Inhibition of Interleukin-1 Responsiveness by Type II Receptor Gene Transfer: a Surface "Receptor" with Anti–interleukin-1 Function

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Summary

The hypothesis that the type II receptor (RII) acts as a decoy for interleukin-1 (IL-1) was tested by gene transfer in cells expressing only the type I receptor (8387 fibroblasts). RII-transfected cells showed defective responsiveness to IL-1 in terms of NFkB activation, cytokine gene expression and production. Blocking monoclonal antibodies against RII restored the capacity of RII-transfected cells to respond to IL-1β. Hence defective IL-1 responsiveness of RII-transfected cells requires surface expression of the molecule. RII-transfected cells showed normal responsiveness to TNF, which shares functional properties and elements in the signal transduction pathway with IL-1. Cells transfected with a deletion mutant of RII missing 26 of 29 amino acids of the cytoplasmic portion of the molecule showed impaired responsiveness to IL-1. Cells transfected with the full-length or the cytoplasmic deletion mutant of RII released copious amounts of RII in the supernatant. However, transfected cells showed defective responsiveness to brief exposure to IL-1, in the absence of measurable released RII. These results indicate that impairment of the responsiveness to IL-1 following RII gene transfer was dependent upon surface expression of the molecule, specific for IL-1 and unaffected by truncation of the cytoplasmic portion. Thus, the type II "receptor" is a decoy surface molecule, regulated by anti-inflammatory signals, whose only known function is to capture and block IL-1.

Interleukin-1 (IL-1) is the term for two polypeptide mediators (IL-1α and IL-1β), which are among the most potent and multifunctional cell activators described in immunology and cell biology. The spectrum of action of IL-1 encompasses cells of hematopoietic origin, from immature precursors to differentiated leukocytes, vessel wall elements, cells of mesenchymal, nervous and epithelial origin (1, 2). Occupancy of few, perhaps one, receptors per cell is sufficient to elicit cellular responses.

The production and action of IL-1 are regulated by multiple control pathways, some of which unique to this cytokine. This complexity and uniqueness is best represented by the term "IL-1 system" (3). The IL-1 system consists of the 2 agonists, IL-1α and IL-1β, a specific activation system (IL-1–converting enzyme [ICE]), a receptor antagonist (IL-1ra) produced in different isoforms (4, 5), and two high-affinity surface binding molecules (reviewed in reference 3).

Two receptors for IL-1, termed type I and II (RI and RII) have been identified and cloned (6–8). RI and RII bind IL-1 independently of each other (9) and their relative amount varies considerably in cells of different lineages (for review see reference 3). In a number of different cell types, IL-1 activity appears to be mediated exclusively by RI (10–13). As illustrated by endothelial cells, RII is dispensable and RI is sufficient to respond to low concentrations of IL-1 with an extremely diversified set of functional alterations (14, 15). Even in myelomonocytic cells, which express predominantly RII, studies with blocking antibodies suggest that IL-1 acts exclusively via minute amounts of RI (10, 13). Antibody blocking experiments suggest that RII acts as a decoy target for IL-1, sequestering the agonist and

[1] *Abbreviations used in this paper:* DSS, disuccinimidyl suberate; ICE, IL-1–converting enzyme; RI and RII, IL-1 receptor I and II.

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 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/04/1841/10 $2.00
Volume 183 April 1996 1841–1850

preventing it from interacting with the true receptor (13). Consistently with this model of function of RII, molecules with anti-inflammatory activity, including glucocorticoid hormones, IL-4 and IL-13, induce augmented expression and release of RII (13, 16, 17) and a RII-related molecule expressed by poxviruses is important for pathogenicity (18, 19). Chemoattractants cause rapid shedding of RII, a phenomenon interpreted as a means to buffer IL-1 leaking from sites of inflammation (20).

The concept of a high affinity binding, nonsignaling molecule, whose only function is to prevent the ligand from interacting with the true receptor on the same cell, is without precedent. In an effort to test the "decoy" model of function of RII directly, we performed gene transfer experiments. Evidence is presented that transfection of RII causes inhibition of responsiveness to IL-1, which is specific for IL-1, mediated by surface expressed RII, and independent of the cytoplasmic portion of the molecule. These results are consistent with the hypothesis that RII acts as a negative regulator of the IL-1 system.

Materials and Methods

Cells and Transfection. The human 8387 sarcoma line (21) was grown in RPMI 1640 (Gibco, Glasgow, Scotland) with 10% heat inactivated fetal calf serum (FCS; Hyclone, Logan, UT). 60% confluent cells were transfected by the calcium precipitated method with 10 μg of the plasmid. After 48 h transfected cells were selected for hygromicin (Boehringer Mannheim, Mannheim, Germany) resistance (40 μg/ml) and the pool of resistant cells was further analyzed.

Constructs. The full-length cDNA for human IL-1 RII, SalI-SalI insert, (7) and a cytoplasmic deletion construct, SalI-BglII insert, (see below) were ligated back into the polylinker of the pCEP4beta expression vector (Invitrogen, San Diego, CA). pCEP4beta is an episomial high copy number expression vector containing the hygromicin resistance gene and the insert cDNA under the control of the CMV promoter. A cytoplasmic deletion construct in which the coding region terminates after the first three amino acids of the cytoplasmic portion (HisArgArg) was constructed, resulting in the insertion of a stop codon after the first three amino acids (aa) of the cytoplasmic portion.

Northern Analysis. Total RNA was isolated by the guanidium isothiocyanate method. 10 μg of total RNA was analyzed by electrophoresis through 1% agarose/formaldehyde gels, followed by Northern blot transfer to Gene Screen Plus sheet (New England Nuclear, Boston, MA). Probes were: a EcoRI-HindIII 477-bp fragment from hIL-1RI cDNA, a EcoRI-SalI 750-bp fragment from hIL-1RII cDNA, full-length cDNA of hMCP-1, a BanII-BanII 620-bp fragment from hIL-6 cDNA, a full-length hIL-8 cDNA, a full-length hc-jun cDNA. Membranes were washed twice with 2× SSC/1% SDS at 60°C and exposed for 18 h at −80°C. RNA transfer to membranes was checked by UV irradiation.

