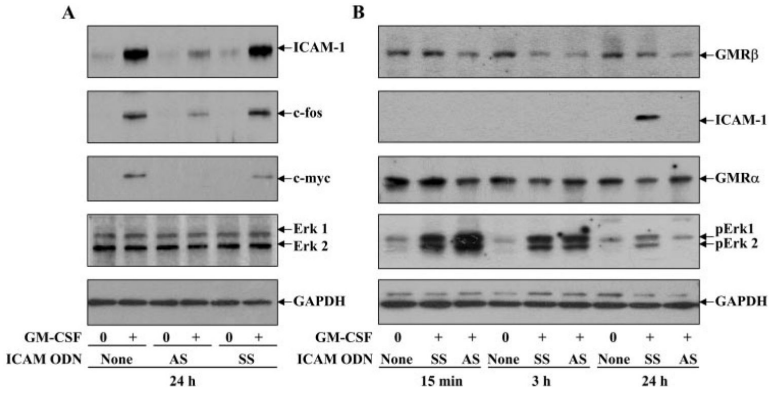

FIGURE 5.

Western blots of antisense oligodeoxynucleotide inhibition of ICAM-1 and its effect on GM-CSF-induced up-regulation of c-fos, c-myc, and phosphorylation of ERK kinases. Freshly isolated eosinophils were treated with antisense oligodeoxynucleotide (AS) against ICAM-1 (50 nM) before stimulation with GM-CSF and analyzed by Western blotting after 24 h of stimulation with GM-CSF. *A*, Antisense oligodeoxynucleotide significantly blocked induction of ICAM-1 while control sense oligodeoxynucleotide (SS) had no effect. Western blotting of c-fos on the same blots revealed a similar effect of ICAM-1 inhibition on c-fos induction. *Bottom panels* show that the level of ERK1/ERK2 proteins was unaffected by ODN treatment and GAPDH detection confirmed equal protein loading. *B*, The effect of pretreatment of eosinophils with ICAM antisense ODN on GM-CSF induced prolongation of ERK1 and ERK2 phosphorylation. Western blotting with an Ab recognizing phosphorylated forms of MAPKs revealed augmented phosphorylation of both ERK1 and ERK2 kinases in response to GM-CSF over baseline alone, correlating with the emergence of ICAM-1 at the t = 24-h time point. Administration of antisense reduces expression of ICAM-1 and likewise reduces phosphorylation of ERK1 and ERK2 to baseline levels. The same blot was stripped and subsequently reprobed with an Ab against GMRβ and GAPDH.

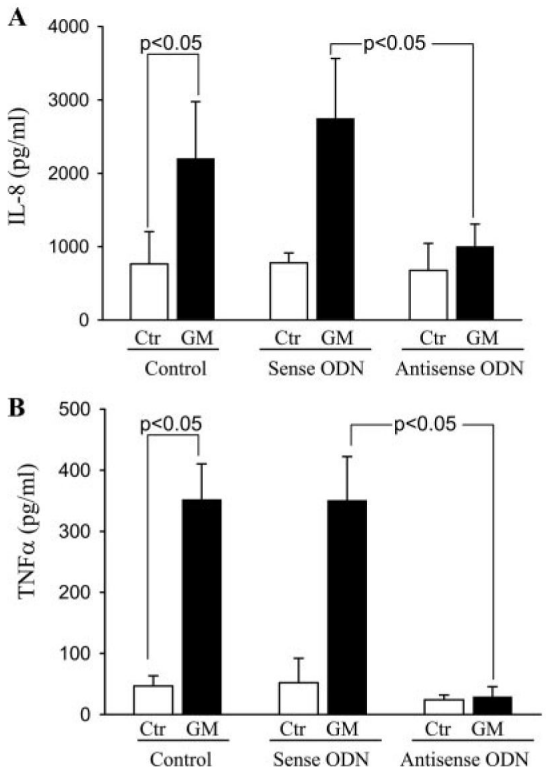

FIGURE 6.

Effect of ICAM-1 inhibition on cytokine release by GM-CSF-stimulated eosinophils. Freshly isolated eosinophils were treated with antisense oligonucleotide against ICAM-1 (50 nM) before stimulation with GM-CSF for 24 h. Cell-free supernatants from 24-h eosinophil cultures were tested for their level of IL-8 (*A*) and TNF-α (*B*) using a multiplex fluorescent bead immunoassay assay. GM-CSF-induced release of IL-8 and TNF-α was significantly inhibited in eosinophils treated with ICAM-1 antisense oligonucleotide. Triplicate experiments were performed. A significant difference was noted ($p < 0.05$) between samples treated with ICAM-1 antisense oligodeoxynucleotides and stimulated eosinophils not treated with ICAM-1 inhibitor. Ctr, Control.

