

# The Soluble Form of IL-1 Receptor Accessory Protein Enhances the Ability of Soluble Type II IL-1 Receptor to Inhibit IL-1 Action

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## Summary

Regulation of the activity of the proinflammatory cytokine IL-1 is complex, involving transcriptional and translational control, precursor processing, a receptor antagonist (IL-1ra), and a decoy receptor. Here we report that the soluble form of the IL-1 receptor accessory protein (AcP) increases the affinity of binding of human IL-1 $\alpha$  and IL-1 $\beta$  to the soluble human type II IL-1 receptor by approximately 100-fold, while leaving unaltered the low binding affinity of IL-1ra. Soluble AcP is present in normal human serum at an average concentration greater than 300 ng/ml. These findings suggest that the soluble form of IL-1R AcP contributes to the antagonism of IL-1 action by the type II decoy receptor, adding another layer of complexity to the regulation of IL-1 action.

## Introduction

The proinflammatory cytokine interleukin-1 (IL-1) is thought to play a role in many diseases, including arthritis, heart disease, pancreatitis, multiple myeloma, and stroke (see Dinarello, 1998, for review). Inhibition of IL-1 is beneficial in many murine models of disease. Two natural regulators of IL-1 activity exist that potentially can be used for this purpose in human therapy. One of these, IL-1 receptor antagonist or IL-1ra (Hannum et al., 1990; Eisenberg et al., 1990), binds to the signaling IL-1 receptor (type I IL-1R) with high affinity but fails to elicit a biological response. By blocking the receptor, however, it interferes with the activity of the agonist IL-1 forms IL-1 $\alpha$  and IL-1 $\beta$ . Treatment of rheumatoid arthritis patients with recombinant IL-1ra is of demonstrated clinical benefit (Dayer et al., 2001). The other natural regulator of IL-1 action is the type II IL-1 receptor (McMahan et al., 1991; Colotta et al., 1993). Although this protein binds tightly to IL-1 $\beta$ , it lacks a cytoplasmic domain and cannot signal; it therefore functions as a decoy receptor. The type II IL-1R also binds IL-1 $\alpha$ , but with significantly lower affinity. It also binds poorly to IL-1ra, with the result that the two regulators of IL-1 activity complement rather than neutralize each other.

The type II IL-1R is initially synthesized as a transmem-

brane protein, but the extracellular, ligand binding domain can be shed from the cell surface via proteolytic cleavage, and remains able to bind IL-1 $\beta$  (Giri et al., 1994). Natural circulating levels of soluble type II IL-1R are approximately 1–5 ng/ml (Giri et al., 1994; Jouvenne et al., 1998). Because the soluble form of type II IL-1R, like the surface form, binds IL-1ra poorly, inhibition of IL-1 is enhanced when both are present, in contrast to the interference that occurs when both soluble type I IL-1R and IL-1ra are present (Burger et al. 1995).

Following binding of IL-1 $\alpha$  or IL-1 $\beta$  to the type I IL-1R, a second protein, the IL-1 receptor accessory protein (AcP), is recruited to the complex (Greenfeder et al., 1995). Both proteins, IL-1R and AcP, are required for signaling. AcP can also be recruited to the transmembrane form of the type II IL-1R, once the latter has bound IL-1 $\beta$  (Lang et al., 1998; Malinowsky et al., 1998). Thus, the type II IL-1R decoys not only IL-1, but also AcP, away from productive association with the type I IL-1R, enhancing its effectiveness as a negative regulator.

In addition to the transmembrane form of AcP, another form exists, encoded by an alternatively spliced mRNA (Greenfeder et al., 1995; Jensen et al., 2000). This form contains only the extracellular domain of AcP and is assumed to be secreted from cells and soluble in blood and extracellular fluids. It has been suggested that soluble AcP might be recruited to the IL-1/IL-1R complex, which would prevent association of the full-length, signal-competent form of AcP. Soluble AcP would thereby function as yet another negative regulator of IL-1 activity. This action of soluble AcP has been difficult to demonstrate, however (Jensen et al., 2000).

In the course of investigating the IL-1 inhibitory ability of soluble human type II IL-1 receptor in vivo, we unexpectedly found that soluble human AcP can associate with ligand-bound soluble IL-1R-II. Association of sIL1R-II with AcP increases the affinity of binding to both human IL-1 $\alpha$  and human IL-1 $\beta$  by approximately two orders of magnitude, which now renders human sIL1R-II an effective inhibitor of the former as well as the latter. In monkeys, efficient binding of IL-1 $\beta$  by soluble type II IL-1R absolutely requires soluble AcP. Circulating levels of soluble AcP are remarkably high, averaging over 300 ng/ml in humans and even higher in monkeys and mice. The mRNA encoding soluble AcP is expressed in almost all tissues.

## Results

### Effect of sIL1R In Vivo in Rhesus Monkeys

Recombinant human soluble type II IL-1 receptor (shu-IL1R-II) was administered to rhesus monkeys, and either 30 min or 2 hr later, the monkeys were injected with LPS from *E. coli*. Two hemodynamic parameters, mean arterial pressure and systemic vascular resistance, suffered precipitous drops over the course of 1–2 hr in animals injected with LPS in the presence of the control

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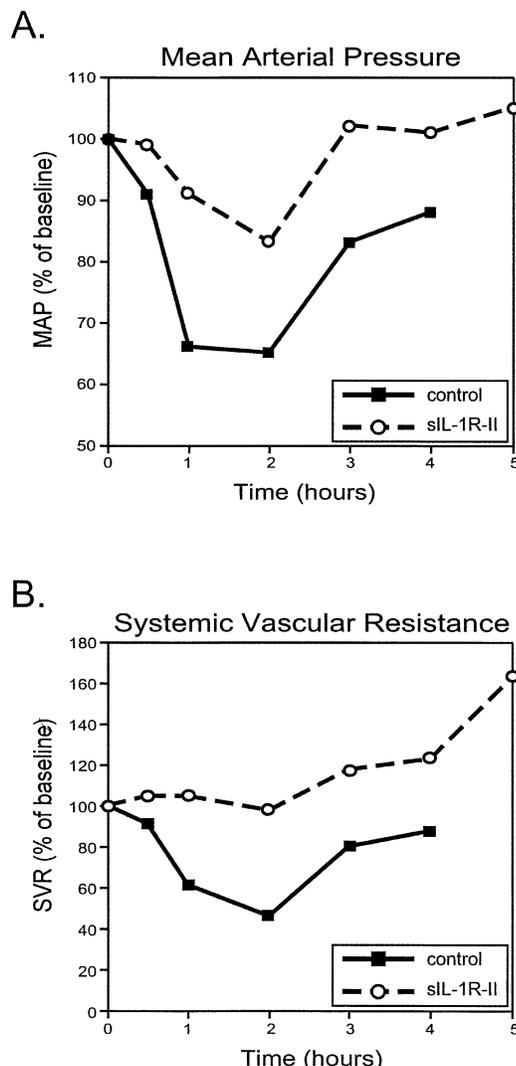


Figure 1. Endotoxin-Induced Hemodynamic Changes in Rhesus Monkeys Are Reduced by sIL1R-II

Changes in (A) mean arterial pressure and (B) systemic vascular resistance after administration of endotoxin, in rhesus monkeys pre-treated with shuIL1R-II or control protein, as indicated. Data are the average of three (SVR) or five (MAP) control animals, and of two animals treated with sIL1R-II.

protein, human serum albumin (Figure 1). In contrast, injection of shuIL1R-II 2 hr before LPS administration blunted the drop in mean arterial pressure (to 83% of baseline, compared to 65% of baseline in the control animals), and completely prevented the drop in systemic vascular resistance (at 2 hr following LPS injection, SVR remained at 98% of baseline in treated animals, compared to 46% of baseline in control animals). Injection of shuIL1R-II 30 min prior to LPS also ameliorated the hemodynamic changes but was less effective (MAP, 71% of baseline; SVR, 86% of baseline) than injection 2 hr prior to LPS. Thus, shuIL1R-II shows clear biological activity in a nonhuman primate model of endotoxemia.

