Counter-Regulation of Interleukin-1α (IL-1α) and IL-1 Receptor Antagonist in Murine Keratinocytes

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Interleukin-1α (IL-1α) is a potent proinflammatory cytokine constitutively expressed by keratinocytes, which also synthesize a specific inhibitor of IL-1 activity, intracellular IL-1 receptor antagonist (IL-1ra). Although homeostatic regulation of the IL-1 system in keratinocytes has long been suspected, there is currently little evidence for this. To explore this issue, the PAM212 murine keratinocyte cell line was exposed to increasing concentrations of either IL-1α or IL-1ra and the opposing ligand was assessed by ELISA. Release of IL-1ra was induced following stimulation by murine IL-1α in a concentration-dependent manner and, conversely, IL-1ra stimulation increased IL-1α release. To determine whether a similar homeostatic circuit operates *in vivo*, epidermis from transgenic mice in which overexpression of IL-1α or IL-1ra was targeted to keratinocytes was analyzed. Epidermal sheets derived from IL-1α transgenic mice released eight times more IL-1ra than those from wild-type mice following *ex vivo* culture and similarly, IL-1α release was increased 3–4-fold in epidermal sheets derived from IL-1ra transgenic epidermis, Use of specific neutralizing antibodies against type I and type II IL-1 receptors indicated that the counter-regulation mechanism is mediated extracellularly through the type I IL-1 receptor alone. Taken together, these observations provide the first demonstration of mutual counter-regulation of IL-1 receptor ligands in keratinocytes.

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Interleukin-1 (IL-1) is a pleiotropic cytokine and primary mediator of cutaneous inflammation (Kupper, 1990; Dinarello, 1996). All biological effects of IL-1 are mediated by two IL-1 receptor ligands, IL-1α and IL-1β, and can be specifically antagonized by a third ligand, IL-1 receptor antagonist (IL-1ra), all of which lie in a 430 kb region of chromosome 2 in humans (Nicklin *et al*, 1994). Both IL-1α and IL-1β are synthesized as 31 kDa molecules lacking signal peptides, and whereas IL-1α remains primarily intracellular in its precursor form, IL-1β is predominantly secreted, following cleavage to a 17 kDa molecule by the cysteine protease caspase-1 at the cell surface (Singer *et al*, 1995). Unlike IL-1β, the 31 kDa form of IL-1α has full biological activity.

IL-1ra exists in four isoforms, one containing a signal peptide and predominantly released from monocytes, whereas the other three lack a leader sequence and are found within keratinocytes and other human epithelial cells (Arend and Guthridge, 2000). IL-1α, IL-1β, and IL-1ra exert their effects through interaction with two specific cell surface receptors. The type I IL-1 receptor (IL-1RI) is an 80 kDa transmembrane molecule with a signal transducing cytoplasmic domain, through which all IL-1-mediated responses are relayed (Sims *et al*, 1989). Thus, IL-1RI-deficient mice fail to respond to IL-1 and display reduced inflammatory responses (Labow *et al*, 1997). The type II IL-1 receptor (IL-1RII) is a smaller molecule (68 kDa) with a truncated cytoplasmic domain, which functions as a non-signalling decoy target for IL-1 receptor ligands (McMahan *et al*, 1991; Colotta *et al*, 1993). Both IL-1 receptors can be cleaved from the cell surface, although soluble-type II IL-1 receptor is the predominant shed form *in vivo* (Arend *et al*, 1994).

The epidermis is physiologically unique in being the only organ in the body to store prodigious quantities of preformed IL-1α (Kupper, 1990) and keratinocytes have been shown to synthesize both IL-1α and -1β molecules *in vitro* (Kupper *et al*, 1986), in addition to both IL-1 receptors (Groves *et al*, 1994). Release of keratinocyte-derived IL-1α is sufficient to trigger cutaneous inflammation in human (Camp *et al*, 1990; Groves *et al*, 1992) and murine (Groves *et al*, 1995b) skin through the induction of secondary cytokines such as IL-8 and upregulation of endothelial adhesion molecules. Presumably, in order to control the potentially harmful effects of excessive IL-1α release in the epidermis, significant quantities of intracellular IL-1ra (icIL-1ra) are detectable in keratinocytes (Bigler *et al*, 1992; Gabay *et al*, 1997). In addition to quenching excessive IL-1α activity, type 1 icIL-1ra may contribute to the inhibition of IL-1 responses by destabilizing messenger RNA (mRNA) of some IL-1 inducible genes such as *gro*-α (Watson *et al*, 1995) and inhibiting IL-1 signal transduction mediators (Garat and Arend, 2003). Moreover, activation of keratin-

Abbreviations: icIL-1ra, intracellular interleukin-1 receptor antagonist; IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist; IL-1RI, interleukin-1 receptor type I; IL-1RII, interleukin-1 receptor type II; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; sIL-1ra, secreted interleukin-1 receptor antagonist; sIL-1RI, soluble interleukin-1 receptor type I

ocytes by mitogens or interferon-γ results in marked up-regulation of IL-1RII *in vitro* and release from the cell surface, adding a further level of control to IL-1 within the epidermis (Groves *et al*, 1995a).

Perturbation of the tightly regulated cutaneous IL-1 network can result in profound inflammatory changes *in vivo*. For example, transgenic mice that overexpress IL-1α under the control of a keratin-14 (K14) promoter display a phenotype characterized by spontaneous focal inflammatory skin lesions (Groves *et al*, 1995b). Further, crossing such animals with mice that overexpress type-1 IL-1 receptor on keratinocytes results in a significantly more severe phenotype than that seen in the IL-1α transgenic mice alone (Groves *et al*, 1996). In contrast, mice that overexpress IL-1RII in basal keratinocytes display a marked reduction in cutaneous inflammatory responses (Rauschmayr *et al*, 1997). Additionally, alterations in the balance of IL-1 molecules have been shown in the human epidermis from individuals with inflammatory skin diseases such as psoriasis, where IL-1α is reduced (Cooper *et al*, 1990) and IL-1ra is increased (Hammerberg *et al*, 1992) and in atopic dermatitis (Terui *et al*, 1998).

The numerous anti-IL-1 molecules present in the epidermis (three isoforms of icIL-1ra, cell surface, and shed type-2 IL-1 receptor) suggest that tightly coordinated regulation of IL-1 activity is essential in the cutaneous microenvironment (Kupper and Groves, 1995). Although counter-regulation of the IL-1 system has long been suspected, formal evidence for this is lacking. In this study, a murine keratinocyte line and mice overexpressing either IL-1α or secreted IL-1ra (sIL-1ra) in basal keratinocytes were used to assess whether homeostatic mechanisms operate both *in vitro* and *in vivo* in the epidermis.

