

# IL-1 $\beta$ Scavenging by the Type II IL-1 Decoy Receptor in Human Neutrophils<sup>1</sup>

Emer Bourke,\* Arianna Casseti,<sup>†</sup> Antonello Villa,<sup>†</sup> Emma Fadlon,<sup>2\*</sup> Francesco Colotta,<sup>3\*</sup> and Alberto Mantovani<sup>4‡</sup>

**IL-1** elicits its cellular effects by binding a heterodimeric receptor consisting of IL-1RI and the accessory protein, IL-1RAcPr. In addition, it binds to IL-1RII, which lacking signaling function has been ascribed a decoy role. The fate of the ligand following interaction with the decoy receptor was examined in human polymorphonuclear cells (PMN), which express predominantly (>90%) IL-1RII. Incubation of PMN with IL-1 $\beta$  results in a rapid decrease in cell surface-associated ligand accompanied by a concomitant increase in internalized IL-1 with 50–60% of IL-1 $\beta$  located intracellularly within 1 h at 37°C. The use of blocking Abs revealed that IL-1 internalization is mediated exclusively by the decoy receptor. The results of inhibitor analysis demonstrate that internalization requires ATP synthesis and involves clathrin-mediated endocytosis. Following removal of the ligand, the receptor was rapidly re-expressed on the cell surface. Cyclohexamide, a protein synthesis inhibitor, had no effect upon the process, suggesting that the re-expressed receptor was recycled. In addition, human keratinocytes stably transfected with IL-1RII (HaCAT 811) also internalized the IL-1RII with 43% cell surface receptor internalized after 90 min. Immunofluorescence microscopy revealed colocalization of the internalized receptor with wheat germ agglutinin-labeled internalized glycoproteins and early endosome Ag-1, a protein associated with the early endosome compartments, indicative of cellular uptake of IL-1RII by endocytosis. In contrast, little or no internalization was observed in other cells of immune origin. These results suggest that the decoy receptor IL-1RII can act as a scavenger of IL-1, representing a novel autoregulatory mechanism of the IL-1 system. *The Journal of Immunology*, 2003, 170: 5999–6005.

**I**nterleukin-1 is one of the most potent proinflammatory cytokines, possessing a wide spectrum of inflammatory, metabolic, hemopoietic, and immunologic properties (1). In addition, elevated levels of the cytokine found in vivo in chronic autoimmune degenerative conditions correlate closely with disease severity and progression, indicating a role for IL-1 as a mediator of disease (2–4). To develop novel therapeutic strategies for the treatment of acute and chronic inflammatory disorders, it is essential to gain a greater understanding of the various regulatory mechanisms controlling the IL-1 system.

One such regulatory mechanism is the endogenous expression of an IL-1R antagonist, which binds to the signaling receptor with similar affinity to the agonist IL-1 $\beta$  but elicits no cellular response of its own (5). Notably, injection of the recombinant form of IL-1R antagonist into patients suffering from rheumatoid arthritis has

been shown to reduce the severe inflammation and bone destruction associated with this disease (6, 7). There are two receptors for IL-1: the type I (IL-1RI), an 80-kDa protein found mainly on T cells fibroblasts, endothelial, and smooth muscle cells and the type II (IL-1RII), a 68-kDa protein, which is the predominant form on B cells, monocytes, and polymorphonuclear cells (PMNs)<sup>5</sup> (8). To date, all evidence suggests that IL-1RI is the main signaling receptor for IL-1 (9–11). Upon binding of IL-1, a trimeric complex forms consisting of ligand, receptor, and accessory protein, which is responsible for transduction of signals to the interior of the cell by engaging a series of intercellular components (12). Although IL-1RII has also been shown to cause formation of this complex (13, 14), it does not seem to mediate any of the cellular responses to IL-1 (15). This is substantiated by the finding that blocking Abs for IL-1RI, but not IL-1RII, inhibit IL-1 activity (10). Hence, a decoy function has been attributed to IL-1RII (8) representing a second mechanism of regulation of the IL-1 system. Its role is to bind IL-1 with high affinity, interfering with binding to the signaling IL-1RI receptor and thus neutralizing the cellular effect normally induced by the ligand.

In addition to the membrane-bound protein, the extracellular domains of the IL-1RII are found as soluble molecules in the circulation and urine under physiological conditions (16, 17). Elevated levels of the soluble IL-1RII are detectable in biological fluids under conditions of immunological disorder (18). It has been shown that rapid shedding of the IL-1RII occurs upon exposure of cells to TNF, PMA, and various chemoattractants and that this shedding is dependent on the action of metalloproteases (19–21). Such release of soluble decoy receptor has been proposed to be an early event in the inflammatory cascade, which acts to limit its

\*Istituto Ricerche Farmacologiche “Mario Negri”, Milan, Italy; <sup>†</sup>Consorzio MIA Dipartimento di Neuroscienze, Università degli Studi di Milano, Bicocca, Milan, Italy; and <sup>‡</sup>Centro IDET, Istituto di Patologia Generale, Università di Milan, Milan Italy.