#### Binding of shuIL1R-II In Vitro to Monkey IL-1s

We attempted to characterize the binding of shuIL1R-II to monkey IL-1s using surface plasmon resonance.

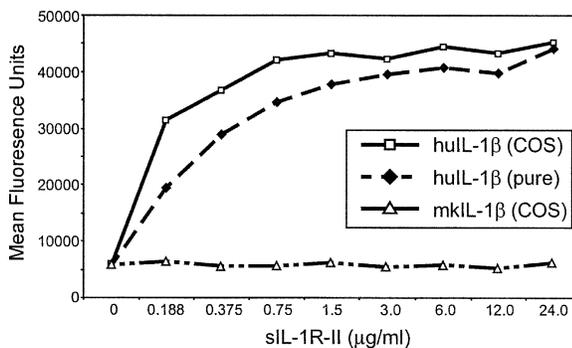


Figure 2. shuIL1R-II Inhibits Growth Arrest of A375 Cells by Human but Not Cynomolgus IL-1β

A375 cells were cultured for 3 days with purified recombinant human IL-1β or with supernatant from COS cells transfected with human or cynomolgus IL-1β. Increasing amounts of shuIL1R-II were added as inhibitor. Metabolic activity was measured by fluorescence 6 hr after addition of Alamar Blue.

Both cynomolgus and rhesus macaque IL-1β were used. The two polypeptides are identical except for the presence of one additional amino acid (serine) at the C terminus of the rhesus protein (Villinger et al., 1995; Totsuka et al., 1998; D.E.S., unpublished data). Binding of shuIL1R-II to human IL-1s was included for comparison. As has been previously demonstrated (McMahan et al., 1991), shuIL1R-II binds to human IL-1β with a  $K_A$  of approximately  $10^9 M^{-1}$  (Table 1). The dissociation rate constant for this interaction is notably slow ( $k_d = 7 \times 10^{-5} s^{-1}$ ). The  $K_A$  values of shuIL1R-II for human IL-1α and human IL-1ra are approximately two orders of magnitude lower (Table 1). In contrast, and despite the biological effect observed in vivo in the rhesus monkeys, the  $K_A$  values of shuIL1R-II for cynomolgus IL-1β and rhesus IL-1β were in the range of  $1-3 \times 10^6 M^{-1}$  (Table 1); indeed, this number is only an approximation, since binding was too low to be measured accurately. The  $K_A$  of shuIL1R-II for cynomolgus IL-1ra was similar to that for human IL-1ra, approximately  $6 \times 10^7 M^{-1}$  (Table 1).

The lack of binding of shuIL1R-II to cynomolgus and rhesus IL-1βs was confirmed in an alternative solid-phase binding assay (not shown) and in a biological assay. Treatment of the human melanoma cell line A375 with human, rhesus, or cynomolgus IL-1β results in growth arrest of the cells in a dose-dependent fashion. Addition of increasing amounts of shuIL1R-II to the assay inhibits the growth arrest induced by human IL-1β but has no effect on arrest induced by cynomolgus IL-1β (Figure 2) or rhesus IL-1β (not shown). The cynomolgus and rhesus IL-1β were active, as judged by their ability to inhibit proliferation of the A375 cells (Figure 2, not shown) as well as their ability to bind human type I IL-1R measured using surface plasmon resonance (not shown). Soluble huIL1R-II failed to bind recombinant cynomolgus IL-1β or to inhibit it in a biological assay, regardless of whether the IL-1β was expressed in *E. coli* or in COS (monkey) cells. In addition, shuIL1R-II failed to bind native cynomolgus IL-1β isolated from LPS-stimulated peripheral blood monocytes.

To be certain that we were not being misled by cross-species effects, we cloned and expressed the type II IL-1 receptors from both cynomolgus and rhesus monkeys

Table 1. Binding Measurements

		human IL-1R type II		
		$k_a$ (1/Ms)	$k_d$ (1/s)	$K_A$ (1/M)
human	IL-1 $\alpha$	$7.10 \times 10^5$	$3.92 \times 10^{-2}$	$1.87 \times 10^7$
human	IL-1 $\beta$	$8.85 \times 10^4$	$6.82 \times 10^{-5}$	$1.41 \times 10^9$
human	IL-1ra	$2.0 \times 10^5$	$5.44 \times 10^{-3}$	$3.67 \times 10^7$
cyno	IL-1 $\beta$	$4.51 \times 10^3$	$3.17 \times 10^{-3}$	$1.42 \times 10^6$
rhesus	IL-1 $\beta$	$9.36 \times 10^3$	$3.66 \times 10^{-3}$	$2.56 \times 10^6$
cyno	IL-1ra	$2.34 \times 10^5$	$3.68 \times 10^{-3}$	$6.34 \times 10^7$
		cyno. IL-1R type II		
		$k_a$ (1/Ms)	$k_d$ (1/s)	$K_A$ (1/M)
cyno	IL-1 $\beta$	$2.75 \times 10^4$	$9.34 \times 10^{-3}$	$2.95 \times 10^6$
rhesus	IL-1 $\beta$	nd	nd	nd
		human IL-1R type II & human AcP		
		$k_a$ (1/Ms)	$k_d$ (1/s)	$K_A$ (1/M)
human	IL-1 $\alpha$	$7.62 \times 10^5$	$3.38 \times 10^{-4}$	$2.28 \times 10^9$
human	IL-1 $\beta$	$9.50 \times 10^4$	$6.78 \times 10^{-7}$	$1.79 \times 10^{11}$
human	IL-1ra	$1.73 \times 10^5$	$5.41 \times 10^{-3}$	$3.19 \times 10^7$
		human IL-1R type II & cyno. AcP		
		$k_a$ (1/Ms)	$k_d$ (1/s)	$K_A$ (1/M)
cyno	IL-1 $\beta$	$4.11 \times 10^4$	$2.33 \times 10^{-4}$	$2.00 \times 10^8$
rhesus	IL-1 $\beta$	$1.76 \times 10^4$	$1.51 \times 10^{-3}$	$1.17 \times 10^7$
cyno	IL-1ra	$2.42 \times 10^5$	$4.51 \times 10^{-3}$	$5.37 \times 10^7$
		cyno. IL-1R type II & cyno. AcP		
		$k_a$ (1/Ms)	$k_d$ (1/s)	$K_A$ (1/M)
cyno	IL-1 $\beta$	$3.98 \times 10^4$	$3.72 \times 10^{-4}$	$1.07 \times 10^8$
rhesus	IL-1 $\beta$	$3.16 \times 10^4$	$1.39 \times 10^{-3}$	$2.28 \times 10^7$

Forward and reverse rate constants for binding of human and monkey cytokines to human and monkey sIL1R-II, in the presence or absence of sAcP, were determined by Biacore as described in the Experimental Procedures. Affinity constants were calculated from the rate constants.

(D.E.S., unpublished data). The two have identical amino acid sequences. The recombinant monkey soluble IL1R-II bound to cynomolgus IL-1 $\beta$  with a  $K_A$  of approximately  $3 \times 10^6 \text{ M}^{-1}$  (Table 1), comparable to the binding of cynomolgus IL-1 $\beta$  by human soluble IL1R-II. It also failed to inhibit the biological activity of cynomolgus IL-1 $\beta$  in the A375 assay (not shown).