Results

Homeostatic regulation of IL-1α and IL-1ra release by murine keratinocytes *in vitro* We initially exposed cells of the PAM212 keratinocyte line to increasing concentrations of recombinant murine IL-1α (rmIL-1α) for 24 h, prior to analysis of supernatants for total IL-1ra immunoreactivity. In the absence of IL-1α, PAM212 cells released negligible quantities of IL-1ra, but this rose in a concentration-dependent manner to 1924 $\pm$ 97 pg per mL following exposure to 10 ng per mL IL-1α (Fig 1*a*).

Following exposure to increasing concentrations (0.03–3 ng per mL) of recombinant IL-1ra, release of IL-1α from PAM212 cells increased from 529 $\pm$ 69 to 1043 $\pm$ 57 pg per mL (Fig 1*b*). The constitutive release of IL-1α by PAM212 cells is in agreement with previous studies (Miyazaki *et al*, 2000) and the increased release of IL-1α and IL-1ra did not occur as a consequence of cell death as no increases in supernatant lactate dehydrogenase (LDH) activity were observed in parallel experiments (data not shown). Moreover, separate experiments revealed no interference between IL-1α and IL-1ra in their respective ELISA assays (data not shown).

Counter-regulation of IL-1α and IL-1ra release from epidermis of transgenic mice that overexpress IL-1 family molecules *in vivo* To extend the *in vitro* data, epidermal sheets were prepared from ears of mice derived from lines overexpressing either IL-1α (IL-1.2) or sIL-1ra (RA1 and RA10), in addition to wild-type animals (FVB strain). Homogenates were prepared and cytokine content was analyzed by ELISA. Epidermis from line IL-1.2 displayed levels of IL-1α approximately 4-fold higher than wild-type (FVB) littermates (21.2 $\pm$ 2.3 ng per mg total protein *vs* 5.5 $\pm$ 0.4 ng per mg, respectively) (Table I). IL-1α levels were unchanged from wild-type levels in both IL-1ra lines. Similarly, IL-1ra concentrations were 3-fold higher than FVB mice in one line overexpressing the secreted keratinocyte isoform, sIL-1ra (RA1), and increased over 4-fold in line RA10 (67.1 $\pm$ 3.3 ng

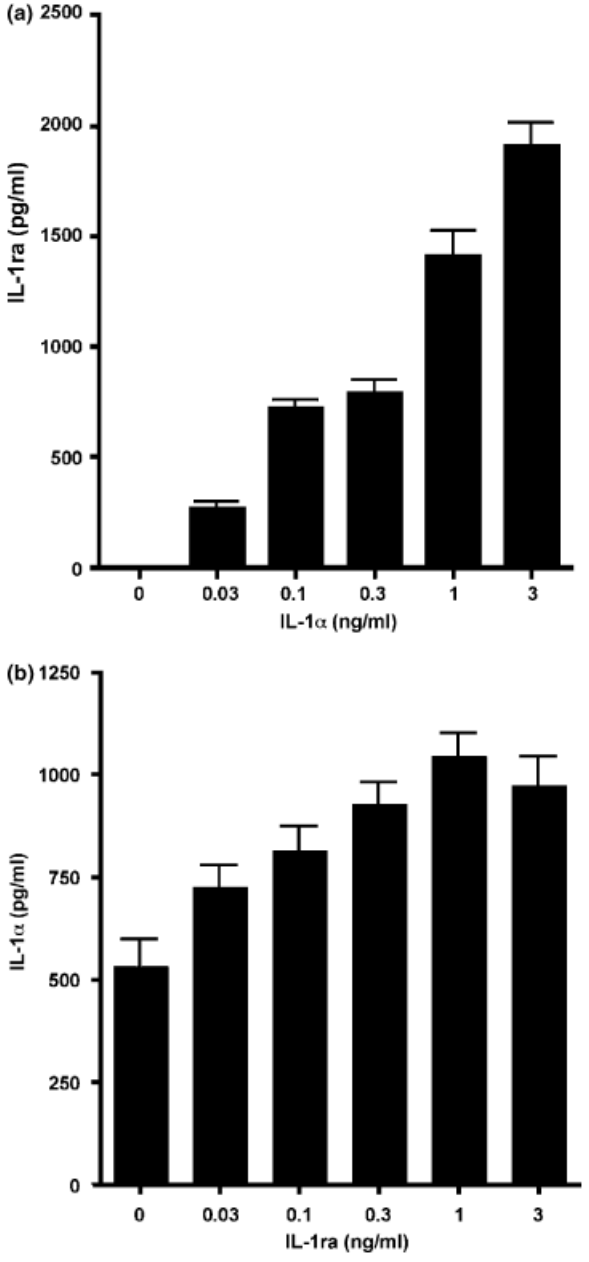

Figure 1
Keratinocytes respond to increasing concentrations of interleukin (IL)-1α or IL-1 receptor antagonist (IL-1ra) by upregulating release of the opposing ligand in a concentration-dependent manner. PAM212 cells were exposed to recombinant murine IL-1α (rmIL-1α) (*a*) or rmIL-1ra (*b*) for 24 h, prior to collection of supernatant and subsequent ELISA for mIL-1ra (*a*) or IL-1α (*b*). Data represent the mean and SEM values for each treatment across three replicate experiments.

per mg total protein *vs* 194.8 ± 3.6 and 284.7 ± 15.0 ng per mg, respectively), whereas no differences were observed in the IL-1.2 line, as expected. Although the ELISA protocol used did not distinguish between the two IL-1ra isoforms, reverse transcription polymerase chain reaction (RT-PCR) analysis of FVB and RA10 epidermal sheets confirmed that the increase in total IL-1ra protein was derived from a substantial upregulation of sIL-1ra mRNA (data not shown). The approximate 10-fold excess of IL-1ra over IL-1α in epidermal homogenates from wild-type mice is in agreement with previous studies analyzing this ratio in keratinocytes (Kutsch *et al*, 1993). These data indicate that transgenic overexpression of IL-1α or IL-1ra in keratinocytes does not affect the total content of the opposing molecule in the epidermis.

We were next interested in determining whether transgenic overexpression of IL-1α or sIL-1ra had any effect on release, as opposed to storage, of the opposing molecule. Thus, epidermal sheets were prepared from transgenic and wild-type mouse ears and placed in organ culture for 24 h, prior to determination of supernatant IL-1α or IL-1ra. As expected, a large increase in IL-1α release was observed from epidermis derived from IL-1.2 mice compared with wild-type littermates (16-fold; 2733 ± 177 *vs* 164 ± 29 pg per mL), consistent with the upregulation seen in the homogenates. Moreover, significant increases in epidermal IL-1α release were also observed in supernatants from epidermal sheets prepared from both RA1 (3-fold; 530 ± 120 pg per mL; $p < 0.01$) and RA10 (4-fold; 678 ± 35 pg per mL; $p < 0.01$) lines (Fig 2*a*). Addition of neutralizing antibody against IL-1ra to the organ culture medium at the onset of incubation significantly reduced IL-1α release from RA10 epidermal sheets (570 ± 42 to 224 ± 9 pg per mL, $p < 0.001$), suggesting that IL-1ra was acting extracellularly (Fig 2*b*). Addition of isotype-matched control antibody was without effect.

