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Intracellular IL-1 Receptor Antagonist Isoform 1 Released from Keratinocytes upon Cell Death Acts as an Inhibitor for the Alarmin IL-1α

Praxedis Martin,*† Gaby Palmer,*† Emiliiana Rodriguez,*† Jennifer Palomo,*†,1 Sylvain Lemeille,* Jérémie Goldstein,*† and Cem Gabay,*†

The inflammatory effects of IL-1α/β are controlled by IL-1R antagonist (IL-1Ra). One IL-1Ra isoform is secreted, whereas three other isoforms (intracellular IL-1Ra [icIL-1Ra] 1, 2, and 3) are supposed to remain intracellular because of the absence of a signal peptide. In contrast to the well-characterized function of the secreted isoform, the biological role of the intracellular isoforms remains largely unclear. icIL-1Ra1 represents the major isoform in keratinocytes. We created icIL-1Ra1−/− mice and investigated the role of icIL-1Ra1 in Aldara (5% imiquimod)-induced psoriasis-like skin inflammation. Naïve icIL-1Ra1−/− mice bred habitually and exhibited a normal phenotype. icIL-1Ra1 deficiency aggravated Aldara-induced skin inflammation, as demonstrated by increased ear thickness and increased mRNA levels of key proinflammatory cytokines. No intracellular effect of icIL-1Ra1 could be detected in isolated keratinocytes using RNA-sequencing analysis; however, Aldara treatment led to caspase 1/11-, caspase 8-, and RIPK3-independent keratinocyte cell death accompanied by the release of both icIL-1Ra1 and IL-1α. Furthermore, blocking IL-1α attenuated the clinical severity of Aldara-induced ear thickening in icIL-1Ra1−/− mice. Our data suggest that upon keratinocyte damage icIL-1Ra1 acts extracellularly as an antagonist of the alarmin IL-1α to immediately counteract its inflammatory effects.

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Psoriasis is a common chronic inflammatory skin disorder affecting ~2% of the world’s population. Its pathophysiology is characterized by epidermal hyperplasia (acanthosis) because of altered keratinocyte proliferation and differentiation, scaling, erythematous plaque formation, and immune cell infiltration (see review in Ref. 1). In mice, the repeated topical application of Aldara (5% imiquimod [IMQ]), an FDA-approved drug for the treatment of genital and perianal warts, actinic keratosis, and basal cell carcinoma, induces skin lesions that resemble human psoriasis. The principal active ingredient of Aldara, the TLR 7 ligand IMQ, but also isostearic acid, a major component of the vehicle, have been previously described to contribute to the observed pathological effects (3). The IL-23/IL-17/IL-22 axis is key for the development of this skin immunopathology (2, 4), but members of the IL-1 family of cytokines are also implicated. Indeed, Aldara-induced skin inflammation is markedly attenuated in IL-36R−/− mice. In addition, mice deficient in the inflammatory cytokines IL-1α and IL-1β or in their signaling receptor IL-1R1 showed reduced neutrophil infiltration and acanthosis (5–7).

IL-1 (referring to both IL-1α and IL-1β) is a master proinflammatory cytokine. However, IL-1α and IL-1β differ in their regulation. Although both cytokines are produced as 31-kDa peptides, pro–IL-1β is biologically inert and requires enzymatic processing to become active, whereas pro–IL-1α is able to bind to IL-1R1 and stimulate inflammatory responses. Pro–IL-1α is a cell-associated protein that can be released following various forms of cell death. Therefore, IL-1α is able to trigger early inflammatory responses upon cell or tissue damage, thereby acting as an alarmin.

The inflammatory effects of IL-1 are tightly controlled by IL-1R antagonist (IL-1Ra), which competitively blocks the binding of IL-1 to IL-1R1. Four isoforms of IL-1Ra exist that are produced from the same Il1rn gene (see review in Ref. 8). The original and best described isoform is secreted IL-1Ra (sIL-1Ra). It is mainly expressed in myeloid cells and synthesized as proprotein sIL-1RA (prosIL-1Ra), containing a signal peptide (9–11). The three other isoforms lack a signal peptide and thus are considered to stay intracellular (intracellular IL-1Ra [icIL-1Ra] 1, icIL-1Ra2, icIL-1Ra3). The icIL-1Ra1 isoform is generated by alternative splicing of an icIL-1Ra1–specific first exon upstream of the sequence for sIL-1Ra into an internal splice-acceptor site within the exon coding for the signal peptide area of sIL-1Ra (12, 13). The cDNA for a second intracellular isoform (icIL-1Ra2) was cloned from human neutrophils and is coded by an extra exon located downstream of the first icIL-1Ra1–specific exon. A

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; EGFP, enhanced green fluorescence protein; ES, embryonic stem; icIL-1Ra, intracellular IL-1Ra; IL-1Ra, IL-1R antagonist; IMQ, imiquimod; iTL, InGenious Targeting Laboratory; K-SFM, keratinocyte serum-free medium; LDH, lactate dehydrogenase; prosIL-1Ra, proprotein sIL-1RA; qRT-PCR, quantitative RT-PCR; rec. m, recombinant mouse; RIPK3, receptor-interacting protein kinase 3; RNA-Seq, RNA-sequencing; sIL-1Ra, secreted IL-1Ra; WT, wild-type.