#### The Soluble Form of IL-1R AcP Enhances Binding of shuIL1R-II In Vitro to Human and Monkey IL-1s

The cell-surface form of type II IL-1 receptor, in the presence of ligand, is capable of interacting with surface-bound IL-1 receptor accessory protein (Lang et al., 1998; Malinowsky et al., 1998) and indeed the presence of AcP is required in order for high-affinity binding of IL-1 $\beta$  to be seen with the mouse type II IL-1R (Malinowsky et al., 1998). In addition, the existence of a naturally occurring, soluble version of AcP has been demonstrated (Greenfeder et al., 1995). We hypothesized that the difference between the considerable beneficial effect of shuIL1R-II in vivo (Figure 1), and the very modest binding affinity in vitro (Table 1; Figure 2) might be due to the presence of circulating, soluble AcP.

We asked whether soluble AcP affected the affinity of binding of IL-1s by type II IL-1 receptors using surface plasmon resonance. The presence of AcP enhanced the binding of cynomolgus IL-1 $\beta$  to cynomolgus sIL1R-II by

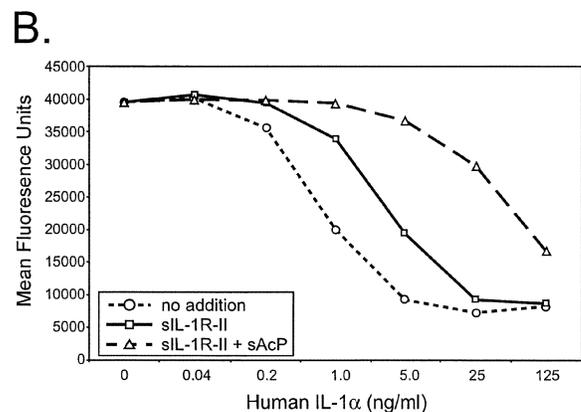
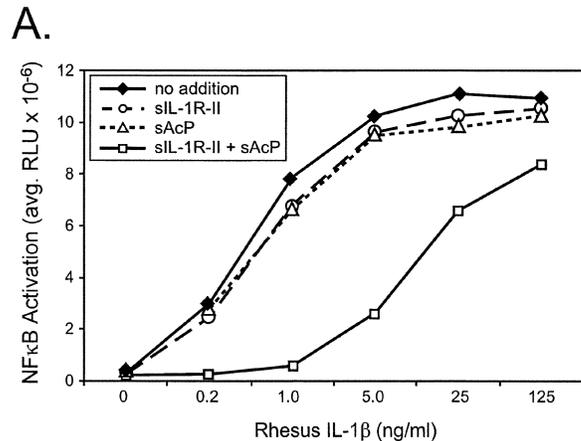


Figure 3. shuIL1R-II Is a Better Inhibitor in the Presence of sAcP

(A) COS7 cells transfected with an NF $\kappa$ B-luciferase reporter plasmid were incubated for 4 hr with varying concentrations of recombinant rhesus IL-1 $\beta$  in the presence of soluble receptors. Cells were lysed and luciferase activity measured to determine NF $\kappa$ B activation.

(B) A375 cells were cultured for 3 days with increasing amounts of purified recombinant IL-1 $\alpha$  alone, or with the addition of 25  $\mu$ g/ml of recombinant sIL1R-II, or with 25  $\mu$ g/ml recombinant sIL1R-II plus 20  $\mu$ g/ml recombinant sAcP. Cell viability was measured by fluorescence 6 hr after addition of Alamar Blue.

36-fold, from  $K_A = 3 \times 10^6 \text{ M}^{-1}$  to  $K_A = 1.1 \times 10^8 \text{ M}^{-1}$  (Table 1). Moreover, although human sIL1R-II is capable by itself of binding human IL-1 $\beta$  with good affinity ( $K_A = 1.4 \times 10^9 \text{ M}^{-1}$ ), its affinity was also enhanced by soluble AcP, by over 100-fold (to  $K_A = 1.8 \times 10^{11} \text{ M}^{-1}$ ) (Table 1). The enhancement in affinity was not restricted to IL-1 $\beta$ ; in the presence of soluble AcP, the affinity of shuIL1R-II for human IL-1 $\alpha$  increased from  $K_A = 1.9 \times 10^7 \text{ M}^{-1}$  to  $K_A = 2.3 \times 10^9 \text{ M}^{-1}$  (Table 1). In both cases, the increased affinity in the presence of AcP was due primarily to a reduction in the dissociation rate constant. Consistent with their physiological roles, there was no change in the affinity of binding of shuIL1R-II to IL-1ra in the presence of AcP, leaving both available to inhibit the agonistic action of IL-1 (Table 1).

#### The Soluble Form of IL-1R AcP Enhances the Ability of shuIL1R-II to Inhibit Human and Monkey IL-1s In Vitro

We wanted to confirm that the increased binding of IL-1 by type II IL-1 receptors, conferred by soluble AcP,

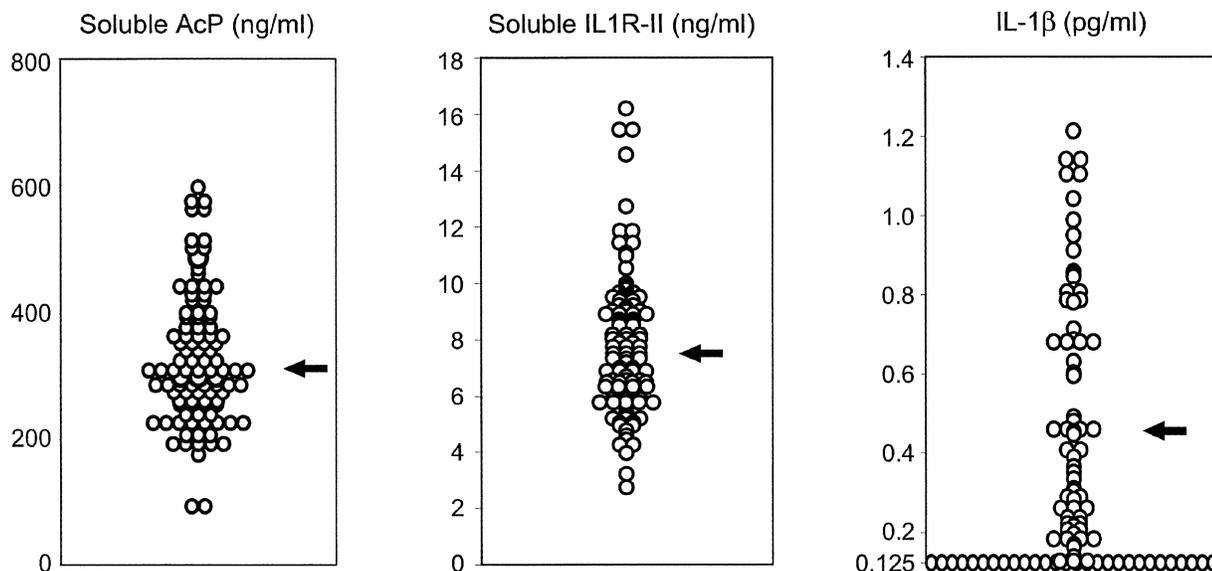


Figure 4. Serum Levels of AcP, sIL1R-II, and IL-1 $\beta$

The concentration, determined by ELISA, of proteins in serum from normal individuals is plotted. Left panel, soluble AcP; middle panel, soluble IL1R-II; right panel, IL-1 $\beta$ . Note that the minimum value in the right-hand panel is 0.125 pg/ml, the detection limit for the IL-1 $\beta$  ELISA. The IL-1 $\beta$  values have not been corrected for the effect of sIL1R-II (see Experimental Procedures). The arrow indicates the median value in each group.

translated into more effective inhibition. To this end, we assessed the ability of monkey and human sIL1R-IIs to inhibit monkey IL-1 $\beta$  and human IL-1 $\alpha$ , respectively, in the absence or presence of soluble AcP. As seen previously (Figure 2), shuIL1R-II by itself showed no inhibition of monkey IL-1 $\beta$  in a biological assay (Figure 3A). AcP by itself also showed no inhibition. In the presence of both shuIL1R-II and soluble AcP, however, a 25-fold increase in IL-1 $\beta$  was required in order to achieve the same biological response (Figure 3A). Soluble huIL1R-II does have a modest inhibitory effect by itself on human IL-1 $\alpha$ , but in the presence of soluble AcP, the inhibition was also approximately 25-fold more effective (Figure 3B). Therefore, soluble AcP confers on soluble IL1R-II not only an increase in the affinity of binding but an increase in effectiveness as an inhibitor as well.