In epidermal sheets derived from sIL-1ra transgenic mice, supernatant IL-1ra levels were markedly increased in both RA1 and RA10 lines (RA1: 9131 ± 396 pg per mL; RA10: 23,060 ± 450 pg per mL; FVB: 611 ± 19 pg per mL). More surprisingly however, an 8-fold increase in IL-1ra release from IL-1.2 epidermal sheets was also observed (5006 ± 140 pg per mL; $p < 0.001$, Fig 3*a*). Addition of a neutralizing antibody against IL-1RI to the organ culture decreased supernatant IL-1ra levels from 5827 ± 89 pg per mL in IL-1.2 sheets to 810 ± 66 pg per mL ($p < 0.001$, Fig 3*b*), again suggesting that the IL-1 was acting extracellularly, whereas isotype control antibody was without effect. To investigate the relative contributions of IL-1ra isoforms to the increase in IL-1ra release from the IL-1.2 epidermal sheets, RT-PCR experiments were performed on ear tissue using specific primers to differentiate icIL-1ra and sIL-1ra transcripts. Although icIL-1ra was found to be the predominant isoform, in agreement with previous studies

Table I. Analysis of epidermal IL-1α and IL-1ra content in IL-1α and IL-1ra transgenic mice[a]

Line	IL-1α (ng per mg total protein)	IL-1ra (ng per mg total protein)
FVB	5.5 ± 0.4	67.1 ± 3.3
IL-1.2	21.2 ± 2.3	62.7 ± 3.6
RA1	4.1 ± 0.5	194.8 ± 3.6
RA10	4.5 ± 0.5	284.7 ± 15.0

IL, interleukin; IL-1ra, IL-1 receptor antagonist.
[a]Epidermal homogenates were prepared from the ears of normal, IL-1α (IL-1.2), and sIL-1ra (RA1 and RA10) transgenic lines, as described and analyzed by ELISA for IL-1α or IL-1ra protein. Data represent mean ± SEM (n = 3).

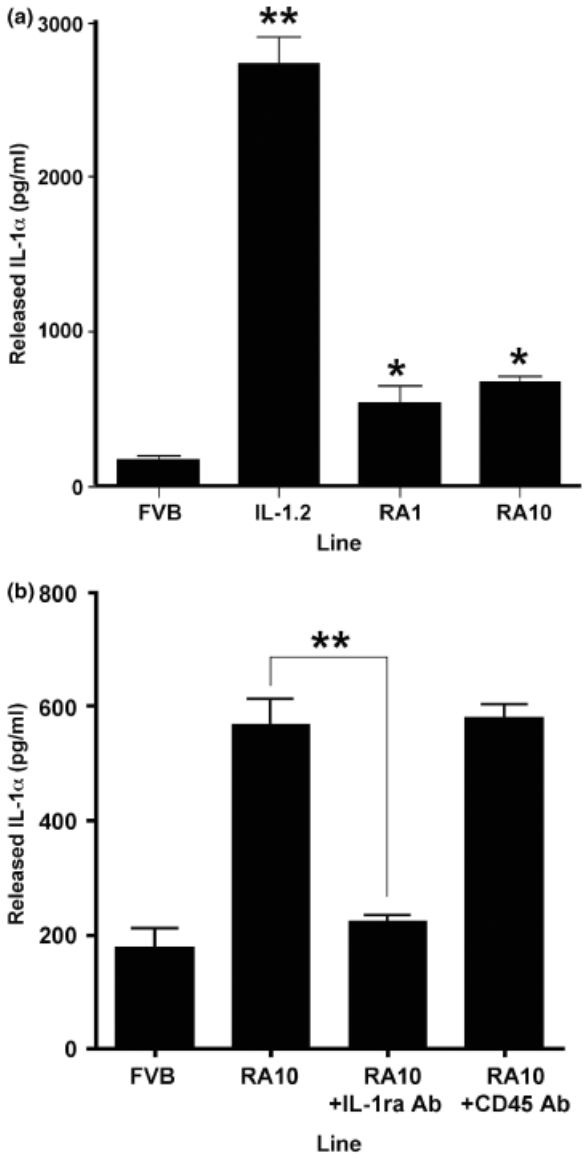

Figure 2
Counter-regulation of interleukin (IL)-1α release in mice transgenic for secreted interleukin-1 receptor antagonist (sIL-1ra). (*a*) Epidermal sheets prepared from three different lines of transgenic mice were subjected to 24 h organ culture and supernatants subsequently assayed by ELISA for murine IL-1α (mIL-1α), as described. *$p < 0.01$ relative to FVB line; **$p < 0.001$ relative to FVB line. (*b*) Epidermal sheets from RA10 (keratin-14–IL-1ra transgenic) mice were co-incubated with either a neutralizing antibody against mIL-1ra or an irrelevant control antibody (CD45) and supernatants subsequently assayed for IL-1α by ELISA. Data represent the mean and SEM values for each treatment across three replicate experiments. **$p < 0.001$.