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<sup>2</sup> Current address: Gendel Ltd., Coleraine, County, Derry, Northern Ireland, U.K.; of Dr. Colotta is Section of Preclinical Pharmacology, Domp S.p.A. Research centre, I-67100 L'Aquila, Italy.

<sup>3</sup> Current address: Section of Preclinical Pharmacology, Domp SpA Research Centre, I-67100 L'Aquila, Italy.

<sup>4</sup> Address correspondence and reprint requests to Dr. Alberto Mantovani, Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62, 20157 Milan, Italy. E-mail address: mantovani@marionegri.it

<sup>5</sup> Abbreviations used in this paper: PMN, polymorphonuclear cell; TCA, trichloroacetic acid; EEA-1, early endosome Ag 1; WGA, wheat germ agglutinin.

severity. The receptor binds tightly to IL-1 $\beta$ , sequestering it within the extracellular compartment and thus preventing its interaction with the signal-transducing IL-1RI. The IL-1RII is also regulated by glucocorticoids and the cytokines IL-4 and IL-13 (11, 22, 23). These well-known anti-inflammatory mediators augment the expression and subsequent release of the IL-1RII, consistent with the idea that the IL-1RII represents an important anti-IL-1 pathway.

It has recently been demonstrated that the membrane-bound form of IL-1RII is itself directly involved in the negative regulation of IL-1 (24) and that it acts as a dominant negative by forming a nonsignaling complex with the accessory protein (8, 13, 14). In the present study, we report that binding of IL-1 to membrane-anchored IL-1RII causes internalization of the receptor-ligand complex, representing a further level of IL-1 autoregulation. Our results suggest that upon exposure of human PMNs to IL-1 $\beta$ , cell surface expression of the IL-1RII is dramatically down-regulated due to internalization of the receptor-ligand complex. In addition, there is recycling of the receptor following internalization. The internalization of IL-1 by IL-1RII and the surface re-expression of the receptors may represent a mechanism by which IL-1 is efficiently removed from inflammatory sites, suggesting that the type II IL-1R not only acts as a decoy but also as a scavenger of IL-1.

## Materials and Methods

### Reagents

All chemicals were supplied by Merck (Darmstadt, Germany) unless otherwise stated. NaCl (0.9%) and distilled water (Bieffe, Bergamo, Italy), Ficoll (Seromed-Biochem, Berlin, Germany), Percoll (Pharmacia, Uppsala, Sweden), RPMI 1640 (Life Technologies, Glasgow, Scotland), FCS (HyClone Laboratories, Logan, UT), glycine (Bio-Rad, Richmond, CA), IL-1 $\beta$  (Domp Research Centre, L'Aquila, Italy),  $^{125}$ I-IL-1 $\beta$  (NEN, Bad Homburg, Germany), BSA (Sigma-Aldrich, St. Louis, MO), cyclohexamide (Sigma-Aldrich), M4 and M22 neutralizing Abs against type I and type II IL-1R, respectively (kind gift from Dr. J. Sims, Amgen, Seattle, WA), and sodium azide (Sigma-Aldrich).

Medium used routinely was RPMI 1640-10% heat-inactivated FCS supplemented with 2 mM L-glutamine (Life Technologies), 20 mM HEPES (Merck), 50 U/ml penicillin, and 50 mg streptomycin (Bristol-Myers Squibb, Sermoneta, Italy). For routine culture of HaCAT811 keratinocytes, DMEM-10% heat inactivated FCS was supplemented with Geneticin G418 (Life Technologies) at a final concentration of 500  $\mu$ g/ml.

### Cells

Human PMNs were separated from peripheral blood of healthy donors using a two-step buoyant density centrifugation as previously described (25). Briefly, buffy coats were centrifuged over a Ficoll gradient. PMNs collected from the pellet were then underlaid and centrifuged with 62% Percoll-RPMI 1640-10% FCS. PMNs of >95% purity, determined by morphology, were resuspended in binding buffer (RPMI 1640-1% BSA, pH 7.4).

The human keratinocyte cell line HaCAT811 was kindly supplied by D. Boraschi (Domp Research Centre). The human keratinocyte cell line HaCAT, which does not express wild-type IL-1RII, was stably transfected with IL-1RII cDNA (26). This cell line was maintained in DMEM supplemented with 2 mM L-glutamine, HIFCS, and 500  $\mu$ g/ml geneticin (used originally to select out transfected cells). Cells were grown to confluency before passage or experimental use.

### IL-1 binding and internalization assay

Briefly,  $4 \times 10^6$  PMN or  $1 \times 10^6$  HaCATs were incubated with 1 nM  $^{125}$ I-IL-1 $\beta$  in the presence or absence of a 200-fold excess of IL-1 $\beta$  in binding buffer at 4°C for 2 h in a final volume of 50  $\mu$ l. Labeled cells were washed twice, resuspended in binding buffer, and incubated at 37°C. Specific binding was calculated by subtracting nonspecific binding, determined in the presence of excess unlabeled ligand, from total binding.