#### Soluble Levels of IL-1R AcP In Vivo

The ability of soluble AcP to enhance the inhibitory capacity of soluble IL1R-II is only biologically relevant if the level of soluble AcP in vivo is sufficient. We therefore wanted to measure the normal circulating level of soluble AcP. An ELISA was developed for this purpose and used to determine sAcP concentrations in the serum of 100 normal individuals. The median soluble AcP concentration in this panel was 311.5 ng/ml (mean 329 ng/ml), with a range of 91.6–600.3 ng/ml (Figure 4). Virtually all samples (98%) are within 2-fold of the mean.

For comparison, we also measured levels of endogenous sIL1R-II, IL-1 $\alpha$ , and IL-1 $\beta$  in this same panel of serum samples. sIL1R-II levels ranged from 2.8 to 16.2 ng/ml, with a median value of 7.6 ng/ml (Figure 4) and a mean of 7.9 ng/ml. IL-1 $\beta$  levels were below the level of detection (0.125 pg/ml) in 28 of the samples; in the other 72 samples, they ranged from 0.126 pg/ml to 1.213

pg/ml, with a median value of 0.43 pg/ml (Figure 4) and a mean of 0.51 pg/ml (but see Experimental Procedures). IL-1 $\alpha$  levels were below the level of detection (3.9 pg/ml) in all samples (not shown). In previous studies, naturally occurring plasma levels of soluble human type II IL-1R were measured in 134 normal individuals by Jouvenne et al. (1998), who found a mean of  $3.6 \pm 2.6$  ng/ml (range <0.6– 10.5 ng/ml). Plasma levels of IL-1 $\beta$  were determined in 21 normal individuals by Eastgate et al. (1988), with a mean of 44.7 pg/ml. We do not know the reason for the large difference in IL-1 $\beta$  levels found in our experiments versus those of Eastgate et al. Nevertheless, the normal circulating concentration of soluble AcP is clearly much greater than that of either IL-1 $\beta$  (approximately 700,000-fold in our measurements) or soluble IL1R-II (approximately 40-fold).

#### Soluble Levels of IL-1R AcP during Inflammation

We sought to determine whether soluble AcP levels would differ between normal individuals and those with inflammatory disease. We measured sAcP in serum from 32 patients suffering from rheumatoid arthritis (not shown). The median value from the arthritis patients was 312 ng/ml (range 103 to 1215.4 ng/ml). The main difference between the arthritis patients and the normals is the existence of a small number of patients with higher levels of circulating soluble AcP (22% of arthritis patients have levels greater than 2 $\times$  the median value).

We also explored the variation in soluble AcP levels in animal models of disease. We examined mouse models of arthritis (in DBA/1 mice) and inflammatory bowel disease (in Balb/c mice). In both strains, soluble AcP levels were approximately 4  $\mu$ g/ml, and declined slightly during the course of the inflammation (data not shown). The same general trend was seen during endotoxemia

in monkeys, although the initial soluble AcP level was approximately 1.1  $\mu\text{g/ml}$  (data not shown). Thus, circulating soluble AcP levels are even higher in monkey and mouse than they are in human, and in all three species, do not appear to change significantly during inflammation.

#### Alternatively Spliced mRNA Encoding Soluble AcP

It has been known for some time that the IL-1 receptor accessory protein gene is transcribed into two splice forms of mRNA (Greenfeder et al., 1995; Jensen et al., 2000). One of these encodes the signaling, transmembrane form of AcP, while the other encodes the soluble form. The latter mRNA has been characterized only in liver cells, where it is regulated modestly (Jensen et al., 2000).

To gain information about the source of circulating soluble AcP, we surveyed a wide variety of cell types by quantitative PCR for their expression of both mRNA isoforms. As can be seen in Figure 5, many tissues express the mRNA isoform encoding soluble AcP. The highest levels in our survey were found in adult liver, skin, and placenta, and in certain fetal organs. Generally, soluble AcP mRNA was present at approximately 5% to 10% the level of mRNA encoding full-length AcP. Notable exceptions are the T84 intestinal cell line and activated HepG2 cells, in which there is a slight excess of mRNA encoding soluble AcP (data not shown). In general, the amount of mRNA encoding soluble AcP does not change much with cell stimulation (not shown). Among the settings in which soluble AcP does show regulation are in human peripheral blood mononuclear cells, in which there is a 1.5- to 4-fold increase upon stimulation with PMA/ionomycin, tetanus toxoid, or PHA, and in human peripheral blood B cells, which show a 10-fold decrease in mRNA when treated with a combination of the Cowan strain of *Staphylococcus aureus*, CD40 ligand, and IL-4. We specifically examined the response of peripheral blood monocytes to anti-inflammatory agents, including IL-4, IL-13, the combination of IL-4 and IL-13, and dexamethasone, since these treatments have been shown to induce expression of the type II IL-1 receptor on monocytes and neutrophils (Mantovani et al. 2001). Although each of these treatments strongly induced IL1R-II mRNA, none of them led to a significant change in the level of the mRNAs encoding either full-length or soluble AcP, at either 4, 7, or 24 hr after stimulation (data not shown).

For many proteins that have both transmembrane and soluble forms, the soluble form is generated by proteolytic cleavage of the membrane-bound precursor (shedding), rather than being encoded by a separate mRNA. We asked whether shedding might also contribute to the circulating pool of soluble AcP. We stained cells with antibodies against AcP and against a control protein known to be shed, before and after treatment with a known shedding stimulus, and compared their profiles by FACS analysis. Figure 6 demonstrates that, as expected, there was considerable shedding of type II IL-1 receptor from HepG2 cells after treatment with PMA. There was no reduction, however, in the amount of surface AcP under the same conditions. Similarly, human neutrophils were induced by either PMA or LPS to shed

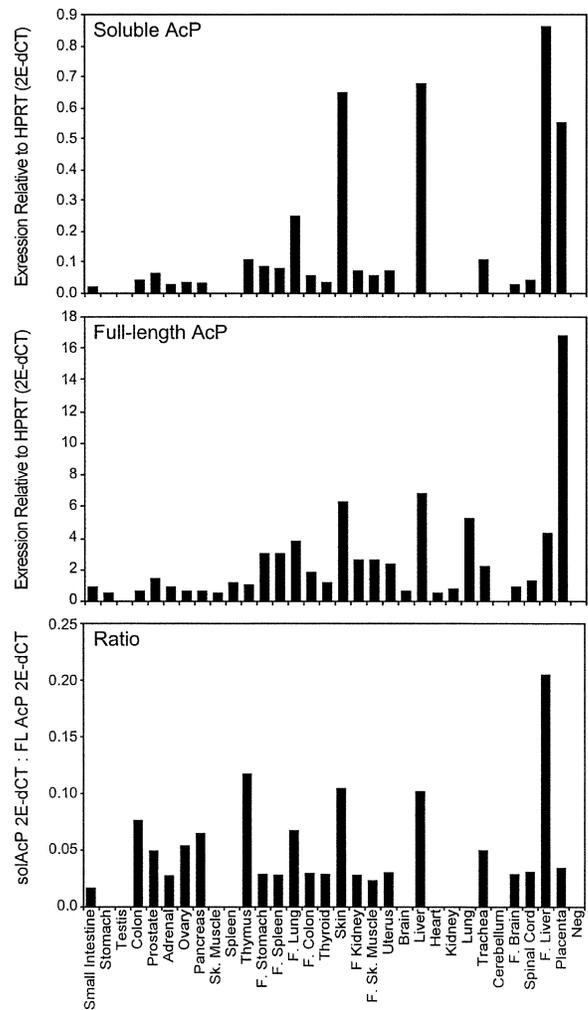


Figure 5. Expression of mRNA Encoding Different Forms of AcP  
Taqman quantitative PCR was performed on a panel of RNAs from normal human tissue, using primers specific for the soluble (top panel) and full-length (middle panel) forms of AcP mRNA. Expression levels are plotted as the ratio of the threshold detection value (CT) of the AcP mRNA to that of a control gene (hypoxanthine-guanine phosphoribosyl transferase). The bottom panel shows the ratio of soluble to full-length mRNA.