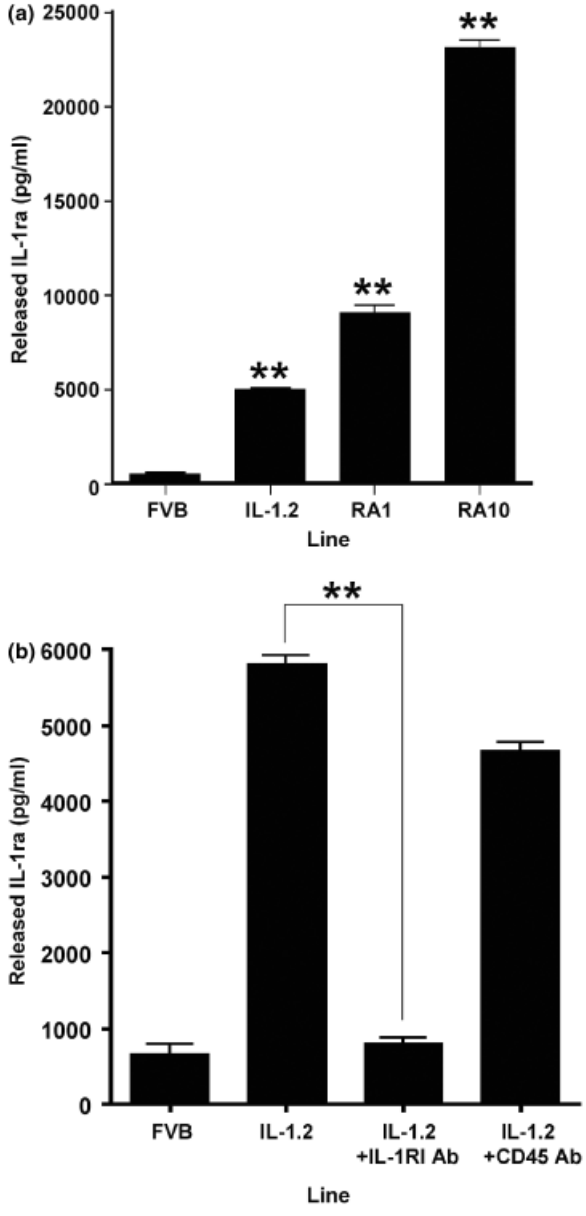

Figure 3
Counter-regulation of interleukin-1 receptor antagonist (IL-1ra) release in mice transgenic for IL-1α. (*a*) Epidermal sheets prepared from three different lines of transgenic mice were subjected to 24 h organ culture and supernatants subsequently assayed by ELISA for murine IL-1ra (mIL-1ra), as described. $^{**}p<0.001$ relative to FVB line. (*b*) Epidermal sheets from IL-1.2 (keratin-14–IL-1α transgenic) mice were co-incubated with either a neutralizing antibody against mIL-1RI or an irrelevant control antibody and supernatants subsequently assayed for IL-1ra by ELISA. Data represent the mean and SEM values for each treatment across three replicate experiments. $^{**}p<0.001$.

(La *et al*, 1999), there was no significant difference in transcription of this molecule between wild-type and IL-1.2 animals, whereas levels of sIL-1ra mRNA increased 179% ± 13% between the two lines (data not shown).

Responses to changes in extracellular IL-1α and IL-1ra concentrations in keratinocytes are mediated through IL-1RI Having shown counter-regulation of IL-1 receptor ligands in keratinocytes, the mechanism of control was investigated by using blocking IL-1R antibodies. PAM212 cells were incubated with 3 ng per mL of either IL-1α or IL-1ra in the presence or absence of monoclonal neutralizing antibodies against either mIL-1RI or mIL-1RII for 24 h prior to ELISA analysis of clarified supernatants. In both cases, the increases observed with the IL-1 receptor ligands were completely abrogated in the presence of anti-IL-1RI antibody (790 ± 8 to 535 ± 19 pg per mL IL-1α and 1001 ± 46 to 361 ± 41 pg per mL IL-1ra), whereas co-incubation with anti-IL-1RII antibody was without effect (Fig 4).

Exposure of keratinocytes to soluble IL-1 receptor (sIL-1R) results in a concentration-dependent increase in IL-1α release *in vitro* Finally, to establish whether manipulation of IL-1RI availability would affect the epidermal IL-1 counter-regulation, PAM212 keratinocytes were incubated overnight at 37°C in the presence of increasing concentrations of recombinant human sIL-1RI, a potent pharmacological IL-1 inhibitor, and supernatants subsequently analyzed by ELISA for IL-1α. Supernatant IL-1α concentrations increased in a concentration-dependent manner from 230 ± 33 to 500 ± 47 pg per mL in the presence of 100 ng per mL sIL-1RI (Fig 5). IL-1α/IL-1R complexes were shown not to interfere with the detection of IL-1α in the ELISA (data not shown).

Discussion

We describe the counter-regulation of IL-1α and IL-1ra in murine keratinocytes, evidenced by increases in IL-1α and IL-1ra release following addition of the opposing ligand, both *in vitro* and in an *ex vivo* organ culture model using epidermal sheets derived from mice that overexpress epidermal sIL-1ra and IL-1α. The complex autoregulatory loops involving released forms of IL-1α and -1β, sIL-1ra, and IL-1 receptors have been extensively studied in leukocytes. For example, binding of IL-1α to IL-1RI results in induction of bio-active IL-1β (Dinarello *et al*, 1987; Manson *et al*, 1989) and sIL-1ra (Jenkins and Arend, 1993), whereas sIL-1ra inhibits both the activity and synthesis of IL-1α and -1β in monocytes (Granowitz *et al*, 1992). Incubation of peritoneal macrophages with sIL-1RI has been reported to induce a significant release of IL-1α in a concentration-dependent manner, consistent with these data and the same study reported a rapid and substantial systemic release of IL-1α following intra-peritoneal injection of sIL-1RI in mice (Netea *et al*, 1999).

In cutaneous cells, icIL-1ra has been shown to be elevated in lysates from human dermal fibroblasts overexpressing proIL-1α, although in the converse experiment, fibroblasts transduced to overexpress icIL-1ra failed to show any increases in IL-1α production (Higgins *et al*, 1999). Additionally, primary murine keratinocytes have been shown to upregulate icIL-1ra mRNA production following IL-1α exposure in a time- and concentration-dependent manner (La *et al*, 1999) and transcription factor binding sites on the icIL-1ra promoter responsible for this increase, including AP-1, have been identified (La and Fischer, 2001; La *et al*, 2002). Our findings extend these observations by the demonstration of a concentration-dependent induction of IL-1ra protein release by IL-1α and our observation that IL-1α release