The distribution of surface-bound and intracellular  $^{125}$ I-IL-1 $\beta$  was determined by washing the cells for 5 min on ice with 50 mM glycine-0.8% NaCl/HCl (pH 3) (285 Osmol) in a volume of 75  $\mu$ l per  $4 \times 10^6$  PMNs or 75  $\mu$ l/ $1 \times 10^6$  HaCAT811s. To separate bound from free cytokine, cells

were centrifuged over sucrose gradient (20% sucrose, 1% BSA) and the activity of the pellets and the supernatants was determined by a gamma counter.

### Determination of degraded and intact $^{125}$ I-IL-1 $\beta$

Degradation status of the IL-1 released into the cell supernatant was determined by trichloroacetic acid (TCA) solubility. Media removed from the cells during the incubation at 37°C were precipitated in a final concentration of 10% TCA on ice for 15 min. These were then centrifuged at 14,000 rpm for 10 min and the soluble (completely degraded IL-1) and precipitate-associated (intact or partially intact IL-1)  $^{125}$ I-IL-1 $\beta$  activity was determined.

### Immunofluorescence microscopy

Medium was removed from HaCAT811 cells (seeded at  $2 \times 10^5$  cells/ml in 10-cm-diameter petri dishes and grown to subconfluency), replaced with medium containing 1 nM IL-1 $\beta$ , and cells were incubated for 10 min. Control cells incubated in medium alone were done in parallel. Cells were fixed for 20 min at room temperature with 4% paraformaldehyde/0.25% glutaraldehyde in 125 mM phosphate buffer (pH 7.4). Cells were scraped and collected by centrifugation. Pellets were washed with the phosphate buffer, infiltrated with concentrated sucrose, and frozen in a 3:1 (v/v) mixture of propane and cyclopentane cooled with liquid nitrogen. Sections (~1- $\mu$ m thick) were prepared using a cryosection apparatus (RMC; Boeckeler Instruments, Tucson, AZ), placed on glass slides, treated briefly with 0.1 M glycine in PBS (pH 7.4) followed by 0.3% Triton X-100, 15% filtered goat serum, 0.45 M NaCl, and 10 mM phosphate buffer (pH 7.4). Sections were incubated overnight at 4°C with mAb early endosome Ag 1 (EEA-1; BD Transduction Laboratories, Lexington, KY) and anti-IL-1RII M22 (see above), washed, and incubated for 1 h at room temperature with the appropriate fluorescein (FITC) and rhodamine (tetramethylrhodamine isothiocyanate) conjugate secondary Abs (Jackson ImmunoResearch, West Grove, PA). Following a final wash, sections were mounted with glycerol. Confocal microscopy was carried out using a Radiance 2100 microscope (Bio-Rad) equipped with a krypton/argon laser. Noise reduction was achieved by kalman filtering during acquisition.

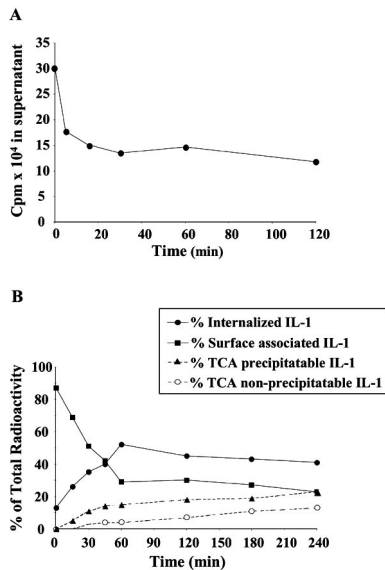
For the endocytosis assay, subconfluent cells grown on coverslips were incubated for 1 h on ice with FITC-wheat germ agglutinin (WGA) conjugates; Molecular Probes, Eugene, OR) in PBS containing 0.1 mM Ca $^{2+}$ , 1 mM Mg $^{2+}$ , and 1% BSA. The cells were washed and incubated at 37°C in the absence and presence of 1 nM IL-1 $\beta$ . Cells were washed and fixed in 4% paraformaldehyde. The coverslips were washed again and the above protocol of immunofluorescence was carried out using anti-human IL-1RII Ab 8.5 (27) (a kind gift from G. Peri, Istituto Mario Negri, Milan, Italy) and rhodamine (tetramethylrhodamine isothiocyanate)-conjugated secondary Ab.

## Results

### IL-1 $\beta$ internalization and scavenging in human neutrophils

To directly measure the ability of freshly isolated human PMNs to sequester active IL-1 from the extracellular environment, we monitored the disappearance over time of radiolabeled IL-1 $\beta$  from medium removed from the cells. Radioactivity within the medium was found to decrease rapidly within the first 10- to 20-min incubation with the PMNs at 37°C indicating scavenging of radioactive IL-1 by the cells (Fig. 1A). We calculated that upon incubation with PMNs,  $2 \times 10^6$  IL-1 $\beta$  molecules/cell were sequestered over a period of 30 min. This was followed by a plateau indicating a state of equilibrium in which further uptake of IL-1 is balanced by release of intact and degraded IL-1 into the medium.