L selectin (CD62L). Neither of these agents induced the shedding of AcP. Other cell lines and conditions examined in which we failed to see shedding of AcP using FACS include primary human monocytes, the human B cell line Namalwa, and the monkey CV-1/EBNA cell line (McMahon et al., 1991) transfected with human type II IL-1 receptor or human AcP, all treated with (or without) PMA.

We explored further the possibility that some sAcP might be generated proteolytically by use of the metalloproteinase inhibitor TAPI (Mohler et al., 1994) with HepG2 cells. This inhibitor and others like it have been demonstrated to inhibit the shedding of a number of cell-surface molecules including pro-TNF $\alpha$ , IL-2R $\alpha$ , and IL-1R type II (Mullberg et al., 1997; Orlando et al., 1997). Spontaneously produced soluble AcP accumulated in the supernatant of the cells to 9.2 ng/ml after 48 hr, and to 12.3 ng/ml after 72 hr. Addition of 50  $\mu\text{M}$  TAPI had

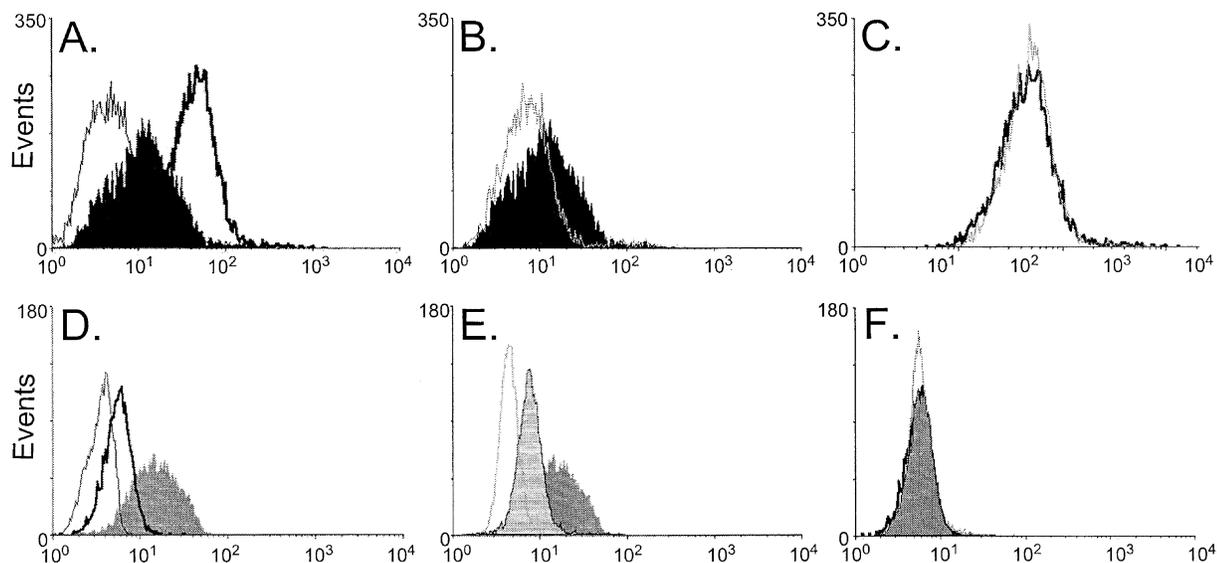


Figure 6. Lack of Shedding of AcP

HepG2 cells express both AcP and type II IL-1R, as demonstrated by FACS analysis (A: light line, isotype control; shaded peak, IL1R-II; heavy line, AcP). Following treatment with PMA, there is a loss of type II IL-1R (B: shaded peak, before; open peak, after) from the cell surface, but no loss of AcP (C). Similarly, primary human neutrophils express cell-surface L-selectin and AcP (D: light line, isotype control; shaded peak, L-selectin; heavy line, AcP). Stimulation with either PMA or LPS leads to loss of L-selectin (E: dark shading, no treatment; light shading, LPS; light line, PMA) but not AcP (F).

no effect on the accumulation (9.9 ng/ml and 13.6 ng/ml, respectively, after 48 and 72 hr). Western blot analysis (not shown) confirmed the lack of effect of TAPI on sAcP accumulation in the medium. Control experiments demonstrated that TAPI was capable of inhibiting completely the PMA-induced shedding of type II IL-1R from the HepG2 cells. Thus, in this limited set of cell types examined, we have found no evidence to support the idea that some of the circulating sAcP is generated by shedding of the transmembrane form.

## Discussion

The experiments presented in this paper reveal a previously unappreciated interaction between the soluble forms of IL1R-II and AcP. Although logically this interaction might have been predicted, given the ability of the cell-surface forms of these proteins to associate, the magnitude of the increase in affinity for the agonist IL-1s (30- to 100-fold) was not expected, nor was the conversion of sIL1R-II from a poor inhibitor of IL-1 $\alpha$  to a reasonably effective inhibitor in the presence of excess sAcP. The improvement in affinity is almost entirely due to a decrease in the dissociation rate constant. Notably, the presence of soluble AcP does not increase the affinity of sIL1R-II for IL-1ra, thus maintaining the ability of both antagonists to regulate IL-1 action.

Also unexpected was the inability of monkey IL-1 $\beta$  to bind to either human or monkey sIL1R-II in the absence of sAcP. We have not yet demonstrated that this is also true for the surface forms of the receptor proteins in monkey. Nevertheless, this finding raises the question of why the regulation of IL-1 activity should be different, at least in detail, between humans and macaques. In

work to be published separately (Smith et al., 2002), we have mapped the difference in behavior of human and monkey IL-1 $\beta$  to a single amino acid change. Regardless, the soluble AcP effect likely explains the ability of sIL1R-II to reduce the effects of endotoxemia in monkeys (Figure 1), despite the poor binding of human (or monkey) sIL1R-II alone to monkey IL-1 $\beta$  in vitro (Table 1; Figure 2).

Yet another surprising finding was the high serum level of soluble AcP (approximately 300 ng/ml in humans, 1  $\mu$ g/ml in monkeys, and higher still in mice). These values can be contrasted to the levels of soluble IL1R-II (approximately 7 ng/ml) and IL-1 $\beta$  (approximately 0.4 pg/ml) found in human serum (Figure 4). It is not obvious why such high levels of sAcP are desirable. One possibility would be to ensure that the binding of IL-1 $\alpha$  or IL-1 $\beta$  by sIL1R-II is driven to completion. Another alternative (not mutually exclusive) is that sAcP interacts with serum components other than ligand-bound IL1R-II. The high degree of sequence conservation of AcP between human and mouse (85% amino acid identity in the extracellular region, compared to 62% amino acid identity between the extracellular regions of human and mouse type II IL-1R and 64% amino acid identity between the extracellular regions of human and mouse type I IL-1R) would be consistent with a need for AcP to interact with several different proteins.