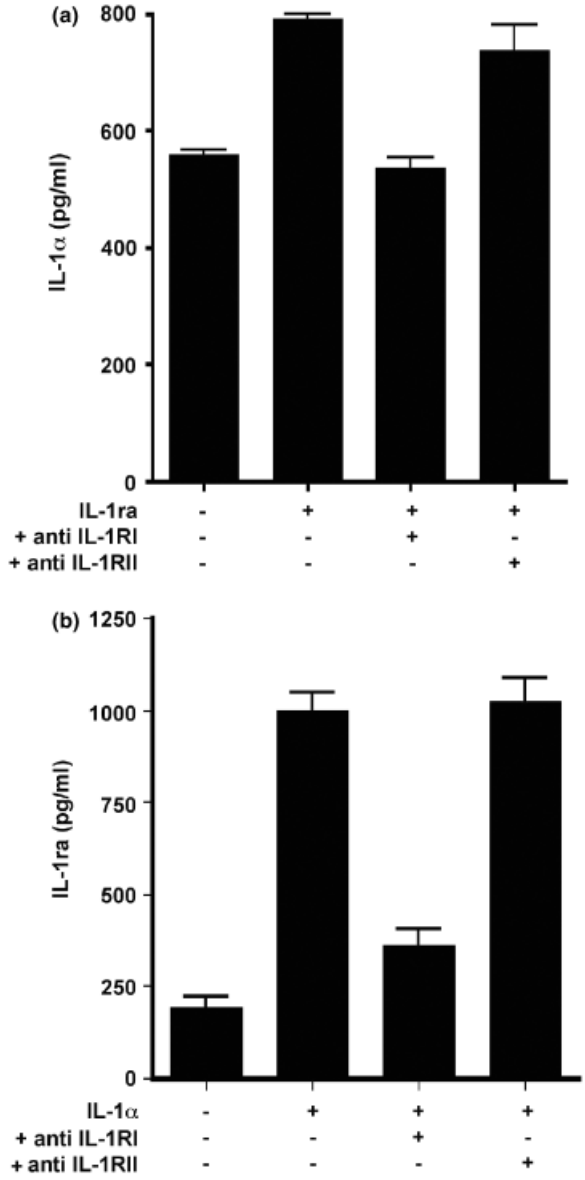

Figure 4
Counter-regulation of interleukin (IL)-1α and IL-1ra is mediated through the type I IL-1 receptor (IL-1R1) in keratinocytes *in vitro*. (*a*) PAM212 cells were incubated with or without IL-1 receptor antagonist (IL-1ra) (3 ng per mL) in the presence or absence of antibodies against either murine IL-1R1 (mIL-1RI) or mIL-1RII (10 μg per mL) for 24 h at 37°C prior to ELISA analysis for IL-1α. (*b*) PAM212 cells were incubated under the same conditions as (a) in the presence of IL-1α (3 ng per mL) and assayed for IL-1ra. Data represent the mean and SEM values for each treatment across three replicate experiments.

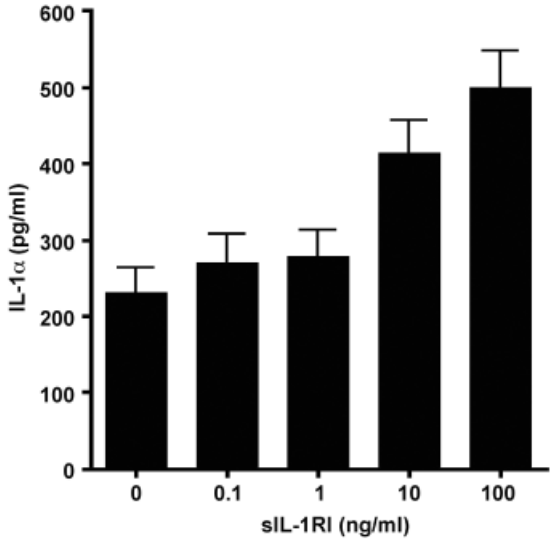

Figure 5
Keratinocytes increase release of interleukin (IL)-1α following exposure to soluble type 1 IL-1 receptor (IL-1R). PAM212 cells were incubated overnight at 37°C in the presence or absence of increasing concentrations of recombinant soluble IL-1 receptor type I (sIL-1RI) and supernatants were subsequently analyzed by ELISA for IL-1α. Data represent the mean and SEM values for each treatment across three replicate experiments.

is stimulated following IL-1ra exposure in keratinocytes is novel.

It is important to note that the effects observed in murine epidermis relate only to extracellular IL-1 molecules. This has several unexpected implications, most notably, that a low level of pericellular IL-1 activity is present around normal keratinocytes. The lack of regulation of opposing IL-1 agonists and antagonists in epidermal homogenates noted in our study strongly suggests that the important site for constitutive IL-1α activity is extracellular, rather than intracellular. Indeed, the release of IL-1α in significant quantities is surprising in itself, as this molecule lacks a signal peptide and the mechanism of its release has been the subject of much speculation. Application of mechanical stress to human keratinocytes, however, has been shown to permit the release of large quantities of IL-1α and IL-1ra in the absence of cell death (Lee *et al*, 1997). Any role for IL-1β in this effect is unlikely because this isoform is produced in negligible quantities by murine keratinocytes (Ansel *et al*, 1988). Further, any IL-1β that is synthesized remains in the bio-inactive pro-form, as a result of deficient caspase-1 processing in this cell type (Mizutani *et al*, 1991) and we found no increase in the release of IL-1β in the epidermal sheets derived from RA1 or RA10 mice (data not shown).

The release of significant quantities of IL-1ra from the epidermal sheets of mice overexpressing IL-1α was unexpected, given the absence of a leader sequence in icIL-1ra isoforms. Although we cannot exclude a contribution from bone marrow-derived cells, for example Langerhans cells, it is most likely that the IL-1ra is keratinocyte-derived. Not only did we observe a similar induction of IL-1ra by IL-1α in our PAM212 cells, but cultured human keratinocytes have been shown previously to release both IL-1α and icIL-1ra into supernatants (Corradi *et al*, 1995). RT-PCR experiments suggested that sIL-1ra accounted for this increase, although it should be noted that very low levels of this transcript were found, relative to the intracellular variant. Indeed, sIL-1ra mRNA was detected only weakly in PAM212 keratinocytes by RT-PCR following treatment with IL-1α (data not shown) and previous studies have found similarly low levels of this isoform in murine skin following lipopolysaccharide administration (Gabay *et al*, 1997). As the increase in total IL-1ra release in IL-1.2 epidermal sheets was more than 8-fold, it is likely that both isoforms contributed to this effect. Irrespective of the cellular source, it is clear that, both in isolated keratinocytes in culture and

in intact epidermis, there is tight regulation of IL-1α by IL-1ra and vice versa.

The observed regulation of keratinocyte IL-1α release by IL-1ra would not be predicted from the known functions of these molecules and underscores the importance of IL-1 homeostasis in the normal cutaneous environment. For a putative IL-1 homeostatic mechanism to function, it is likely that keratinocytes signal an excess or deficit of extracellular IL-1 molecules through the type-1 IL-1 receptor, and our data indicate that this is indeed the case. We hypothesize that low-level ligation of type-1 IL-1 receptor occurs in normal keratinocytes both *in vitro* and *in vivo*. Should there be perturbation of this low-level receptor occupancy, either by the presence of excess receptor agonists or antagonists, keratinocytes respond by releasing the opposing molecule (Fig 6). It should be stressed that this model does not suggest a novel signaling role for IL-1ra, only that IL-1RI occupancy by IL-1ra molecules blocks the signal transduced by IL-1α, which has the effect of reversing the induction of IL-1ra and repression of IL-1α induced by agonist ligand binding.

The physiologic role of constitutive cutaneous release of IL-1α is uncertain. One hypothesis is that keratinocyte-derived IL-1 is required for the production of other molecules involved in tissue homeostasis such as keratinocyte growth factor and granulocyte macrophage colony stimulating factor from dermal fibroblasts (Szabowski *et al*, 2000). Alternatively, it may simply act to maintain a "tick-over" of the cutaneous IL-1 system, ensuring that keratinocytes are primed for IL-1 release in case of injury or other noxious stimulus.