To further characterize the fate of IL-1 $\beta$  bound to IL-1 surface receptors, PMNs were incubated with saturating amounts of  $^{125}$ I-IL-1 at 4°C in the absence and presence of a 200-fold excess of unlabeled cytokine. Unbound cytokine was then removed and the IL-1 $\beta$ -bound cells were incubated at 37°C in fresh medium to allow receptor internalization. At various time points, aliquots were removed, medium was collected, and cells were treated with acidic glycine buffer. This acid wash removes surface bound IL-1 without affecting the internalized cytokine. Preliminary experiments carried out at 4°C showed that the acid wash was 85–95% effective in removal of cell surface-associated ligand (results not shown). The levels of radioactivity contained in the supernatant,



**FIGURE 1.** A, Disappearance of  $^{125}\text{I}$ -IL-1 from medium removed from the cells. PMNs ( $5 \times 10^5$  cells/ $50 \mu\text{l}$ ) were incubated with  $^{125}\text{I}$ -IL-1 $\beta$  for the indicated periods of time. Medium was then removed from the cells and cpm were determined by a gamma counter. Total radioactivity added was measured by adding  $3 \times 10^{-12}$  mol of  $^{125}\text{I}$ -IL-1 $\beta$  to  $50 \mu\text{l}$  of medium in the absence of cells. Results are expressed as number of molecules  $\times 10^{11}$  of  $^{125}\text{I}$ -IL-1 $\beta$  within the medium. B, Internalization of IL-1 $\beta$  in PMNs. Human PMNs ( $4 \times 10^6$  cells/ $50 \mu\text{l}$ ) were incubated with 1 nM  $^{125}\text{I}$ -IL-1 $\beta$  in the presence or absence of a 200-fold excess of cold IL-1 $\beta$  in binding buffer at  $4^\circ\text{C}$  for 2 h. Labeled cells were washed, resuspended in binding buffer, and incubated at  $37^\circ\text{C}$  for the indicated time periods. Cells were transferred to  $4^\circ\text{C}$ , the medium was removed, and the cell pellet was acid washed to remove surface-bound  $^{125}\text{I}$ -IL-1 $\beta$ . The distribution of surface-bound and intracellular  $^{125}\text{I}$ -IL-1 $\beta$  was determined by separating acid wash removable and cell pellet-associated counts by sucrose gradient. Medium removed from the cells was analyzed by TCA precipitation to determine the degradation status of the IL-1 $\beta$  released. The radioactive counts of the cell pellets (internalized IL-1), the acid wash (surface-associated IL-1), TCA-precipitable (intact or partially degraded IL-1), and TCA-soluble (degraded IL-1) fractions from the supernatants were determined by a gamma counter. Data are presented as a percentage of specific bound  $^{125}\text{I}$ -IL-1 $\beta$  on the PMN surface after 2 h of binding and are representative of five experiments.

acid wash, and cell pellet, representing released, surface-bound, and internalized ligand, respectively, were determined for each time point. Fig. 1B illustrates the rate of internalization of  $^{125}\text{I}$ -IL-1 $\beta$  into human PMNs. The IL-1-IL-1R complex was rapidly internalized with 50–60% of radioactivity associated with the pellet after 1 h. The percentage of receptor within the cell remained relatively unchanged up to 4 h of incubation. Over the same 60-min period, cell surface-bound cytokine decreased to 25% and remained constant thereafter. This was accompanied by a concomitant accumulation within the medium of both TCA-precipitable and nonprecipitable radioactive fractions. The nonprecipitable fraction represents fully degraded IL-1, whereas the precipitable fraction represents both intact and partially degraded molecules which are indistinguishable from each other using the TCA method. Indeed SDS-PAGE analysis of the medium (results not shown) revealed bands representing partially degraded IL-1 in addition to intact and fully degraded IL-1. Thus, much of the IL-1 released back into the medium following internalization has been rendered inactive within the cell.

### Internalization of $^{125}\text{I}$ -IL-1 $\beta$ is mediated by the IL-1 type II receptor

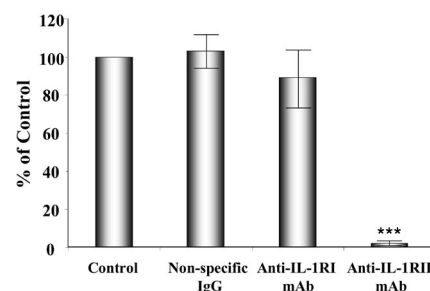
Previous studies have demonstrated that human PMNs express >90% IL-1RII (28). This would suggest that the IL-1 internalization observed in these cells is primarily mediated by the IL-1RII. To determine the exact contributions of the type I and the type II receptors to internalization, binding and internalization experiments were carried out in the presence of blocking mAbs against the IL-1RI or IL-1RII. Anti-type I Ab (M4) had no effect on binding and internalization, whereas the anti-type II Ab (M22) inhibited both binding and internalization by up to 98% (Fig. 2). These data indicate that the IL-1 type I receptor, and thus signaling by IL-1, is not required to mediate internalization of the type II receptor.