There is little difference in the distribution of serum soluble AcP levels between normal individuals and rheumatoid arthritis patients. In contrast, soluble AcP levels drop by about half over the course of several acute inflammatory models (monkey endotoxemia, mouse collagen-induced arthritis, and mouse colitis). The reason for the decrease is unknown, as is the biological implication. In our preliminary explorations, we have found iso-

lated examples in which mRNA levels encoding soluble AcP are regulated by cell stimuli. However, we have not found evidence for the kind of dramatic and substantial changes in expression level seen with IL-1ra or type II IL-1 receptor. We have also shown that shedding of the cell-surface form does not appear to be a major means of generating soluble AcP.

When the soluble form of AcP was first discovered, it was attractive to hypothesize that this molecule could associate with ligand-bound cell-surface type I IL-1 receptor, displacing full-length AcP and thereby contributing to the control of IL-1 responses. Jensen et al. (2000), however, were unable to demonstrate such an association. Whether soluble AcP might associate with the ligand-bound form of cell-surface type II IL-1 receptor to enhance the affinity of IL-1 binding is unknown. The ligand-bound form of cell-surface type II IL-1 receptor is able to bind and sequester full-length AcP in a nonproductive complex (Lang et al., 1998; Malinowsky et al., 1998). One might imagine that ligand-bound soluble type II IL-1R could also tie up full-length AcP. However, the data in Figure 2 suggest that this is not the case; if it were, then sIL1R-II should also be able to inhibit cynomolgus IL-1 $\beta$  (Figure 2; Table 1).

The findings reported here enhance our understanding of the complexity of IL-1 regulation. Whether the large amount of circulating soluble AcP contributes to the regulation of additional cytokines remains an open question.

## Experimental Procedures

### Protein Reagents

Multiple forms of all proteins were used. The cloning of IL-1 $\beta$  and type II IL-1 receptor from rhesus and cynomolgus monkeys is described elsewhere (Smith et al., 2002). Descriptions of the other proteins are as follows.

#### Type II IL-1 Receptors

Biacore measurements utilized Ig fusions (Baum et al., 1994) of human, rhesus, and cynomolgus soluble IL1R-II that were expressed by transient transfection of COS cells and purified by chromatography on protein A sepharose. The primate endotoxemia experiments, as well as biological assays with the A375 cell line, utilized untagged soluble human IL1R-II made by stable expression in CHO cells and purified by affinity chromatography using human IL-1 $\beta$  covalently attached to Affigel-10. Concentration was determined by amino acid analysis. Specific amino acid residues included in the soluble constructs are as follows: human IL1R-II, residues 1-346 of Genbank NM\_004633; cynomolgus IL1R-II, residues 1-346 of Genbank AY172102; rhesus IL1R-II, residues 1-346 of Genbank AY172100. Cynomolgus and rhesus type II IL-1 receptors are identical.

#### IL-1 Ligands

Human IL-1 $\alpha$ , IL-1 $\beta$ , and IL-1ra used in Biacore experiments were generated at Amgen and purified by conventional means. Human ligands used in the A375 assays were purchased from R&D Systems (Minneapolis, MN). Cynomolgus IL-1ra cDNA was cloned following PCR amplification of mRNA derived from peripheral blood lymphocytes (kindly provided by SNBL, Everett, WA) that had been cultured for 3.5 hr in LPS and human CD40 ligand (both at 1  $\mu$ g/ml). PCR primers were designed from the 5' and 3' untranslated regions of human IL-1ra. The cynomolgus and human IL-1ra amino acid sequences are 96% identical. The cloning of cynomolgus and rhesus IL-1 $\beta$  ligands is described elsewhere (Smith et al., 2002). PCR primers were designed based on Genbank accession numbers D63353 (cynomolgus; the sequence we subsequently derived differs from the Genbank version at one amino acid) and U19845 (rhesus). Full-length cynomolgus IL-1ra, and the mature forms of cynomolgus and rhesus IL-1 $\beta$ , were expressed by transient transfection and purified

by affinity chromatography using recombinant human sIL1R-I covalently attached to Affigel-10 resin. Concentrations were determined by amino acid analysis.

### Accessory Proteins

Cynomolgus IL-1 receptor accessory protein cDNA was cloned from the same RNA source used to clone cynomolgus IL-1ra. Rhesus IL-1 receptor accessory protein cDNA was cloned following PCR amplification of mRNA derived from tonsil. PCR primers were designed from the 5' and 3' untranslated regions of human AcP. The protein sequences of cynomolgus and rhesus AcP proteins were identical to each other, and 99% identical to human AcP. Biacore measurements utilized Ig fusions (Baum et al., 1994) of human and cynomolgus sAcP that were purified by chromatography on protein A sepharose. Soluble human sAcP tagged at its C terminus with a peptide containing the FLAG epitope followed by six histidine residues (...RSGSSDYKDDDDKGGSSHHHHH\*), expressed in COS cells and purified by immobilized metal affinity chromatography, was used in A375 assays. The human ELISA standard was untagged and was purified by conventional chromatography. COS cell expression supernatants were exposed in batch to Heparin Sepharose 6 Fast Flow (Pharmacia), previously equilibrated in 50 mM Tris-HCl, 7.5) at a resin:supernatant ratio of 1:12, overnight at 4°C. Under these conditions AcP does not bind to the Heparin resin. Unbound material was then batch exposed to a wheat germ agglutinin (WGA) resin (Vector), previously equilibrated in 50 mM Tris-HCl (pH 7.5) containing 50 mM N-acetylglucosamine (binding buffer), at a resin:supernatant ratio of 1:25, overnight at 4°C. Following overnight binding the WGA resin was collected in a column, washed with 10 column volumes of binding buffer, and the sAcP eluted with a linear gradient of 50 mM-1.0 M N-acetylglucosamine in 50 mM Tris-HCl (pH 7.5). Fractions containing sAcP were pooled and concentrated and then applied onto a Superdex 75 HiLoad 26/60 column (Pharmacia) previously equilibrated with PBS at a flow rate of 2 ml/min. Soluble AcP behaved as a monomer, displaying a retention time consistent with a molecular weight of ~60 kDa. Fractions were analyzed by SDS PAGE. Pure fractions were pooled and concentration determined by amino acid analysis. To generate standards for the nonhuman ELISAs, soluble AcP proteins from cynomolgus and mouse were tagged at their C termini with a sequence encoding the FLAG epitope followed by six histidine residues (...RSGSSDYKDDDDKGGSSHHHHH\*) and purified by immobilized metal affinity chromatography. Specific AcP amino acid residues included in the various constructs are as follows: human AcP, residues 1-354 of Genbank AF029213; cynomolgus AcP, residues 1-359 of Genbank AY182233; mouse AcP, residues 1-359 of Genbank X85999.

### Endotoxemia in Monkeys

Domestic born, male rhesus monkeys, *Macaca mulatta*, mean weight 4.5  $\pm$  0.5 kg, from the Veterinary Resource Facility at the University of Maryland, Greenebaum Cancer Center, were used in these studies. Animals were anesthetized with ketamine (10 mg/kg, im) and immobilized in a well-padded, specifically designed restraint system to allow placement of arterial and venous catheters. Following line placement, the animals were allowed to awaken, and baseline hemodynamic measurements taken. Animals received an infusion over 10 min of *E. coli* endotoxin (O111:B4), 6 mg/kg bw, mixed in 20 ml of normal saline. At 30 min and every hour up to 5 hr after endotoxin, hemodynamic measurements were obtained on the restrained and resting but unanesthetized animals. Once measurements were completed, animals were anesthetized with ketamine, venous and arterial lines removed, and the animals returned to their cages under observation by technical staff until they were awake, alert, and responsive. Experimental animals received a subcutaneous injection of soluble IL-1 receptor type II (sIL1R-II) at 200  $\mu$ g/kg either 2 hr or 30 min prior to endotoxin. In addition, the animals were infused with sIL-1RII (200  $\mu$ g/kg/hr, mixed in normal saline) via continuous iv drip starting 5 min prior to LPS infusion and continuing over the next 4 hr. Control animals were treated identically except that they received human serum albumin (Cappel, catalog #55912) instead of sIL1R-II.