Electrophoresis Mobility Shift Assay. Nuclear extracts were prepared as described (22). Double strand oligonucleotide (5′ TGACAGAGGGGACTTTCCGAGAGGA 3′) was labeled with [α-^{32}P]dCTP and polynucleotide kinase and eluted from a G25 column. For binding reactions, 10 μg nuclear proteins were mixed with an aliquot of the labeled oligonucleotide (5 × 10^4 CPM) and 1 μg/ml poly(dldC) in 10 μl of a 10 mM Tris, pH 7.5, buffer containing 50 mM NaCl and 6% (vol/vol) glycerol for 20 min at room temperature. Mixtures were then electrophoresed through a 5% acrylamide, 0.5× TBE gel; gel was then dried and exposed for autoradiography for a few hours.

IL-1 Binding. The number and affinity of surface IL-1 receptors were determined as described (13). Cells were detached from the plate with 5 mM EGTA and washed with binding buffer (RPMI 1640-0.2% BSA, pH 7.4) and 2 × 10^6 or 2 × 10^5 cells were incubated with different concentrations of ^{125}I-IL-1β (spec. activity 145 μCi/μg for 8C and 8C7, 95 μCi/μg for 83C7 and 83M, 111 μCi/μg for 83C; NEN, Bad Homburg, FRG) in the presence or absence of a 200-fold molar excess of cold cytokine in 50 μl binding buffer at 4°C for 4 h in polystyrene round-bottomed 96-well microplates (Falcon, Lincoln Park, NJ) on a shaking platform. To separate bound from free ^{125}I-IL-1β, cells were layered on the top of a 200-μl cushion of 20% sucrose (Merck)-1% BSA in 400-μl polypropylene tubes (Beckman Instruments, Palo Alto, CA) and centrifuged at 10,000 rpm for 30 s. The cellular pellets were counted in a γ-counter. Next, Scatchard analysis was performed by the LIGAND program to determine the kD and numbers of receptors for IL-1β.

Affinity Cross-linking. Cross-linking of surface and released receptors was performed as described (13). Briefly, for surface affinity cross-linking, 1 × 10^7 cells were incubated in binding buffer with 1 nM ^{125}I-IL-1β for 4 h at 4°C. After addition of 1 mM disuccinimidyl suberate (DSS; Pierce, Rockford, IL) at 4°C for 4 h, the cell pellet was lysed in 100 ml lysis buffer (0.5% Triton X-100, 25 mM Hepes, 1 mM PMSF). The debris-free supernatant was analyzed by 10% SDS-PAGE under reducing conditions and dried gels were exposed to autoradiography for 3 h. For soluble covalent cross-linking, medium was recovered and 100 μl were added to 1 nM ^{125}I-IL-1β, with or without a 200 M excess of cold IL-1β or 10 μg/ml M1 (blocking mAb anti IL-1 RI) or M22 (blocking mAb anti IL-1 RII), and incubated at 4°C for 4 h. After addition of 1 mM DSS at 4°C for 30 min, samples were analyzed by gel electrophoresis as above and dried gels exposed to autoradiography for 18 h.

Cytokine Assays and Reagents. 3 × 10^4 cells were plated in 96 flat-bottomed plates in 200 μl RPMI + 10% heat-inactivated FCS (culture medium). After 1 d medium was changed with culture medium containing stimuli (recombinant IL-1β; from Dompè, L'Aquila, Italy, specific activity 10^7 U/mg; recombinant TNFα; Basf Knoll, Ludwighafen, Germany, specific activity 6.6 × 10^6 U/mg) and incubation was continued for an additional 24 h. The supernatants were then harvested and kept at −20°C. In some experiments, recombinant soluble RII expressed in CV-1 EBNA-1 cells (9) was added to the wells before IL-1β.

IL-6 was measured using a specific ELISA or as hybridoma growth factor activity on the 7TD1 cell line obtained through the courtesy of Dr. J. Van Snick (Ludwig Institute, Bruxelles, Belgium) as described (23). Briefly, 2 × 10^3 cells in 200 μl (four replicates per dilution) were cultured for 72 h with different dilutions of the culture supernatants to be tested or with control medium. The number of cells was assessed by the MTT colorimetric test as described (23). HGF activity resulting in half maximal stimulation of target cell growth was arbitrarily defined as 1 unit. The reference standard used in these experiments was human recombinant IL-6.

MCP-1 was determined using a recently developed sandwich ELISA, with a sensitivity limit of 30 pg/ml (24). IL-8 was measured using a specific ELISA with mAb and a polyclonal serum (gifts from Dr. M. Ceska, Sandoz, Wien, Austria). Cytokine assays were conducted with four replicates and results are mean ± SE.

The properties and use of blocking mAb directed against the type I (M4 or M1) and the type II (M22) receptor have been described previously (9, 13). A novel specific ELISA was used to measure release of RII from transfected cells. The assay is based on a poyclonal antiserum and mAb (clone no. 8) raised against the RII expressed in 8387 cells. It has a sensitivity of ~300 pg/ml (Peri, G., unpublished data).

Results

Characterization of Cells Transfected with the IL-1 Decoy RII. The 8387 cell line was selected for these studies because it expresses only RI by Northern and PCR analysis (Fig. 1 *A* and reference 15). Following transfer of a full-length RII cDNA under the control of the CMV promoter, high levels of RII mRNA were detected (Fig. 1 *A*). It is important to note that cells transfected with the RII continued to express RI at levels comparable to those of parental or vector transfected cells (Fig. 1 *A*). RII gene transfer was associated with a marked increase in the capacity of 8387 cells to bind IL-1β. For instance, in the experiment shown in Fig. 1 *B*, cells transfected with the empty vector had 167 IL-1β binding sites/cell with a kD of 3.4×10^{-10} M, compared to 3.7×10^4 binding sites/cell (kD 7.1×10^{-10} M) for transfected cells. Following gene transfer, cross-linking of surface IL-1 binding molecules revealed a major band of ~87 kD, which, after taking into account the labeled ligand, corresponds to the molecular mass of RII (68 kD) (Fig. 1 *C*).