### Effects of different agents upon the internalization of IL-1RII

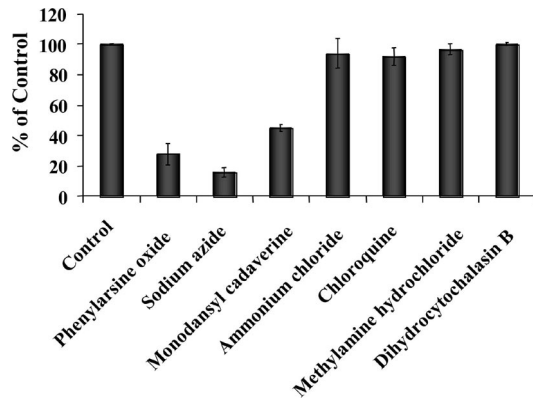
To examine the underlying mechanism of IL-1R-mediated internalization of ligand, we tested the effects of a range of known inhibitors of receptor endocytosis on IL-1 internalization in human PMNs. Phenylarsine oxide, a general inhibitor of receptor-mediated endocytosis, inhibited internalization in the PMNs by 70% (Fig. 3). The metabolic blocker sodium azide inhibited IL-1 uptake by 84%, indicating the involvement of ATP synthesis in the internalization process. Monodansylcadaverine, a potent inhibitor of transglutaminases, is thought to block clathrin-mediated endocytosis at the receptor-invasion step. Pretreatment of the PMNs with this agent caused 45% inhibition of internalization, indicating the possible involvement of clathrin-mediated mechanisms in IL-1 internalization in these cells. This is corroborated by results of immunofluorescent microscopic analysis (see below), which show colocalization of IL-1RII with the early endosome compartments known to be downstream during clathrin-mediated endocytosis. Lysosomotropic agents (e.g., ammonium chloride, chloroquine, methylamine hydrochloride, and, an inhibitor of actin assembly, dihydrocytochalasin B) all failed to inhibit receptor-mediated IL-1 internalization at  $37^\circ\text{C}$  (Fig. 3).

### Recycling of the IL-1R after internalization

PMNs were first incubated with saturating amounts of unlabeled IL-1 $\beta$  at  $37^\circ\text{C}$  to allow internalization of IL-1RII. Then unbound ligand was removed and the cells were further incubated at  $37^\circ\text{C}$  for various periods of time to evaluate the reappearance of the receptor at the cell surface. At each time point, cells were washed

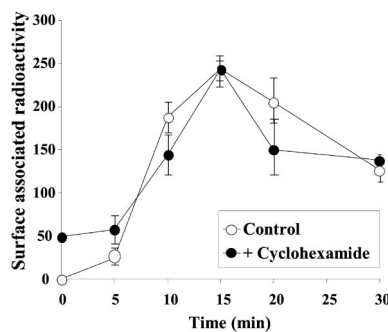


**FIGURE 2.** Internalization of  $^{125}\text{I}$ -IL-1 $\beta$  by PMNs is mediated by the IL-1 type II receptor. Binding and internalization were carried out as described previously in the absence and presence of blocking Abs to IL-1RI (M4), IL-1RII (M22), or an irrelevant IgG2b control at  $10 \mu\text{g/ml}$ . Following 1-h internalization, cells were removed and subjected to an acid wash and the percentage of internalized IL-1 was determined. Data are expressed as the percentage inhibition relative to untreated cells and is representative of three independent experiments. Statistical analysis was carried out using the student *t* test with \*\*\* representing a  $p < 0.01$ .



**FIGURE 3.** Effects of different agents upon the internalization of IL-1RII. Briefly,  $4 \times 10^6$  PMNs were incubated for 30 min at 37°C in binding buffer in the presence of inhibitors at the following concentrations: phenylarsine oxide (50  $\mu$ M), monodansylcadaverine (100  $\mu$ M), sodium azide (0.1%), ammonium chloride (20 mM), chloroquine (100  $\mu$ M), methylamine hydrochloride (10 mM), and dihydrocytochalasin B (10  $\mu$ M). These concentrations were maintained throughout the course of the experiment. A control without addition of any agent was carried out in parallel. Saturation binding followed by internalization was carried out as described previously. Results are pellet-associated radioactivity in the presence of inhibitors expressed as percentage of control.

and subjected to binding with  $^{125}$ I-IL-1 $\beta$  at 4°C. As shown in Fig. 4, IL-1 binding gradually increased with time, peaking at 15 min and decreasing again possibly due to another cycle of receptor internalization. The IL-1 binding capacity of PMNs re-expressing IL-1RII was found to be approximately 60–70% of that of the same cell population before the internalization process. Since reappearance of the protein could be due to either de novo synthesized molecules and/or re-expression of the internalized receptors, we carried out this experiment in the absence and presence of the protein inhibitor cycloheximide. The re-expression was unaffected by the presence of this agent, suggesting that the receptors reappearing at the surface are preformed and de novo protein synthesis is not required. Since the IL-1RII does not signal, functional properties of this molecule other than binding could not be measured.