### Cynomolgus Peripheral Blood Monocytes

Cynomolgus primary cells were isolated from whole blood (kindly provided by Shin Nippon Biomedical Laboratories in Everett, Wash-

ington). PBMCs were isolated from heparinized blood by density centrifugation. The cells were cultured in RPMI1640 with 10% FBS for 2 hr at 37°C at a density of  $5 \times 10^6$ /ml. Nonadhering cells were washed away and the remaining cells cultured for 2 hr in the presence of 1  $\mu$ g/ml LPS and  $^{35}$ S Cys + Met to metabolically label newly translated proteins. ATP was then added to the culture to 6 mM and the cells incubated an additional 2.5 hr. Exported native IL-1 $\beta$  was collected in the conditioned medium and used for direct binding studies with recombinant IL-1 receptors.

#### Affinity Measurements

Affinities were determined by surface plasmon resonance, using a Biacore 3000 instrument at 25°C with research-grade CM5 sensor chips. A capture system was employed with goat anti-human IgG Fc-specific antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) immobilized using standard amine-coupling chemistry. Immobilized capture antibody alone was used as a reference surface. sIL1R-II.Fc at 7.5  $\mu$ g/ml in HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20 [pH 7.4] from Biacore AB) with 0.1 mg/ml BSA or a mixture of sIL1R-II.Fc and sAcP.Fc (7.5  $\mu$ g/ml each, in the same buffer) was injected over the captured antibody surface at 10  $\mu$ l/min for 3 min to obtain a density of 300–600 resonance units. Recombinant IL-1 $\alpha$ , IL-1 $\beta$ , or IL-1ra in HBS-EP with 0.1 mg/ml BSA was injected over both the receptor and reference surfaces at concentrations ranging from 1000 nM down to 1 nM at a flow rate of 50  $\mu$ l/min. The complex was allowed to associate for 2 to 3 min and dissociate for 3 to 7 min. If necessary, both surfaces were regenerated with a single injection of 10 mM glycine HCl (pH 1.5) for 30 s at a flow rate of 50  $\mu$ l/min. Duplicate injections of each sample and triplicate injections of a buffer blank were injected in random order. The data were fit globally to a simple 1:1 interaction model with local Rmax using BIAevaluation 3.1 Software.

#### A375 Assay

A375.S2 cells (ATCC catalog number CRL 1972) at  $1 \times 10^4$  cells per well in 96-well flat-bottom tissue culture-treated plates (Costar #3596) were incubated with the specific cytokines and soluble receptors indicated in the figures in a total volume of 100  $\mu$ l of DMEM containing 5% fetal bovine serum. After 72 hr at 37°C, 10% CO $_2$ , plate wells were washed once with PBS and a solution of Alamar Blue fluorescent metabolic indicator dye (BioSource Int., Camarillo CA) was added to all wells and incubated for another 4–6 hr. Fluorescence was determined using a TECAN SPECTRAFluor plate reader at 560 nm excitation wavelength and 595 nm emission wavelength. The mean values from at least two replicate wells are plotted. In Figure 2, the purified recombinant human IL-1 $\beta$  standard was used at 3 ng/ml, and supernatant from COS cells expressing either human or cynomolgus IL-1 $\beta$  was used at a final dilution of 1:2500. Metabolic labeling experiments suggested that comparable amounts of human and monkey IL-1 $\beta$  were present in the COS cell-conditioned media.

#### COS Cell Assay

COS7 cells in DMEM plus 5% fetal bovine serum were transfected using Fugene 6 reagent (Roche Molecular Systems, Pleasanton CA) in 24-well plates with 200 ng of an NF $\kappa$ B luciferase plasmid. Twenty hours later, soluble receptors were added to cells in a final volume of 0.4 ml (final concentrations: 25  $\mu$ g/ml of human sIL1R-II and 20  $\mu$ g/ml of cynomolgus sAcP-FlagHis). Affinity-purified recombinant rhesus IL-1 $\beta$  was added and the assay was allowed to proceed for 4 hr. Cells were washed three times with PBS and then lysed with 150  $\mu$ l Passive Lysis Buffer (Promega). The luciferase activity in 8  $\mu$ l of lysate was measured using a Lumat LB 9507 luminometer (EG&G Berthold). All samples were assayed in quadruplicate and average RLU values were plotted.

#### Measurements of Soluble AcP Levels in Serum

##### Human AcP ELISA

AcP concentrations were estimated by an ELISA employing AcP-specific monoclonal antibodies to capture and detect soluble AcP in serum. Wells of 96-well microtiter plates ("Maxisorp," Nunc, Roskilde, Denmark) were coated with anti-huAcP mouse IgG1 monoclonal antibody M355 (Amgen Corporation, Seattle, WA) and incubated overnight at 2–8°C. The coated wells were washed with

PBS (pH 7.2) with 0.05% Tween-20 (PBST) prior to use. Samples and reagents were dispensed into the wells in the following sequence, each separated by an incubation period and wash step: (1) serum samples and CHO-derived human sAcP concentration standards diluted in a sample buffer composed of PBST with 0.01% casein and 0.5 mg/ml purified rat IgG; (2) biotinylated anti-huAcP mouse IgG1 monoclonal antibody Clone 60 (Amgen Corporation) diluted in PBST; and (3) peroxidase-labeled streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBST. Color was developed with TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reaction was quenched with acid and optical densities (ODs) were determined at a wavelength of 450 nm. The dose-response curve, constructed of concentration standards in the range of 4–256 ng/mL, was fitted to a four-parameter logistic model. Sample concentrations were interpolated from the fitted curve and corrected for dilution factor. Control experiments examined the ability of the assay to detect both free sAcP and sAcP complexed with sIL1R-II and IL-1 $\beta$ . The addition of up to a 100-fold molar excess of both sIL1R-II and IL-1 $\beta$  did not affect the measured soluble sAcP levels (not shown).

##### Cynomolgus Macaque AcP ELISA

sAcP levels in cynomolgus sera were determined by a modified version of the human sAcP ELISA described above. The two methods were identical except that purified rat IgG was omitted from the sample buffer and concentration standards were prepared from cynomolgus sAcP-FlagHis in the range of 2–128 ng/mL. Pilot experiments showed that the ELISA values obtained with an untagged, conventionally purified sAcP standard were identical to those obtained with sAcP-FlagHis. Control experiments indicated that the presence of sIL1R-II and IL-1 $\beta$  reduced the measured sAcP values, as follows: at a 1:1:1 ratio of sAcP:sIL1R-II:IL-1 $\beta$ , the measured AcP value was 87% of control (obtained in the absence of sIL1R-II and IL-1 $\beta$ ); at a 1:10:10 ratio, the value was 62% of control; at a 1:100:100 ratio, the value was 8% of control. Ratios of 1:10:1 and 1:1:10 each gave 100% of the control value for sAcP. Since sAcP levels are typically orders of magnitude higher than sIL1R-II or IL-1 $\beta$  levels, interference was thought not to be an issue.