Responsiveness to IL-1 after RII Gene Transfer. Fig. 2 shows typical experiments in which cells transfected with RII were exposed to graded concentrations of IL-1β (from 10 pg/ml to 100 ng/ml) and the response was measured in terms of cytokine (IL-6, IL-8, and MCP-1) production. Parental 8387 cells or cells transfected with the empty vector were exquisitely sensitive to IL-1β, with a minimal active concentration for IL-6 production of 10 pg/ml and an ED50 of 361 ± 84 pg/ml (mean ± SE) in 12 different experiments. RII gene transfer resulted in a marked impairment of the capacity of 8387 cells to respond to IL-1β. Inhibition was observed in three series of experiments corresponding to three independent transfections performed over a period of 2 yr. The inhibition of IL-1 responsiveness was related to the numbers of transfected RII receptors. As illustrated in Fig. 2 *A*, with 37×10^3 RII/cell, high concentrations of IL-1β (100 ng/ml) reversed the defective responsiveness of RII-transfected cells. With 400×10^3 sites/cell, only a weak response, not reaching the half-maximal value was observed. Defective responsiveness of RII-transfected 8387 cells was not confined to IL-6 production, since similar results were obtained when IL-8 (nine experiments) and MCP-1 (13 experiments) release were measured (Fig. 2 *B*). Defective cytokine production by RII-transfected cells was evident after different culture periods ranging from 18 to 48 h (data not shown). RI gene transfer under similar conditions augments the responsiveness of cells to IL-1 (reference 6 and unpublished).

Effect of Anti-IL-1 R mAb. Previously characterized and used blocking mAbs (9,13) were used to investigate the role of the IL-1 receptors in control and RII-transfected cells. As expected, the anti-RI mAb M4 inhibited the capacity of 8387 cells to respond to IL-1 (Fig. 3 *A*). The same mAb also inhibited the weak responsiveness to IL-1 of cells expressing high levels of RII after gene transfer. Blocking mAb directed against RII reverted the defect in IL-1 responsiveness of cells in which expression of this molecule was forced by gene transfer (Fig. 3 *A*). Anti-RII mAb did not affect cytokine production in parental or vector-transfected cells. Anti-RII mAb also restored the defective capacity of transfected cells to respond to IL-1 in terms of rapid induction of cytokine mRNA (not shown). In an effort to further test the possibility that an undefined "right"

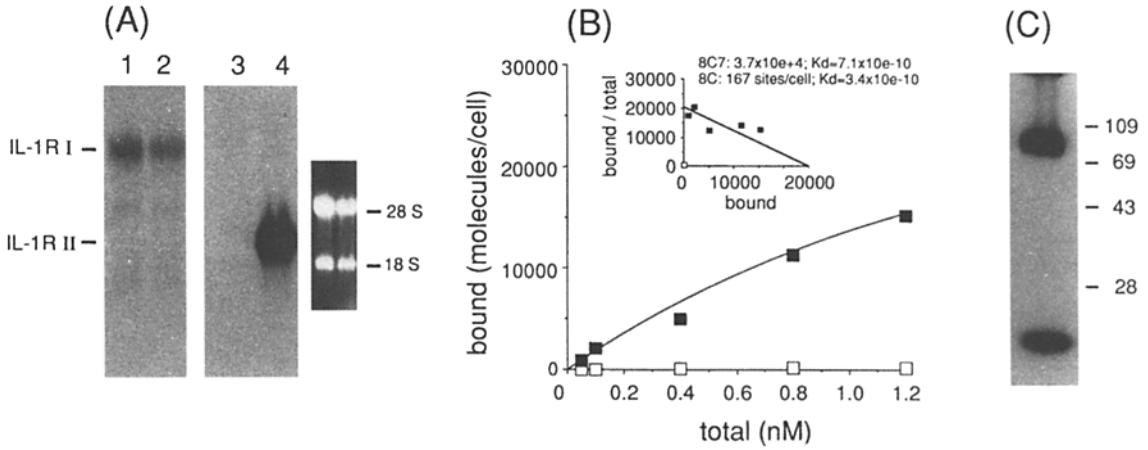


Figure 1. Characterization of RII-transfected cells. (*A*) Northern blot analysis of the IL-1 RI (lanes *1* and *2*) or RII (lanes *3* and *4*) expression in cells transfected with the empty vector (8C, lanes *1* and *3*) or with RII (8C7, lanes *2* and *4*). The ethidium bromide stained membrane is shown in the right part of the panel. (*B*) Saturation curve and Scatchard analysis of ^{125}I-IL-1β binding to cells transfected with empty vector (8C, □) or with decoy RII (8C7, ■). Number and affinity of sites were calculated by LIGAND program. The scale on the ordinate does not allow showing individual points for control cells (8C, □) in the insert. (*C*) Affinity cross-linking of surface RII by ^{125}I-IL-1β in RII-transfected cells (line 8C7). Molecular mass standards are indicated.

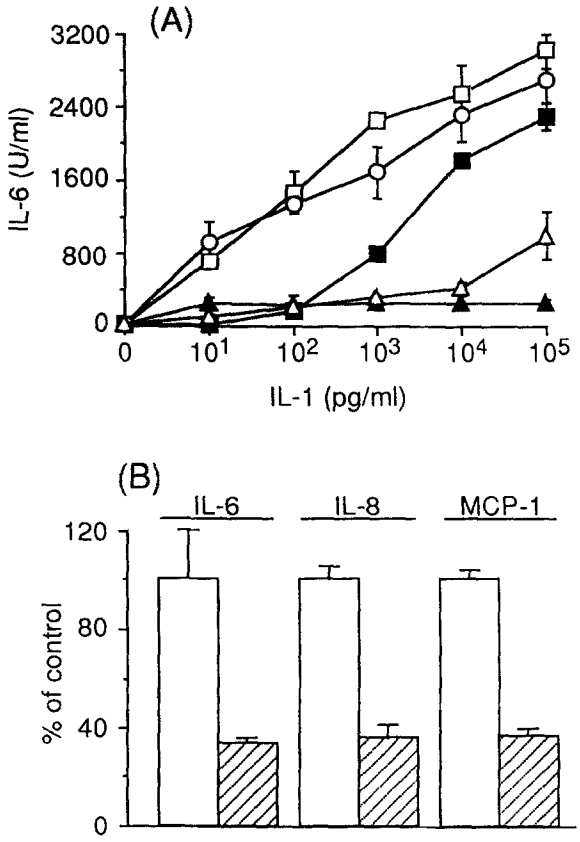


Figure 2. Cytokine production after RII gene transfer. Cells were incubated with recombinant IL-1β for 24 h. (*A*) Production of IL-6. (○) parental 8387; (□) cells transfected with empty vector (8C line); (■) RII-transfected cells (8C7; 3.7 × 10^4 sites/cell; full-length RII); (△) Δ372-398 RII-transfected cells (83M; 25 × 10^4 sites/cell); (▲) RII-transfected cells (83C7; 40 × 10^4 sites/cell full-length RII). Results are representative of 12 experiments performed over a period of 24 mo. (*B*) Production of IL-6, IL-8 and MCP-1. Results are percent of control at a concentration of 1 ng/ml IL-1β. Open bars, vector control (8C); hatched bars, RII transfected cells (8C7).