**FIGURE 4.** Recycling of the IL-1RII after internalization. Human PMNs ( $4 \times 10^6$  cells) were incubated for 2 h at 4°C in binding buffer containing 10 nM unlabeled IL-1 $\beta$  in the absence (○) and presence (●) of 10  $\mu$ g/ml cyclohexamide and then transferred to 37°C for 1 h. Cells were acid washed, resuspended in binding buffer, and further incubated at 37°C for the indicated times. Cells were then subjected to binding with  $^{125}$ I-IL-1 $\beta$  at 4°C in the absence and presence of 200-fold excess of IL-1 $\beta$ . The pellet-associated activity was then determined by a gamma counter. Cyclohexamide concentration was kept constant throughout the course of the experiment. Data are representative of three independent experiments.

### Internalization of IL-1RII in different cellular contexts and localization in early endosomes

The type II decoy receptor is the main IL-1-binding molecule expressed in monocytes (29); therefore, the study was extended to include this cell type. Levels of internalization in freshly isolated human monocytes were low, with a maximum of 10–25% of radioactivity detectable within the cell pellet after a 2-h incubation at 37°C (results not shown). No internalization was detectable in the monocyte cell lines THP-1 or MonoMac (results not shown). In addition, we tested other cell types of immune origin such as macrophages and dendritic cells (mature and immature) in which little or no internalization was detectable. In contrast, when we carried out the same experiment with the human keratinocyte cell line HaCAT811, significant levels of internalization were observed. This line was stably transfected to overexpress the gene encoding IL-1RII, ensuring high levels of decoy receptor surface expression. Similar to results in the PMNs, this cell type also rapidly internalized the ligand receptor complex, peaking at 42% internalization after 1.5 h incubation (Fig. 5A).

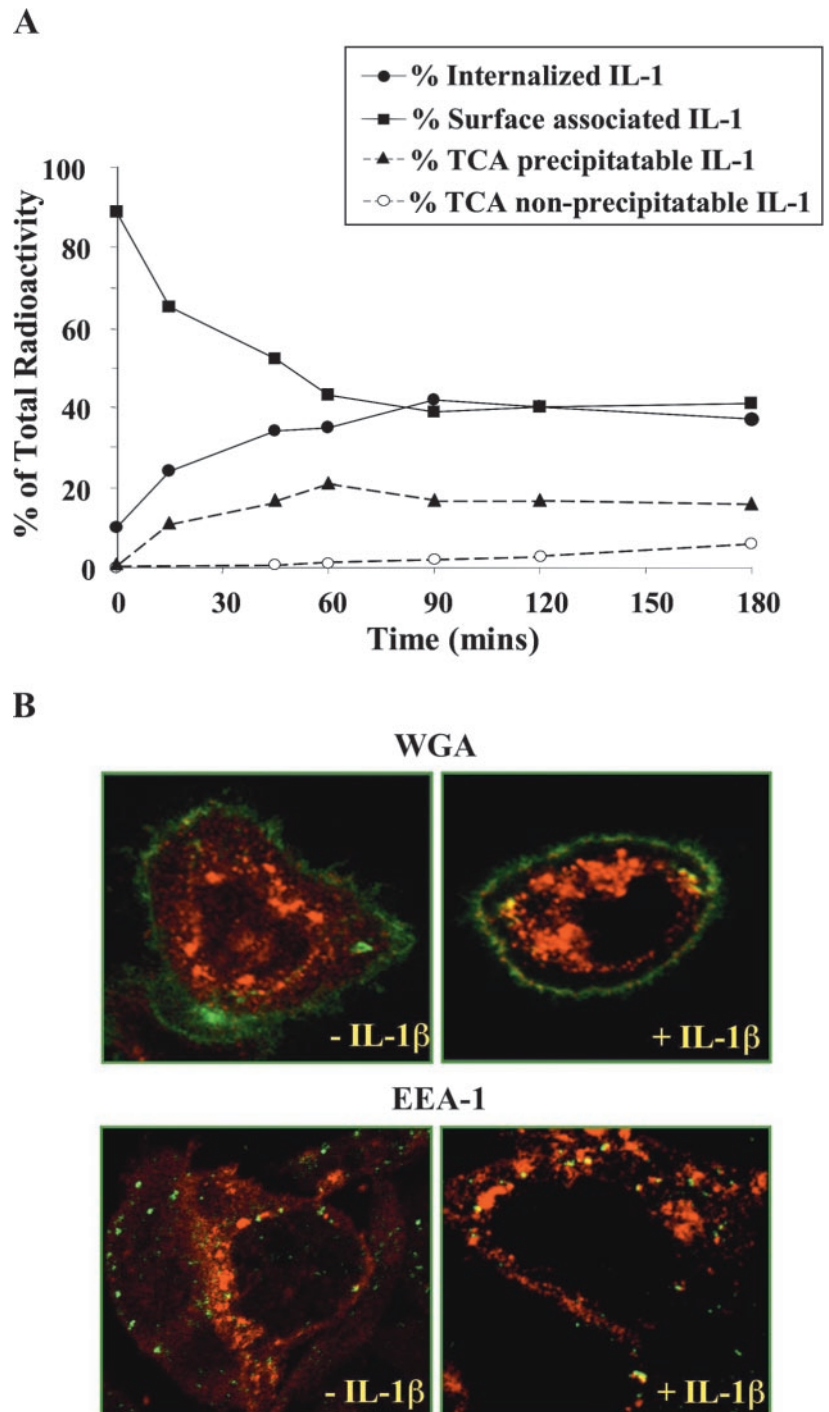
The process of IL-1R internalization was also examined immunohistochemically. The HaCAT811 cell line was used in preference to the PMNs to ensure sufficiently high, consistent levels of IL-1RII expressed at the cell surface, albeit this also resulted in highly elevated levels of receptor detectable within the cytoplasm in transit to cell membrane (Fig. 5B). Following a 10-min incubation with IL-1 $\beta$ , IL-1RII receptors were found to colocalize with internalized WGA-labeled surface glycoproteins, indicating the involvement of receptor-mediated endocytosis during internalization of IL-1 $\beta$  in these cells (30, 31). At later time points (results not shown), similar colocalization was also observed occurring in some cells even in the absence of IL-1 $\beta$ . Furthermore, IL-1RII colocalized extensively with EEA-1 (an established marker of early endosomes (32)) in IL-1 $\beta$ -treated cells when compared to controls. Early endosomes are the first cellular compartments into which endocytosed molecules pass during the process of endocytosis (33). Therefore association with these compartments further corroborates the finding that the IL-1-IL-1RII complex is readily internalized by endocytosis into HaCAT811s upon exposure to IL-1.