If such a homeostatic role exists, IL-1α and IL-1ra are unlikely to be the sole players in the pathway as neither IL-1α nor IL-1ra knockout mice show any gross cutaneous abnormalities (Horai *et al*, 1998; Nicklin *et al*, 2000), although deletion of the IL-1ra gene specifically on a BALB/c background has been demonstrated recently to induce cutaneous inflammation on ear pinnae (Shepherd *et al*, 2004). Six new members of the IL-1 superfamily have been cloned (Dunn *et al*, 2001) and, although none has been reported to exhibit high-affinity binding to cell surface IL-1RI, three members (IL-1F6, IL-1F8, and IL-1F9) have been shown recently to activate the pathway leading to nuclear factor κB translocation following binding to an IL-1 receptor homolog (Towne *et al*, 2004). Whether these new molecules influence the delicate balance of IL-1 in the epidermis awaits further investigation.

The data presented here represent a description of mutual counter-regulation of IL-1 receptor ligands in keratinocytes and underscore the necessity for tight control of this cytokine system in the extracellular microenvironment around epidermal keratinocytes. Our data indicating that IL-1ra can induce a significant release of IL-1α in keratinocytes may explain the cutaneous inflammatory response that follows therapeutic administration of IL-1ra (Bresnihan, 1999) and illustrate the importance of fully understanding the biology of the IL-1 system in the cutaneous microenvironment.

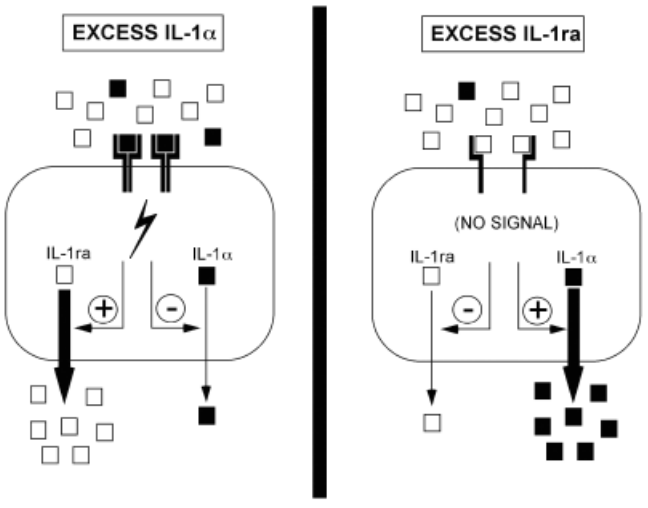

Figure 6
Schematic illustration of the homeostatic mechanism involved in maintaining extracellular interleukin (IL)-1 activity in murine keratinocytes.

Materials and Methods

Reagents PAM212 keratinocytes were a gift from Dr Stuart Yuspa (NIH, Bethesda, Maryland). rmIL-1α, mIL-1ra, and goat anti-mouse IL-1ra antibody were purchased from R&D Systems (Abingdon, UK). Anti-CD45 antibody was purchased from Calbiochem (Nottingham, UK). Recombinant human sIL-1RI was a gift from Dr John Sims (Amgen Corp., Seattle, Washington). The monoclonal antibodies against mIL-1RI (35F5) and mIL-1RII (4E2) were kind gifts from Dr Richard Chizzonite (Hoffman-La Roche, Nutley, New Jersey).

Animals Transgenic mice used in these studies overexpressing IL-1α (IL-1.2) have been described previously (Groves *et al*, 1995b). To generate mice that overexpress IL-1ra in basal epidermis, cDNA encoding the secreted form of mIL-1ra (a gift from Dr Alexander Whitehead, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; (Zahedi *et al*, 1991)) was cloned by blunt-end ligation into the *Bam*H1 site of the K14-hGH vector as previously described (Groves *et al*, 1995b). Transgenic mice were generated in the FVB strain by pronuclear microinjection of the K14–IL-1ra-hGH construct and two founder lines were identified by Southern blot analysis, designated RA1 and RA10. Both lines passed the transgene in a Mendelian fashion and their skin was both grossly and histologically normal.

Eight- to 12-wk-old mice were used in all experiments, which were carried out under the provisions of the Animals (Scientific Procedures) Act, 1986 (UK).

Culture of PAM212 cells PAM212 murine keratinocytes were maintained in RPMI 1640 medium supplemented with 10% newborn calf serum, glutamine (2 mM), penicillin (100 U per mL), streptomycin (100 μg per mL), and HEPES buffer (10 mM) (all reagents from Invitrogen, Paisley, UK). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Confluent layers of PAM212 cells in 48-well plates were incubated in triplicate with increasing concentrations of IL-1α, IL-1ra, or sIL-1RI in a total volume of 0.5 mL per well in the presence or absence of anti-IL-1R antibodies at a final concentration of 10 μg per mL for 24 h at 37°C. Subsequently, supernatants were collected, cleared by centrifugation, and stored at −20°C prior to ELISA analysis. For cell viability experiments, supernatant LDH levels were assessed using a kit (Sigma-Aldrich, Gillingham, UK), according to the manufacturer's instructions.

Preparation of epidermal homogenates Mouse ears were removed and split into dorsal and ventral halves prior to incubation at

37°C for 60 min in the presence of dispase I (2.5 U per mL in phosphate-buffered saline (PBS); Roche, Lewes, UK). Epidermis was separated from the dermis and washed thoroughly in PBS. Epidermal sheets were then homogenized in 500 μL of extraction buffer (50 mM Tris base, 150 mM NaCl) using a motorized mixer for 90 s. Homogenates were cleared by centrifugation, and stored at −20°C. The total protein content was assessed using a modified Bradford assay, according to the manufacturer's instructions (Sigma).

Organ culture of epidermal sheets Epidermal sheets prepared by dispase separation as described above were placed in duplicate into wells of a 24-well plate with 1 mL of RPMI 1640 medium (Invitrogen) and incubated for 24 h at 37°C. Supernatants were subsequently aspirated, clarified by centrifugation, and stored at-20°C. For neutralization studies, anti-IL-1RI (35F5) or anti-IL-1ra antibody were added to the culture medium at a final concentration of 10 μg per mL at the onset of incubation. An isotype-matched, irrelevant control antibody was used at the same concentration in all experiments.