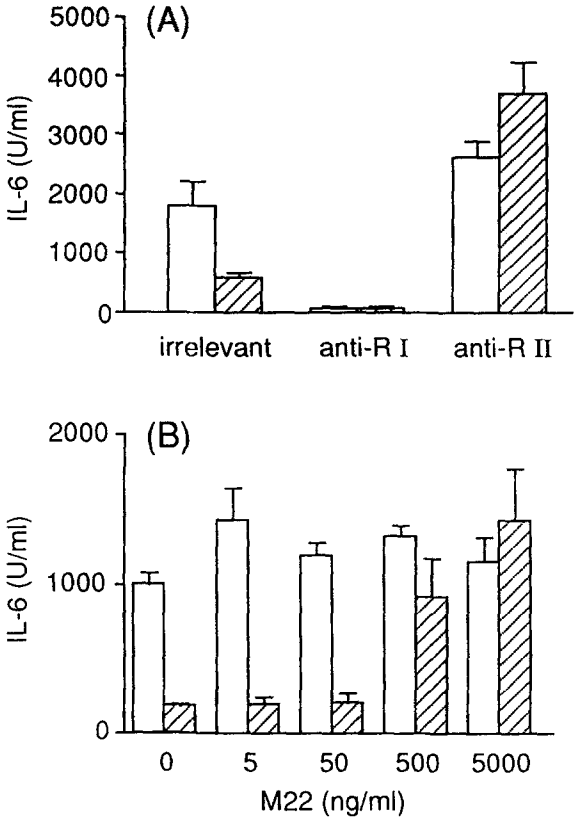


Figure 3. Effect of anti-IL-1 R mAb IL-1 responsiveness of RII-transfected cells. (*A*) Cells transfected with empty vector (8C, *open bars*) or RII (8C7, *hatched bars*) were stimulated with 100 pg/ml IL-1β in the presence of an irrelevant mAb, or anti-RI mAb (M4), or anti-RII mAb (M22) for 24 h and IL-6 production was measured. (*B*) Cells transfected with empty vector (8C, *open bars*) or RII (8C7, *hatched bars*) were stimulated with 100 pg/ml IL-1β in the presence of graded doses of M22 mAb (from 5 ng/ml to 5 μg/ml) for 24 h and IL-6 production was measured.

number of RII may present the ligand to the signal transducing RI, in analogy with ligand passing by the p75 TNF R (25), we studied the effect of graded concentrations of anti-RII mAb on IL-1 responsiveness. A similar approach was used after over-expression of the p75 TNF R in p55 expressing HeLa cells, and showed amplification of the response to TNF in the presence of suboptimal anti-p75 (25). As shown in Fig. 3 *B*, at concentrations ranging from 5 ng to 5 μg/ml, anti-RII mAb only inhibited and never amplified responsiveness to IL-1 in RII-transfected cells versus cells expressing only the RI.

These results indicate that inhibition of the response to IL-1 after RII gene transfer is reversed by blocking anti-RII mAb. Hence, defective IL-1 responsiveness in transfectants requires surface expression of RII.

Defective NFkB Activation and Induction of Gene Expression in RII-transfected Cells. It was important to ascertain whether RII gene transfer affected early events following interaction of IL-1 with cells. Activation of AP-1 and NFkB translocation to the nucleus are involved in IL-1–activated cellular responses (12, 26, 27). Therefore, we investigated NFkB activation and expression of immediate early genes in RII-transfected cells. As shown in Fig. 4, NFkB activation as well as induction of cytokine mRNA expression, was drastically reduced in RII-transfected cells exposed to IL-1β for 1/2–4 h. Similar results were obtained when c-*jun* mRNA induction was examined (not shown). Thus, RII gene transfer compromises early events that follow interaction of IL-1 with signaling RI.

Specificity of Defective IL-1 Responsiveness. TNF has a spectrum of action and a signal transduction pathway remarkably similar to that of IL-1 (28–31). It was therefore important to ascertain the responsiveness of RII-transfected cells to TNF. As shown in Fig. 5, RII-transfected cells, with defective response to IL-1, produced normal amount of IL-6 (*A*) and IL-8 (*B*) when exposed to TNF. Thus, RII gene transfer selectively impairs the response to IL-1, but not that to the functionally related cytokine TNF.

Inhibition of IL-1 Responsiveness by Truncated RII. The results discussed so far indicate that surface expression of the RII following gene transfer specifically inhibits the capacity