### Discussion

The present study shows that following binding to IL-1RII on human PMNs, IL-1 $\beta$  is internalized. The rate of internalization is slow compared to other cytokine receptors, such as IL-8 or GM-CSF, which have been shown to achieve maximal internalization by 10 min (34–36). It is however comparable to the kinetics of internalization of the type I IL-1R observed previously (37–39). In fact, Heguy et al. (40) showed that deletion mutants of the IL-1RI lacking most of the cytoplasmic domain fail to engage the signaling apparatus but retain the ability to bind and internalize IL-1 concurrent with our results on IL-1RII, the structure and function of which resembles a truncated IL-1RI.

Characterization of IL-1 internalization in PMNs revealed absolute temperature dependency since no internalization can be detected when the temperature of incubation is lowered to 4°C as indicated by elution of 95% of  $^{125}$ I-IL-1 $\beta$  by acid washing of the cell surface. Internalization is followed by an accumulation in the medium of intact, partially, and fully degraded IL-1 molecules. Anti-IL-1RI blocking Abs had no effect upon the internalization process, confirming that IL-1 internalization is mediated exclusively by the IL-1RII in this cell type. In addition, we observed that IL-1 is rapidly taken up by the PMNs from medium spiked with a fixed amount of radioactive IL-1. These results indicate that in

**FIGURE 5.** Internalization of IL-1RII in the IL-1RII-transfected human keratinocyte cell line HaCAT811. *A*, HaCAT811 cells ( $1 \times 10^6/50 \mu\text{l}$ ) were incubated with 1 nM  $^{125}\text{I}$ -IL-1 $\beta$  in the presence or absence of a 200-fold excess of cold IL-1 $\beta$  in binding buffer (DMEM-0.1% BSA, pH 7.4) at 4°C for 4 h. Labeled cells were washed, resuspended in binding buffer, and incubated at 37°C for the indicated time periods. Cells were transferred to 4°C, the medium was removed, and the cell pellet was acid washed to remove surface-bound  $^{125}\text{I}$ -IL-1 $\beta$ . Cell-associated and acid-removable counts were separated by sucrose gradient. Medium removed from the cells was analyzed by TCA precipitation to determine the degradation status of the IL-1 $\beta$  released. The radioactive counts of the cell pellets (internalized IL-1), the acid wash (surface-associated IL-1), TCA-precipitable (intact or partially degraded IL-1), and TCA-soluble (degraded IL-1) fractions from the supernatants were determined by a gamma counter. Data are presented as a percentage of specific bound  $^{125}\text{I}$ -IL-1 $\beta$  on the PMN surface after 2 h of binding and are representative of five independent experiments. *B*, Colocalization with WGA: Subconfluent HaCAT811 cells were labeled with FITC-WGA (a marker for membrane transport) at 0°C and incubated at 37°C in the absence (-IL-1 $\beta$ ) and presence (+IL-1 $\beta$ ) of 1 nM IL-1 $\beta$  before fixation and staining with anti-human IL-1RII Ab. In the presence of IL-1 $\beta$  the confocal image shows colocalization (yellow) of IL-1RII (red) with the internalized labeled glycoproteins (green), which is not apparent in untreated cells. Colocalization with EEA-1: HaCAT811 cells incubated at 37°C in the absence (-IL-1 $\beta$ ) and presence (+IL-1 $\beta$ ) of 1 nM IL-1 $\beta$  were fixed, collected, and permeabilized. Cells were incubated with anti-EEA-1 (a marker for early endosomes) Ab and anti-IL-1RII Ab. The presence of the yellow staining in cells treated with IL-1 $\beta$  shows colocalisation of IL-1RII with EEA-1 which indicates the presence of IL-1RII within the early endosomes of the cell.