ELISA For the measurement of total mIL-1ra protein, an ELISA was developed using antisera raised against rat IL-1ra protein. Ninety-six-well plates ("Maxisorp", NUNC, Roskilde, Denmark) were coated with a polyclonal sheep anti-rat IL-1ra antibody at 2 μg per mL in bicarbonate coating buffer (0.1 M $NaHCO_3$, 0.1 M NaCl, pH 8.2) overnight at 4°C. Non-specific binding sites were blocked with 1% bovine serum albumin (Sigma) for 1 h at room temperature. Samples or standards prepared from rmIL-1ra (R&D Systems) were added at 100 μL per well and incubated for 2 h at room temperature. Following thorough washing (wash buffer; 0.5M NaCl, 2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 0.1% vol/vol Tween 20), 100 μL of biotinylated polyclonal sheep anti-rat IL-1ra antibody (1:1000 dilution in wash buffer) was added to each well and incubated for 1 h at room temperature. Detection was achieved with 100 μL avidin–HRP (1:5000 dilution in wash buffer; Dako Cytomation, Ely, UK) for 30 min, followed by addition of 100 μL of substrate solution (1 OPD tablet (Sigma) in 10 mL substrate buffer (34.7 mM citric acid, 66.7 mM Na_2HPO_4) + 2 μL H_2O_2) for 20 min and termination with 150 μL 1M H_2SO_4 prior to measurement of absorbance at 490 nm. The IL-1ra ELISA was sensitive to 50 pg per mL and did not distinguish between secreted and intracellular isoforms. IL-1ra concentrations obtained from the ELISA were normalized to total protein content in each sample for comparison.

mIL-1α was assessed using a similar protocol, using a polyclonal sheep anti-mouse IL-1α antibody at 0.5 μg per mL for coating and a biotinylated polyclonal sheep anti-mouse IL-1α antibody at 1:1000 dilution in wash buffer as a secondary antibody. Standards were prepared from rmIL-1α (R&D Systems) and the IL-1α ELISA was sensitive to 30 pg per mL. IL-1α and IL-1β at concentrations up to 1 μg per mL were not detected in the ELISA for mouse IL-1ra, and IL-1β and IL-1ra at concentrations up to 1 μg per mL were not detected in the ELISA for mouse IL-1α.

RT-PCR For the measurement of isoform-specific IL-1ra, whole ears were harvested and snap-frozen in liquid nitrogen, prior to total RNA extraction using 3 mL Trizol (Invitrogen) per ear pair, according to the manufacturer's instructions. Reverse transcription and PCR were performed as described elsewhere (Mee *et al*, 2000) using murine-specific primers directed against hypoxanthine phosphoribosyltransferase (sense: GCTTCCTCCTCAGACCGCTTTTT, antisense: AGGCTTTGTATTTGGCTTTTCCAGT; 60°C annealing temperature, 30 s extension time, 24 cycles), sIL-1ra (sense: CCTCGGGATGGAAATCTG, antisense: CTGGTTGTTTCTCAGGTAAAAGG; 55°C annealing temperature, 30 s extension time, 35 cycles), and icIL-1ra (sense: GCTCCTTTATACACAGCAAGTCTCT, antisense: as sIL-1ra; 55°C annealing temperature, 30 s extension time, 30 cycles). Following electrophoresis, gels were scanned and analysis was performed on a Macintosh computer using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/).

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References

Ansel JC, Luger TA, Lowry D, Perry P, Roop DR, Mountz JD: The expression and modulation of IL-1α in murine keratinocytes. J Immunol 140:2274–2278, 1988

Arend WP, Guthridge CJ: Biological role of interleukin 1 receptor antagonist isoforms. Ann Rheum Dis 59 (Suppl. I):i60–i64, 2000

Arend WP, Malyak M, Smith MF, *et al*: Binding of IL-1α, IL-1β, and IL-1 receptor antagonist by soluble IL-1 receptors and levels of soluble IL-1 receptors in synovial fluids. J Immunol 153:4766–4774, 1994

Bigler CF, Norris DA, Weston WL, Arend WP: Interleukin-1 receptor antagonist production by human keratinocytes. J Invest Dermatol 98:38–44, 1992

Bresnihan B: Treatment of rheumatoid arthritis with interleukin 1 receptor antagonist. Ann Rheum Dis 584:I96–I98, 1999

Camp RDR, Fincham NJ, Ross J, Bird CR, Gearing AJH: Potent inflammatory properties in human skin of interleukin-1 alpha-like material isolated from normal skin. J Invest Dermatol 94:735–741, 1990

Colotta F, Re F, Muzio M, *et al*: Interleukin-1 type II receptor: A decoy target for IL-1 that is regulated by IL-4. Science 261:472–475, 1993

Cooper KD, Hammerberg C, Baadsgaard O, *et al*: IL-1 activity is reduced in psoriatic skin. Decreased IL-1α and increased nonfunctional IL-1β. J Immunol 144:4593–4603, 1990

Corradi A, Franzi AT, Rubartelli A: Synthesis and secretion of interleukin-1α and interleukin-1 receptor antagonist during differentiation of cultured keratinocytes. Exp Cell Res 217:355–362, 1995

Dinarello CA: Biologic basis for interleukin-1 in disease. Blood 87:2095–2147, 1996

Dinarello CA, Ikejima T, Warner SJC, Orencole SF, Lonnemann G, Cannon JG, Libby P: Interleukin 1 induces interleukin 1. Induction of circulating interleukin 1 in rabbits *in vivo* and in human mononuclear cells *in vitro*. J Immunol 139:1902–1910, 1987

Dunn E, Sims JE, Nicklin MJH, O'Neill LAJ: Annotating genes with potential roles in the immune system: Six new members of the IL-1 family. Trends Immunol 22:533–536, 2001

Gabay C, Porter B, Fantuzzi G, Arend WP: Mouse IL-1 receptor antagonist isoforms: Complementary DNA cloning and protein expression of intracellular isoform and tissue distribution of secreted and intracellular IL-1 receptor antagonist *in vivo*. J Immunol 159:5905–5913, 1997

Garat C, Arend WP: Intracellular IL-1ra type I inhibits IL-1-induced IL-6 and IL-8 production in Caco-2 intestinal epithelial cells through inhibition of p38 mitogen-activated protein kinase and NF-κB pathways. Cytokine 23: 31–40, 2003

Granowitz EV, Clark BD, Vannier E, Callahan MV, Dinarello CA: Effect of interleukin-1 (IL-1) blockade on cytokine synthesis. 1. IL-1 receptor antagonist inhibits IL-1-induced cytokine synthesis and blocks the binding of IL-1 to its type II receptor on human monocytes. Blood 79:2356–2363, 1992

Groves RW, Giri J, Sims JE, Dower SK, Kupper TS: Inducible expression of type 2 IL-1 receptors by cultured human keratinocytes. Implications for IL-1 mediated processes in epidermis. J Immunol 154:4065–4072, 1995a

Groves RW, Mizutani H, Kieffer JD, Kupper TS: Inflammatory skin disease in transgenic mice that express high levels of interleukin 1α in basal epidermis. Proc Natl Acad Sci USA 92:11874–11878, 1995b