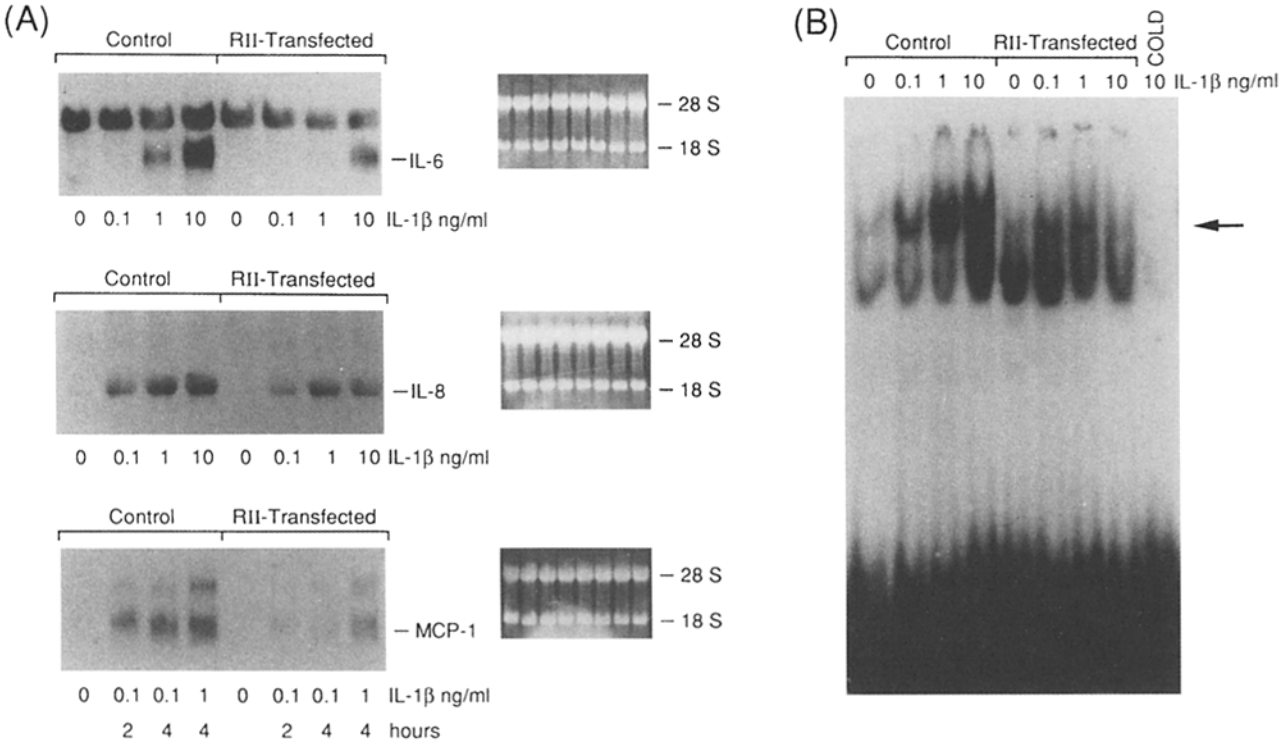
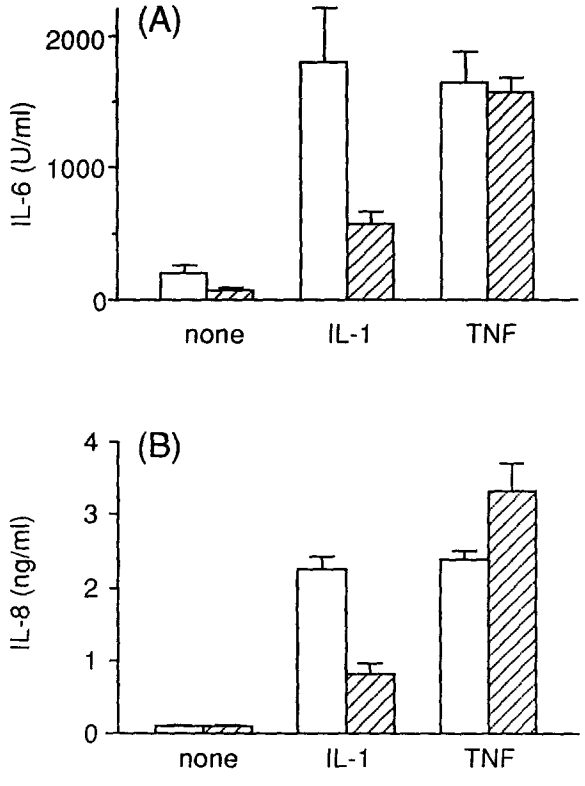


Figure 4. NFkB translocation and gene expression in RII-transfected cells. (*A*) Cytokine gene expression. Cells were exposed to IL-1β for 2–4 h and gene expression was assessed by northern analysis with total RNA using IL-6, IL-8 or MCP-1 probes. The amount of RNA blotted was checked by ethidium bromide staining of the membranes. (*B*) NFkB translocation. EMSA with nuclear extracts from control (83C) and RII-transfected cells (83C7) stimulated with IL-1β for 30 min. 100× excess of cold oligonucleotide was added to control (cold).



of cells to respond to IL-1 via RI. It was important to investigate whether the cytoplasmic portion of the RII, consisting of 29 amino acids (7), played any role in blocking IL-1 activity. Cells were transfected with an RII cDNA construct encoding the extracellular and membrane portions and missing the cytoplasmic tail from aa 372 to 398. As shown in Fig. 6 (*A*), transfer of the Δ372-398 RII gene resulted in an increased capacity to bind IL-1, with 2.5×10^5 sites/cell and a kD of 8.8×10^{-9} M. Cells transfected in parallel with a construct encoding the full-length RII had 4×10^5 sites/cell and a kD of 5×10^{-9} M. The different surface binding obtained reflected the levels of mRNA in the two transfectants (not shown). As shown in Fig. 6 (*B*), expression of a truncated version of RII, missing the last 26 cytoplasmic amino acids, resulted in a marked impairment of the capacity of the cells to respond to IL-1 evaluated as MCP-1 or IL-6 production. Taking into account the different number of binding sites in Δ372-398 versus intact RII transfectants (2.5×10^5 versus 4×10^5 sites/cell), it is apparent that the cytoplasmic truncation did

Figure 5. Selectivity of defective IL-1 responsiveness. Cells transfected with empty vector (8C, *open bars*) or RII (8C7, *hatched bars*) were stimulated with 100 pg/ml IL-1β or 10 U/ml TNFα and IL-6 (*A*) or IL-8 (*B*) were measured.