##### Murine AcP ELISA

Murine serum sAcP concentrations were also determined by ELISA. Wells of Nunc Maxisorp microtiter plates were coated with a solution of anti-muAcP rat IgG1 monoclonal antibody M215 (Amgen Corporation, Seattle, WA) and incubated overnight at 2–8°C. The coated wells were washed with PBST prior to use. Samples and reagents were dispensed into the wells in the following order, each separated by an incubation period and wash step: (1) serum samples and murine sAcP-FlagHis concentration standards diluted in PBST with 0.1% BSA (PBSAT); (2) rabbit anti-muAcP antiserum (Amgen Corporation, Seattle, WA) diluted in PBSAT; and (3) peroxidase-labeled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in PBSAT. Color was developed with TMB peroxidase substrate. The reaction was quenched with acid and optical densities (ODs) were determined at a wavelength of 450 nm. The dose-response curve, constructed of concentration standards in the range of 0.125–8 ng/mL, was fitted to a four-parameter logistic model. Sample concentrations were interpolated from the fitted curve and corrected for dilution factor. Pilot experiments showed that the sAcP ELISA values obtained with an untagged, affinity-purified sAcP standard were approximately double those obtained with the sAcP-FlagHis standard. In control experiments, the measured values for sAcP were unaffected by a 100-fold excess of sIL1R-II and IL-1 $\beta$  (not shown).

#### Other ELISAs

##### Soluble IL1R-II

Wells of 96-well microtiter plates ("Maxisorp," Nunc, Roskilde, Denmark) were coated with anti-shuL-1R-II monoclonal antibody M25 (Amgen Corporation, Seattle, WA) and incubated overnight at 2–8°C. The coated wells were washed with PBS (pH 7.2) with 0.05% Tween-20 (PBST) prior to use. Samples and reagents were dispensed into the wells in the following sequence, each separated by an incubation period and wash step: (1) serum samples and CHO-derived shuL-1R-II concentration standards diluted in a sample buffer composed of PBST with 0.01% casein, 5 mM EDTA, and 5% normal rat serum;

and (2) peroxidase-labeled anti-shuIL-1R-II monoclonal antibody M4 (Amgen Corporation) diluted in PBST with 0.01% casein and 5 mM EDTA. Color was developed with TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reaction was quenched with acid and optical densities (ODs) were determined at a wavelength of 450 nm. The dose-response curve, constructed of concentration standards in the range of 78.1-5000 pg/mL, was fitted to a four-parameter logistic model. Sample concentrations were interpolated from the fitted curve and corrected for dilution factor. The presence of IL-1 $\beta$  and/or soluble AcP did not interfere with the assay.

#### IL-1 $\alpha$

IL-1 $\alpha$  concentrations were measured using the Quantikine human IL-1 $\alpha$  Immunoassay (Catalog #DLA50), manufactured by R&D Systems (Minneapolis, MN). The manufacturer states that soluble IL-1 receptors do not interfere with the assay unless present at greater than 10 ng/ml (sIL1R-I) or greater than 30 ng/ml (sIL1R-II).

#### IL-1 $\beta$

IL-1 $\beta$  concentrations were measured using the Quantikine HS (high sensitivity) human IL-1 $\beta$  Immunoassay (Catalog #HSLB50), manufactured by R&D Systems (Minneapolis, MN). The assay is reported to show no interference by either sIL1R-I or sIL1R-II, when present at 10 ng/ml. In our hands, however, addition of 10 ng/ml recombinant sIL1R-II to the assay reduced the apparent concentration of the IL-1 $\beta$  standard (4 pg/ml) by 65%. Further addition of 500 ng/ml recombinant soluble AcP had no additional effect. The values presented in the text and in Figure 4 are the directly measured values and have not been corrected for the inhibitory effect of sIL1R-II.

#### ELISA Samples

Normal human sera were obtained from the BioclinicalPartners division of Impath, Inc. (Franklin, MA). Serum from normal cynomolgus monkeys was obtained from SNBL USA, Ltd. (Everett, WA). Serum from rheumatoid arthritis patients was obtained at baseline from volunteers enrolled in an Amgen-sponsored clinical trial. The murine colitis experiment was carried out according to Sivakumar et al. (2002), while the murine arthritis experiment used the protocol of Durie et al. (1993).

#### Quantitative PCR

Quantitative PCR was performed using a Taqman 7700 real-time PCR instrument (Applied Biosystems, Foster City, CA) for 40 cycles under standard conditions. To detect full-length AcP, the following reagents were used: forward primer (GGGCAGGTTCTGGAAGCA) at 900 nM, reverse primer (GCTAGACCGCCTGGGACTTT) at 900 nM, and FAM probe (TCACTGGCATGGCCACCTGCAG) at 200 nM. To detect soluble AcP: forward primer (GGACAAAAGGAGAGATTGA GAACAA) at 300 nM, reverse primer (TCATTTAAGTTTGATTTCAT TACTATGGGTTA) at 900 nM, and FAM probe (AGC TCCAGCACC TAGCCTGACGGC) at 200 nM. The amplification efficiencies for each AcP-specific reaction were determined by plotting the cT value vs. Log (pg control template) and calculating the slope (data not shown). Based on a perfect amplification efficiency of -3.3, the AcP reactions were both found to be 95% efficient (full-length AcP =  $-3.436 \pm 0.096$ , soluble AcP =  $-3.433 \pm 0.106$ ). AcP levels were normalized to a control gene (HPRT) present in all cDNA sources. Threshold (cT) levels were set at 0.12 for the FAM layer (AcP) and 0.16 for the VIC layer (HPRT). RNAs were either generated at Amgen or obtained from commercial sources (Ambion, Austin, TX; Clontech Laboratories, Palo Alto, CA; and Stratagene, La Jolla, CA). They were treated with DNase (Ambion #1906) and reverse transcribed using TaqMan Reverse Transcription Reagents (#N808-0234, Applied Biosystems, Foster City, CA) and random hexamers according to the manufacturers instructions. Samples were run in triplicate.

#### Shedding Analysis

Human neutrophils were separated from peripheral blood of healthy human donors by Ficoll gradient centrifugation (GIBCO, Rockville MD) and purified by dextran sedimentation. Human hepatoma cells (HepG2) were obtained from the American Type Culture Collection (Manassas, VA). Both neutrophils and HepG2 cells were incubated in endotoxin-free RPMI 1640 (GIBCO) supplemented with 10% (v/v) fetal bovine serum at 37°C in the presence of 5% CO<sub>2</sub>. Cells were

treated with 100 ng/ml PMA (Sigma, St. Louis, MO) or LPS (*Escherichia coli* 005:B5; Difco, Detroit, MI) for 40 min. After washing and blockade of nonspecific binding using 200  $\mu$ g/ml normal goat IgG (Sigma), they were stained with 5  $\mu$ g/ml mouse IgG1 monoclonal antibodies directed against human AcP (M355, Amgen), type II IL-1 receptor (M2, Amgen), or L selectin (CD62L, R&D Systems, Minneapolis, MN), or with control mouse IgG1 (MOPC-21 protein, Pharmingen, San Diego, CA), then with PE-conjugated goat anti-mouse antiserum (Sigma), and analyzed by flow cytometry. For the TAPI experiments,  $3 \times 10^6$  HepG2 cells were seeded in 10 ml of medium in 10 cm dishes, with or without 50  $\mu$ M TAPI (Amgen Corporation). Medium was collected at 48 and 72 hr and assayed by ELISA for the presence of soluble human AcP. As a control, PMA (100 ng/ml) was added to some cells 24 hr after seeding, with or without 50  $\mu$ M TAPI, and the cells analyzed by FACS to confirm that shedding of type II IL-1R was completely inhibited by TAPI (not shown).

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#### Accession Numbers

The sequences for newly cloned proteins used in this paper have been deposited with GenBank and have been assigned the following accession numbers: cynomolgus IL-1ra, AY182232; cynomolgus AcP, AY182233; rhesus AcP, AY182234.