Groves RW, Rauschmayr T, Nakamura K, Sarkar S, Williams IR, Kupper TS: Inflammatory and hyperproliferative skin disease in mice that express elevated levels of the IL-1 receptor (type I) on epidermal keratinocytes: Evidence that IL-1 inducible secondary cytokines produced by keratinocytes *in vivo* can cause skin disease. J Clin Invest 98:336–344, 1996

Groves RW, Ross E, Barker JNWN, Ross JS, Camp RDR, MacDonald DM: Effect of *in vivo* interleukin-1 on adhesion molecule expression in normal human skin. J Invest Dermatol 98:384–387, 1992

Groves RW, Sherman L, Mizutani H, Dower SK, Kupper TS: Detection of interleukin-1 receptors in human epidermis. Induction of the type II receptor after organ culture and in psoriasis. Am J Pathol 145:1048–1056, 1994

Hammerberg C, Arend WP, Fisher GJ, *et al*: Interleukin-1 receptor antagonist in normal and psoriatic epidermis. J Clin Invest 90:571–583, 1992

Higgins GC, Wu Y, Postlethwaite AE: Intracellular IL-1 receptor antagonist is elevated in human dermal fibroblasts that overexpress intracellular precursor IL-1α. J Immunol 163:3969–3975, 1999

Horai R, Asano M, Sudo K, *et al*: Production of mice deficient in genes for interleukin (IL)-1α, IL-1β, IL-1α/β, and IL-1 receptor antagonist shows that IL-1β is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med 187:1463–1475, 1998

Jenkins JK, Arend WP: Interleukin 1 receptor antagonist production in human monocytes is induced by IL-1α, IL-3, IL-4 and GM-CSF. Cytokine 5: 407–415, 1993

Kupper TS: Immune and inflammatory processes in cutaneous tissues: Mechanisms and speculations. J Clin Invest 86:1783–1789, 1990

Kupper TS, Ballard DW, Chua AO, *et al*: Human keratinocytes contain mRNA indistinguishable from monocyte interleukin 1α and β mRNA. J Exp Med 164:2095–2100, 1986

Kupper TS, Groves RW: The interleukin-1 axis and cutaneous inflammation. J Invest Dermatol 105:62S–66S, 1995

Kutsch CL, Norris DA, Arend WP: Tumor necrosis factor-α induces interleukin-1α and interleukin-1 receptor antagonist production by cultured human keratinocytes. J Invest Dermatol 101:79–85, 1993

La E, Fischer SM: Transcriptional regulation of intracellular IL-1 receptor antagonist gene by IL-1α in primary mouse keratinocytes. J Immunol 166:6149–6155, 2001

La E, Muga SJ, Locniskar MF, Fischer SM: Altered expression of interleukin-1 receptor antagonist in different stages of mouse skin carcinogenesis. Mol Carcinog 24:276–286, 1999

La E, Rundhaug JE, Pavone A, Fischer SM: Regulation of transcription of the intracellular interleukin-1 receptor antagonist gene by AP-1 in mouse carcinoma cells. Mol Carcinog 33:237–243, 2002

Labow M, Shuster D, Zetterstrom M, *et al*: Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. J Immunol 159:2452–2461, 1997

Lee RT, Briggs WH, Cheng GC, Rossiter HB, Libby P, Kupper TS: Mechanical deformation promotes secretion of IL-1α and IL-1 receptor antagonist. J Immunol 159:5084–5088, 1997

Manson JC, di Giovine FS, Poole S, Duff GW: Autoregulation of interleukin-1 production. Eur J Immunol 19:261–265, 1989

McMahan CJ, Slack JL, Mosley B, *et al*: A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J 10:2821–2832, 1991

Mee JB, Alam Y, Groves RW: Human keratinocytes constitutively produce but do not process interleukin-18. Br J Dermatol 143:330–336, 2000

Miyazaki Y, Yokozeki H, Awad S, *et al*: Glucocorticoids augment the chemically induced production and gene expression of interleukin-1α through NF-κB and AP-1 activation in murine epidermal cells. J Invest Dermatol 115: 746–752, 2000

Mizutani H, Black RA, Kupper TS: Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Different strategies of IL-1 production and processing in monocytes and keratinocytes. J Clin Invest 87:1066–1071, 1991

Netea MG, Kullberg BJ, Boerman OC, Verschueren I, Dinarello CA, Van der Meer JWM: Soluble murine IL-1 receptor type I induces release of constitutive IL-1α. J Immunol 162:4876–4881, 1999

Nicklin MJH, Hughes DE, Barton JL, Ure JM, Duff GW: Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. J Exp Med 191:303–311, 2000

Nicklin MJH, Weith A, Duff GW: A physical map of the region encompassing the human interleukin-1α, interleukin-1β and interleukin-1 receptor antagonist genes. Genomics 19:382–384, 1994

Rauschmayr T, Groves RW, Kupper TS: Keratinocyte expression of the type 2 interleukin 1 receptor mediates local and specific inhibition of interleukin 1-mediated inflammation. Proc Natl Acad Sci USA 94:5814–5819, 1997

Shepherd J, Little MC, Nicklin MJH: Psoriasis-like cutaneous inflammation in mice lacking interleukin-1 receptor antagonist. J Invest Dermatol 122: 665–669, 2004

Sims JE, Acres RB, Grubin CE, McMahan CJ, Wignall JM, March CJ, Dower SK: Cloning the interleukin 1 receptor from human T cells. Proc Natl Acad Sci USA 86:8946–8950, 1989

Singer II, Scott S, Chin J, *et al*: The interleukin-1β-converting enzyme (ICE) is localised on the external cell surface membranes and in the cytoplasmic ground substance of human monocytes by immuno-electron microscopy. J Exp Med 182:1447–1459, 1995

Szabowski A, Maas-Szabowski N, Andrecht S, Kolbus A, Schorpp-Kistner M, Fusenig NE, Angel P: c-Jun and JunB antagonistically control cytokine-mediated mesenchymal–epidermal interaction in skin. Cell 103:745–755, 2000

Terui T, Hirao T, Sato Y, *et al*: An increased ratio of interleukin-1 receptor antagonist to interleukin-1 alpha in inflammatory skin diseases. Exp Dermatol 7:327–334, 1998

Towne JE, Garka KE, Renshaw BR, Virca GD, Sims JE: Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-κB and MAPKs. J Biol Chem 279:13677–13688, 2004

Watson JM, Lofquist AK, Rinehart CA, Olsen JC, Makarov SS, Kaufman DG, Haskill JS: The intracellular IL-1 receptor antagonist alters IL-1-inducible gene expression without blocking exogenous signaling by IL-1β. J Immunol 155:4467–4475, 1995

Zahedi K, Seldin MF, Rits M, Ezekowitz RAB, Whitehead AS: Mouse IL-1 receptor antagonist protein. Molecular characterization, gene mapping, and expression of mRNA *in vitro* and *in vivo*. J Immunol 146:4228–4233, 1991