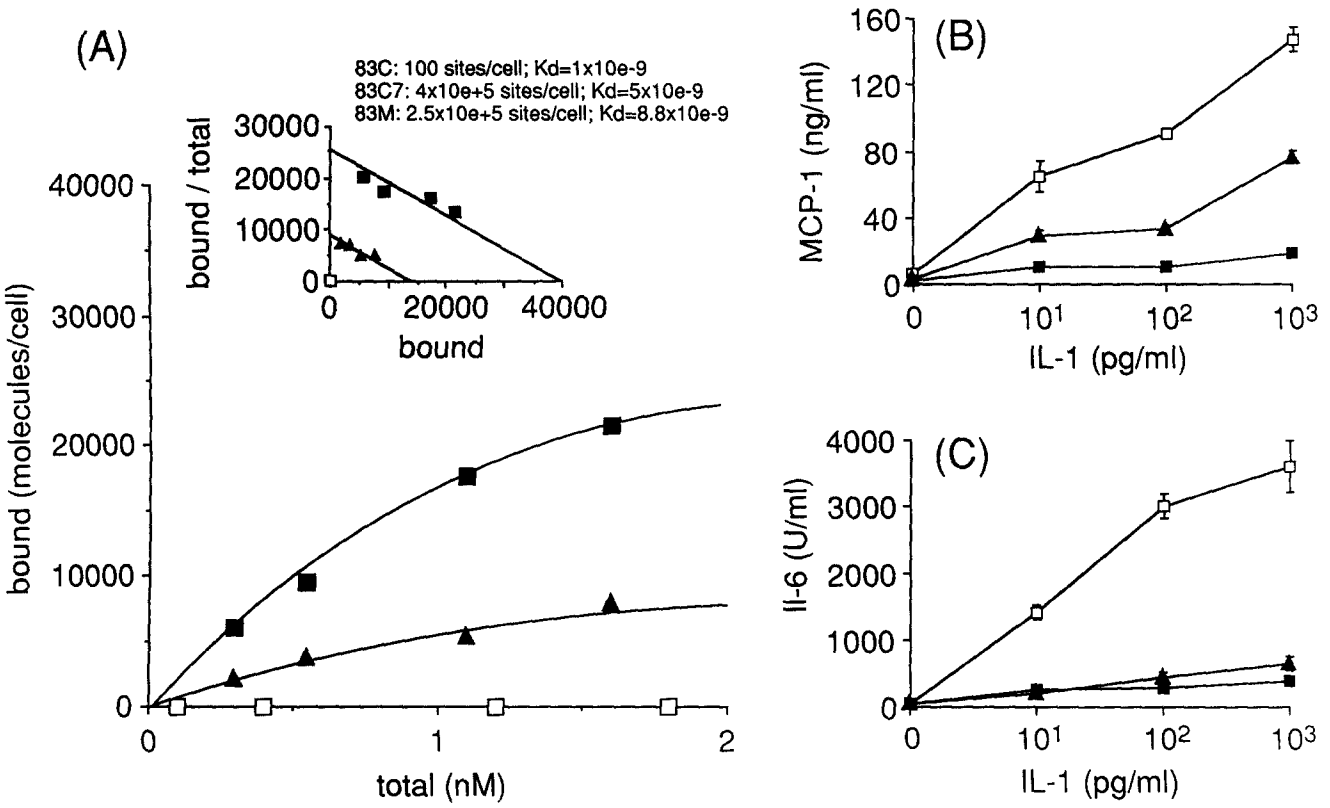


Figure 6. Inhibition of IL-1 responsiveness by Δ372-398 RII gene transfer. (*A*) Saturation curve and Scatchard analysis of IL-1β binding to cells transfected with empty vector (83C □), with RII (83C7 ■) or Δ372-398 RII (83M ▲). Number and affinity of sites were calculated by LIGAND program. The scale on the ordinate does not allow showing individual points for control cells (8C □) in the insert. (*B*) Cells transfected with empty vector (83C □), with RII (8C7 ■) or Δ372-398 RII (83M ▲) were stimulated with graded doses of IL-1β for 24 h and MCP-1 was measured. (*C*) Cells transfected with empty vector (83C h), with RII (8C7 ■) or Δ372-398 RII (83M ▲) were stimulated with graded doses of IL-1β for 24 h and IL-6 was measured.

not affect the capacity of the RII to block IL-1 activity. Thus, the cytoplasmic domain is not involved in the capacity of RII to inhibit responsiveness to IL-1.

Release of RII after Gene Transfer. RII is found in soluble form in biological fluids and culture supernatants (32–39). Release of the RII by myelomonocytic cells is augmented by glucocorticoids, IL-4 and IL-13 (13, 16, 17) and induced rapidly by chemoattractants (20). It was therefore important to investigate whether cells transfected and overexpressing RII released this molecule and whether released RII accounted for the block in responsiveness to IL-1. Using a specific ELISA, we determined that transfected 8387 cells released the RII with, in a representative experiment out of three performed, 17.6 ± 5.9 ng/ml/15 × 10^4 cells/24 h (Fig. 7 *A*). The Δ372-398 RII was also released from transfected cells in amounts comparable to the full length RII (24 ng/ml in 24 h). The released soluble decoy RII, was detectable after 9 h of incubation and continued to accumulate thereafter.

In an effort to molecularly identify the immuno-reactive RII released from transfected cells and to determine its capacity to bind IL-1β, cross-linking experiments were performed. As shown in Fig. 7 *B*, supernatants from transfected cells contained an IL-1β binding molecule with a molecular mass of 60 kD after taking into account the ligand. Competition for cross-linking using blocking anti-RI and anti-RII mAb confirmed release of RII from transfected cells. Based on quantitative estimates by ELISA and on the molecular mass of the released R, we calculated that the number of released receptors/cell in 24 h was 13.5 × 10^5 (mean of two experiments) for cells expressing 4 × 10^5 sites/cell and 2.3 × 10^5 for cells expressing 0.37 × 10^5 sites/cell (one experiment). We conclude that nonhematopoietic cells transfected with RII release substantial amounts of a soluble 60-kD version of the molecule, which retains ligand binding capacity, and that release does not require the cytoplasmic portion of the molecule.