addition to its decoy receptor function IL-1RII serves to inhibit the actions of IL-1 by shuttling active IL-1 into the cell away from the extracellular environment, thus preventing its interaction with the signaling IL-1RI molecule.

The nonspecific inhibitor of internalization, phenylarsine oxide, which reacts with sulfhydryl groups, forming stable ring structures, was found to strongly inhibit internalization of the IL-1R. The process was found to be ATP synthesis dependent since it was inhibited by the metabolic inhibitor sodium azide. Monodansyl cadaverine, a known inhibitor of clathrin-mediated endocytosis, was found to partially inhibit IL-1 internalization. In contrast, lysototropic agents such as ammonium chloride, methylamine hydrochloride, and chloroquine, which act by neutralizing the cyto-

solic environment, had no significant inhibitory effects on internalization. In addition, dihydrocytochalasin B had no effect on internalization, suggesting that actin polymerization is not involved in the process. This is in fact in agreement with the observations made in a previous study on ligand-induced internalization of the IL-8R (35).

Rapid reappearance of the receptor was observed reaching peak levels after only 15 min, then rapidly decreasing once more possibly due to another cycle of internalization. This indicates that following internalization, there is constant turnover of the IL-1RII receptor, ensuring the constant scavenging of active IL-1, neutralizing its effects on the signaling receptor. De novo protein synthesis was not associated with this event since cyclohexamide had no

effect; however, whether these receptors are obtained from a preformed intracellular pool, as for TNF- $\alpha$  (41), or from recycled receptors cannot be determined.

In an effort to assess whether IL-1 $\beta$  internalization and scavenging was restricted to PMNs, similar experiments were conducted in monocytes, which predominantly express the type II decoy receptor, as well as in a IL-1RII-transfected cell line, HaCAT811. Internalization was modest or absent in fresh human monocytes and monocyte-derived cell lines. In contrast, a substantial fraction of IL-1RII-bound radioactive IL-1 $\beta$  was internalized by HaCAT811 cells. Therefore, the cellular context dictates whether the IL-1 type II receptor, in addition to inhibiting and neutralizing IL-1 $\beta$ , also serves as a scavenging mechanism.

Further analysis of IL-1RII internalization by confocal immunofluorescence microscopy, using HaCAT811 as a model, revealed that in the presence of IL-1 $\beta$  IL-1RII is found intracellularly, closely associated with WGA. This plant lectin glycoprotein is a marker of membrane transport (30) as it readily binds cell surface glycoproteins colocalizing with them upon endocytosis. Moreover, upon exposure of cells to IL-1, IL-1RII associates with the early endosome protein, EEA-1. Early endosomes are the main sorting compartment from which ligands may then be released and transported to lysosomes and receptors recycled to the membrane or from which the entire ligand-receptor complex is transported to lysosomes or recycled to the plasma membrane (42). The presence of IL-1RII within the early endosomes reveals a process whereby the IL-1-IL-1RII complex is endocytosed by clathrin-coated pits. These invaginate forming coated vesicles, which uncoat and transport the entire complex to the early endosomes (33). We therefore suggest this to be the underlying mechanism of IL-1RII-mediated IL-1 internalization in HaCAT811 cells. Indeed, our observation that the inhibitor of clathrin-mediated endocytosis, monodansyl cadaverine, can inhibit IL-1 internalization in human PMNs is suggestive of a similar mechanism in this cell type.

Our findings support the hypothesis that the IL-1RII is a potent anti-inflammatory molecule not only in its actions as a decoy receptor but also by its ability to scavenge IL-1 molecules at sites of inflammation. Shuttling into the cell of the receptor-ligand complex reduces the concentration of free IL-1 available for interaction with the IL-1RI signaling receptor, effectively neutralizing the effects of IL-1. Modulation of such a physiological autoinhibitory mechanism may provide a means to intervene therapeutically in the initiation and development of inflammatory disorders.

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