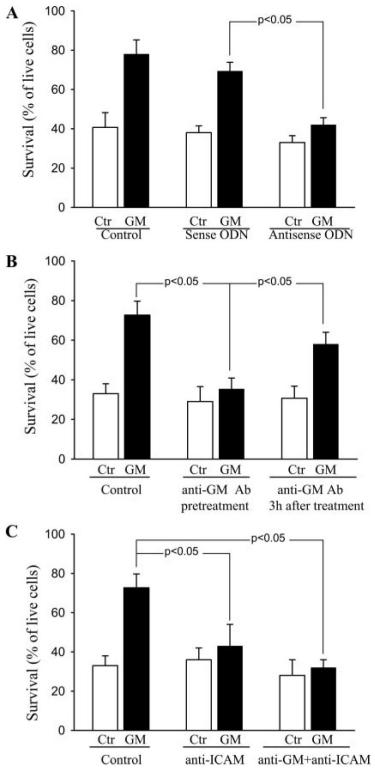

FIGURE 7.

Flow cytometric analysis of eosinophil survival after treatment with ICAM-1 antisense oligonucleotides and anti-GM-CSF- and anti-ICAM-1- neutralizing Abs. *A*, Freshly isolated eosinophils were treated with antisense oligonucleotide against ICAM-1 (50 nM) before stimulation with GM-CSF. Comparison with live (7-ADD-negative/annexin V-negative) eosinophils showed prolongation of eosinophil survival upon stimulation with GM-CSF for 48 h. Inhibition of ICAM-1 expression with antisense oligonucleotide showed abrogation of eosinophil survival when compared with GM-CSF-stimulated control (Ctr) and sense ODN-treated cells. *B*, Preincubation with GM-CSF-blocking Ab (VB22) for 30 min before cell stimulation showed complete neutralization of eosinophil prosurvival activity; however, the results with the same anti-GM-CSF added 3 h later showed significantly less efficiency in blocking the antiapoptotic effect of GM-CSF. *C*, Treatment of eosinophils with neutralizing anti-ICAM-1 mAb 3 h after GM-CSF stimulation showed significant reduction of viable cells (43%) while the combination of anti-GM-CSF and anti-ICAM-1 treatment showed complete reduction of GM-CSF-induced prolongation of eosinophil viability.

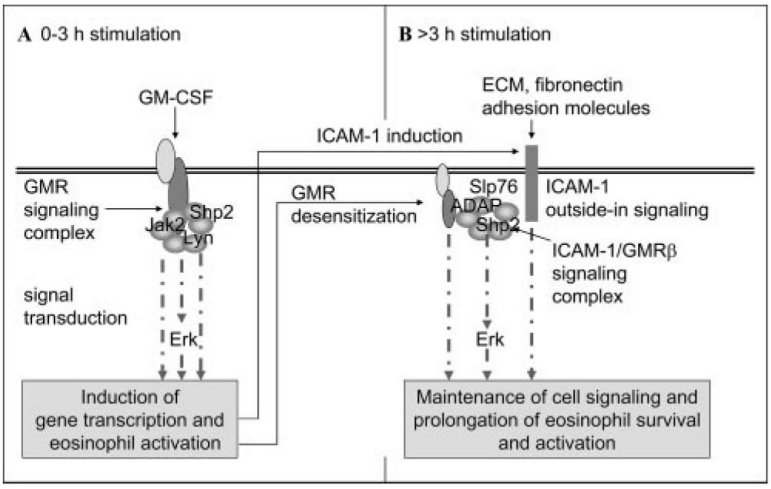

FIGURE 8.

Schematic representation highlighting ICAM-1's novel role in supporting GM-CSF-induced signaling and survival in later stages of eosinophil activation. *A*, Early stage of eosinophil activation includes binding of GM-CSF to GMR resulting in the formation of signaling complexes and activation of several signaling pathways leading to the induction of gene transcription (ICAM-1 and antiapoptotic molecules), cell activation, and desensitization of the GMR furthering GM-CSF stimulation. *B*, Late stage of eosinophil activation includes interaction of newly expressed ICAM-1 with ligands present in the extracellular matrix or with adhesion molecules present on other cells leading to formation of signaling complexes containing Shp2, ADAP, and Slp76 adaptor molecules bridging ICAM-1 with the GM-CSF receptor and hence providing signaling cross-talk with GMR. Thus, ICAM-1 "outside-in" signaling maintains GM-CSF-induced eosinophil activation and inhibition of apoptosis during the period of relative insensitivity of GMR to GM-CSF stimulation.