A series of experiments was performed in an effort to establish whether release of RII completely accounted for defective responsiveness to IL-1. First, cells transfected with vector alone or with the RII gene were exposed to different concentrations of IL-1β for 1 or 24 h. Since accumulation of the released RII required several hours, if the released molecule played a dominant role in inhibiting IL-1, the prediction was that transfected cells should retain a relatively higher response to brief (1 h) versus long (24 h) exposure to IL-1. Long (24 h) exposure of control cells to IL-1 resulted in higher responses (Fig. 7 *C*, *open squares* versus *open triangles*), with a minimal effective concentration of >100 pg/ml and 10 pg/ml for incubation with IL-1 for 1

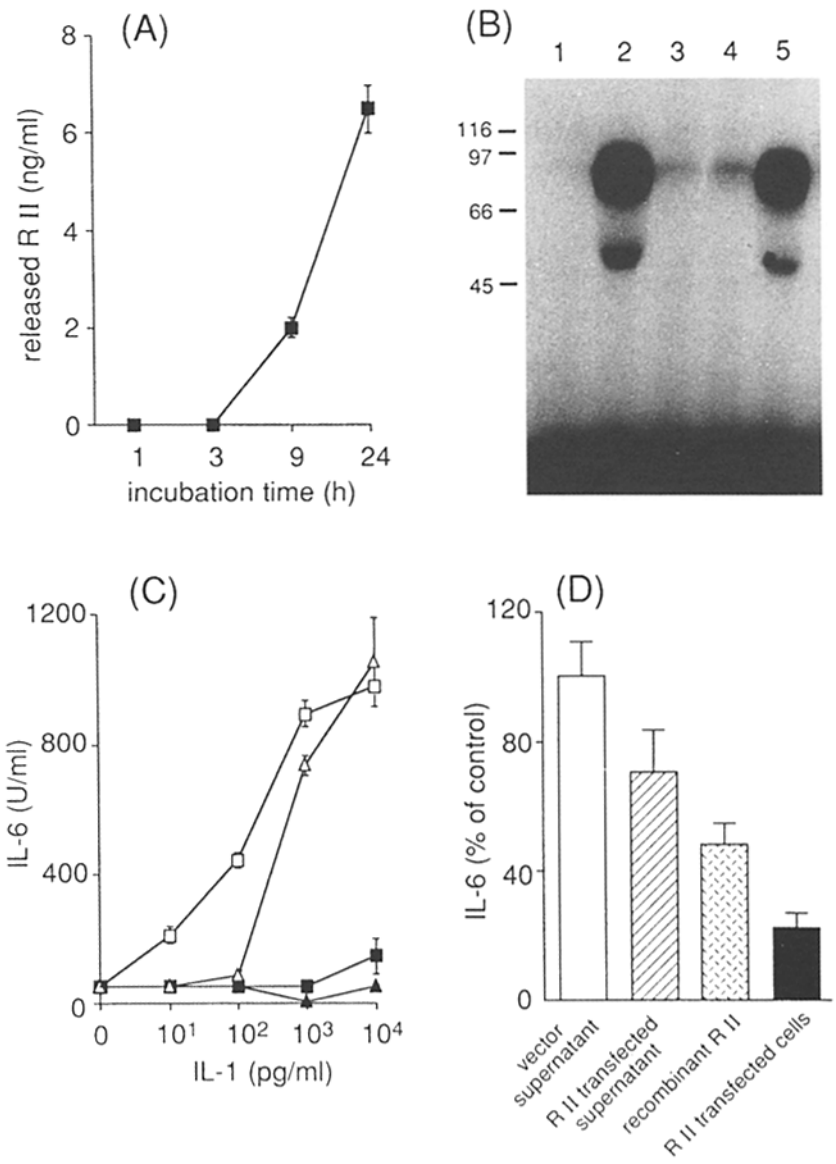


Figure 7. Role of released versus cell-associated RII in defective responsiveness of RII-transfected cells. (*A*) Release of RII. Supernatants were collected at different times of incubation of RII-transfected cells (83C7). Released RII was measured using a specific ELISA. (*B*) Cross-linking of released RII with ^{125}I-IL-1β. Affinity cross-linking of IL-1β to supernatants of cells transfected with empty vector (lane *1*) or RII (from lane *2* to lane *5*). The binding was competed with cold IL-1β (lane *3*), M22 mAb (anti-RII, lane *4*) or M1 mAb (anti-RI, lane *5*). Molecular mass standards are indicated. (*C*) Defective responsiveness is unaffected by the length of exposure to IL-1β. Control and RII transfected (83C and 83C7) cells were incubated with different concentrations of IL-1β for 1 h, washed extensively and further incubated in IL-1–free medium for 23 h. Alternatively cells were exposed to IL-1β for 24 h: (△), Control cells exposed to IL-1β for 1 h; (▲) RII-transfected cells (83C7) exposed for 1 h; (□), Control cells exposed for 24 h; (■), RII-transfected cells (83C7) exposed for 24 h. (*D*) Effect of released RII on IL-1 responsiveness. The 24 h supernatant of vector (8C) or RII transfected cells (83C7) was added to control 8387 cells. IL-1β (1 ng/ml) was added to the culture for 24 h and response was measured as IL-6 production. The supernatant of RII-transfected cells contained 27 ng/ml of released RII. By way of comparison, recombinant RII (100 ng/ml) was added to 8387 cells with IL-1β 1 ng/ml. The defective responsiveness to IL-1β (1 ng/ml) of the same cells used as a source of the supernatant is shown in parallel.

and 24 h, respectively. The defect in the capacity to respond to IL-1β of RII-transfected cells was similar after 1 or 24 h of exposure to the agonist (Fig. 7 *C*, *closed symbols*). Finally, 24 h conditioned medium from RII transfected cells or amounts in excess of recombinant RII were added to control cells. As shown in Fig. 7 *D* the conditioned medium containing 27 ng/ml of soluble RII caused a 29.6% inhibition of the activity of 1 ng/ml IL-1β, and recombinant RII (100 ng/ml) caused a 52% of inhibition of the activity of 1 ng/ml IL-1β. Under the same conditions, transfected cells (the source of conditioned medium) showed a more dramatic impairment of the response to IL-1β, with a 77.9% reduction at 1 ng/ml relative to control cells.

These results indicate that the defective capacity to respond to IL-1 of RII-transfected cells cannot be completely accounted for by release of a soluble version of the molecule and that membrane-bound RII contributes to sequestration of IL-1.

Discussion

The study presented here was designed to test the "decoy receptor" model of function of IL-1 RII. Based on the model (13), it was predicted that expression of the RII obtained by gene transfer in cells expressing only RI should result in a decreased responsiveness to IL-1. Consistently with this prediction, it was found that transfer of RII gene in cells expressing only RI resulted in defective responsiveness to IL-1. The 8387 sarcoma system was analyzed in detail, but similar results were obtained with cell lines of different lineage, including the myelomonocytic cell line U937 (not shown). U937 cells only express RI transcripts

and gene transfer resulted in expression of RII numbers similar to those present on monocytes. RII-transfected U937 showed defective induction of IL-8 mRNA (not shown). Therefore these results provide strong direct evidence, which complements previous data obtained using blocking mAb (10, 13), that RII is not a signaling molecule and that it acts as a molecular trap for IL-1.

RII-transfected cells expressed normal levels of RI. Moreover, blocking mAb directed against RII restored the capacity of transfected cells to respond to IL-1. Conversely, blocking mAb directed against RI inhibited the capacity of control and RII-transfected cells to respond to IL-1. These results exclude that the impairment of IL-1 responsiveness of RII-transfected cells is caused by sequestration or inhibition of expression of RI and demonstrate that the defect depends upon surface expression of RII.

TNF is an inflammatory cytokine whose spectrum of action, effects, and signal transduction pathways are remarkably similar to those of IL-1 (28–31). Cells transfected with IL-1 RII retained responsiveness to TNF. Moreover, blocking mAb directed against RI or RII did not affect responsiveness to TNF. Thus, the impairment of IL-1 responsiveness of transfected cells is specific for IL-1 and does not reflect interference with common transduction pathways.

TNF interacts with two receptors, p55 and p75 (40, 41). The role of p75 in the regulation of TNF activity is complex (42). Under certain conditions it has been shown that p75 captures TNF and presents it to the signal transducing p55 R (25). Transfer of the IL-1 RII gene was never associated with amplified responsiveness to IL-1, even with lines or clones expressing low levels of surface RII (not shown). Moreover, following an approach used for TNF receptors (25), we treated RII-transfected cells with graded concentrations of blocking mAb, in search of a putative "right" concentration of RII suitable to present the ligand to the few RI and thus to amplify response to IL-1. These efforts failed and cell lines expressing low levels of RII never showed augmented responsiveness to IL-1. Thus, all in all, gene transfer and mAb studies gave no indication that the RII can capture the ligand and present it to the signal transducing RI.

RII is found in the supernatants of mitogen activated mononuclear cells, B lymphoblastoid cells and myelomonocytic cells, keratinocytes (13, 32–34, 36, 39), as well as in human plasma from healthy donors or patients with sepsis (35, 37) or inflammatory synovial fluids (35, 38). Anti-inflammatory agents that augment expression of RII in PMN and monocytes (glucocorticoids, IL-4, IL-13), also cause increased release of the molecule (references 13, 16, 17 and Colotta, F., and A. Mantovani, unpublished). More recently, it was found that chemoattractants cause rapid release of RII by activating a performed protease (20). In the present study we found that transfected cells release RII in the absence of deliberate stimulation. The released RII was 60 kD in size and retained the capacity to bind IL-1β, as assessed by cross-linking. Intriguingly, the classic soluble RII described in culture supernatants is 45 kD (13, 16, 17, 32–35). The 60-kD RII found in the supernatant of transfected 8,387 cells resembles the molecule released from monocytes (Colotta, F., and A. Mantovani, unpublished). The molecular basis and mechanisms for heterogeneity of soluble RII as well as its biological significance remain to be defined.

Several lines of evidence suggest that release of RII does not account for the defective IL-1 responsiveness of RII-transfected cells. First, the poor IL-1 responsiveness in terms of cytokine production was equally evident after short (1 h) and long (24 h) exposure to the agonist, i.e., in the absence and presence of substantial amounts of released RII. Second, the supernatant of RII-transfected cells and amounts in excess of recombinant RII did not inhibit the response to IL-1β to an extent as profound as that observed in cells expressing surface RII. Finally, carefully washed RII-trasfected cells showed a profound defect in IL-1β induced NFkB activation, protooncogene and cytokine gene expression, rapid responses that occur in the absence of measurable released RII. Thus, released RII does not account for defective IL-1 responsiveness and surface expression of the molecule plays an important role in unresponsiveness of RII transfected cells. One may speculate that the surface RII may protect myelomonocytic cells, which express this molecule as predominant IL-1 receptor and produce copious amounts of IL-1β, from excessive autocrine stimulation.

The mechanism(s) through which surface RII inhibits IL-1 responsiveness are in part a matter of speculation. In addition to binding IL-1, RII could internalize and scavenge the molecule, acting as scavenger receptor. Evidence compatible with a scavenger function of RII was recently obtained in PMN (F. Colotta, unpublished observation). In addition surface RII may compete for yet undefined components of the signal transducing RI receptor complex. An accessory molecule which cooperates with RI in binding IL-1β (but not IL-1ra) was recently identified (43). Since truncation of the cytoplasmatic portion does not influence the inhibitory capacity, one could speculate that cognate recognition of the ligand by RII and by an accessory molecule could at the same time prevent the ligand and the accessory component from interacting with the signaling RI. The transfected cell lines generated in the present study represent an invaluable tool to further investigate the inhibitory action of RII.

The IL-1ra competes with IL-1 for binding to the receptor and antagonizes its action. Three isoforms of IL-1ra have been described, two of which are intracellular (5, 44–47). Molecules with anti-inflammatory activity such as glucocorticoids, IL-4 and IL-13, induce augmented expression of both IL-1ra (48, 49) and RII (13, 16, 17). IL-1ra binds the RI signaling receptor and, with much lower affinity, the membrane-associated and released RII (7, 17, 38, 50, 51). Therefore IL-1ra and RII represent pathways of negative regulation unique to the IL-1 system which minimally cross neutralize each other.

The results presented here directly demonstrate that RII is a nonsignaling molecule that captures IL-1 and blocks its

action. The inhibitory capacity of transfected RII depends on surface expression, is specific for IL-1 and is independent of the cytoplasmic domain of the molecule. Thus, surface RII is a unique pathway of negative regulation of the IL-1 system, potentially a tool and a target for therapeutic intervention.

This work was supported by strategic project "Cytokines" from CNR, by the 6th AIDS project from Institut Superiore di Sanitá, and by AIRC. M. Muzio is an AIRC fellow.

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Received for publication 11 December 1995 and in revised form 6 February 1996.

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