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corresponding protein has not yet been described (14). icIL-1Ra3 is produced by alternative translation initiation from sIL-1Ra mRNA (15). Despite the lack of a signal peptide, the three intracellular isoforms can be passively released by dying cells or actively released by a yet unknown leaderless pathway (16–19). In vitro assays using recombinant proteins corresponding to the intracellular isoforms demonstrated that they can compete extracellularly for IL-1R1 binding in a manner similar to sIL-1Ra (13, 20, 21).

Although the collective role of all four IL-1Ra isoforms has been well characterized using Il1rn knockout mice and in humans deficient in IL-1Ra (22, 23), the specific function(s) of the intracellular isoforms in vivo remains unknown. We thus generated a knock-in mouse line by insertion of an enhanced green fluorescence protein (EGFP) cassette into the icIL-1Ra1-specific exon, which resulted in the deletion of icIL-1Ra1 without affecting the other IL-1Ra isoforms.

Because icIL-1Ra1 is constitutively expressed and represents the major IL-1Ra isoform in both mouse and human keratinocytes (12, 24, 25), we investigated the role of icIL-1Ra1 in an experimental model of skin inflammation. We show in this study that icIL-1Ra1 has a beneficial effect on the severity of Aldara (5% IMQ)-induced skin inflammation. No intracellular effect of icIL-1Ra1 could be detected using isolated keratinocytes; however, addition of Aldara led to keratinocyte cell death, accompanied by the release of both icIL-1Ra1 and IL-1α. Furthermore, blocking IL-1α attenuated the clinical severity of Aldara-induced ear thickening in icIL-1Ra1−/− mice. Taken together, our results suggest that icIL-1Ra1 is stored in keratinocytes and that upon cell death, icIL-1Ra1 is coreleased with the alarmin IL-1α and immediately counteracts its inflammatory potential, thus acting as a natural anti-alarmin.

Materials and Methods

Mice

Mice specifically deficient for the icIL-1Ra isoform 1 (icIL-1Ra1−/−; B6.129-Illrn<sup>GFP</sup>/CemG<sup>entC</sup>) were generated by InGenious Targeting Laboratory (ITL; Stony Brook, NY). An EGFP knock-in targeting vector was designed (ITL) to delete the icIL-1Ra1–specific first exon of the Il1rn gene and replace it by an EGFP reporter gene (Fig. 1A). A 6.14-kb region used to construct the targeting vector was first subcloned from a C57BL/6 BAC clone (RP23: 159M23) into a 2.4-kb backbone vector (pSP72; Promega, Madison, WI) prior to electroporation into iTL BA1 (C57BL/6 × 129/SvEv) hybrid embryonic stem (ES) cells. The cassette consisting of the

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**FIGURE 1.** Generation of icIL-1Ra1−/− mice. (A) Targeting strategy used for the introduction of an EGFP–Neo cassette into the icIL-1Ra1–specific first exon (icIl1rn1) of the mouse Il1rn gene. This approach does not interfere with the expression of the other transcript variants. Shown from top to bottom are the WT icIl1rn1 genomic locus (WT allele), the targeting vector and the targeted genomic locus (Mutant allele). Bold lines indicate arms of homology for recombination. Exon 1 is represented as a gray box and numbered. The neomycin resistance gene (Neo) is flanked by two loxP sites (triangles). The

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**TABLE 1.** Analysis of IL-1Ra isoforms in lysates of BMDC generated from naive WT and icIL-1Ra1−/− (KO) mice. IL-1Ra was detected using a polyclonal goat anti-mouse IL-1Ra Ab recognizing all IL-1Ra isoforms. 2×/2× mice. (B) Southern blot analysis of WT (+/+), heterozygous (+/−), and homozygous (−/−) PCR genotyping to distinguish WT (+/+), heterozygous (+/−), and homozygous (−/−) PCR genotypes. DNA from 2×/2× mice. (C) 2×/2× PCR products are 300 and 842 bp, respectively. (D) Male (left panel) and female (right panel) WT (male: n = 13/n = 6 [10/17 wk]; female: n = 11/n = 6 [10/17 wk]) and icIL-1Ra1−/− (male: n = 13/n = 13 [10/17 wk]; female: n = 11/n = 9 [10/17 wk]) mice were weighed at 10 and 17 wk of age. Data are shown as the mean ± SEM. No significant differences in body weight were observed between the genotypes (unpaired two-tailed Student t test). (E) Analysis of IL-1Ra isoforms in lysates of BMDC generated from naive WT and icIL-1Ra1−/− (KO) mice. IL-1Ra was detected using a polyclonal goat anti-mouse IL-1Ra Ab recognizing all IL-1Ra isoforms. Protein loading was assessed by the determination of GAPDH expression. One representative sample per genotype out of n = 3 per genotype is shown. Arrows indicate different IL-1Ra isoforms.
EGFP coding sequence, followed by a loxP-Flanked Neo sequence in reverse orientation, was inserted immediately upstream of the ATG start codon of the Il1rn gene, thereby replacing the coding sequence and splice donor site of the icl-1Ra1 allele. The long homology arm extends 3.94 kb 5’ of EGFP. The short homology arm extends 1.95 kb 3’ of the Neo cassette. The deletion of exon 1 does not interfere with the expression of the other isoforms with the transcription start site in exon 2, located 12.06 kb downstream of the WT Il1rn allele. Male and female mice were weighed at the age of 10 and 17 wk (Fig. 1D). The deletion of exon 1 does not interfere with the expression of other Il-1Ra isoforms (sIl-1Ra, icIl-1Ra3) (Fig. 1C). PCR transcription start site in exon 2 is located 10 kb downstream of the icl-1Ra1 allele.

Mice lacking all isoforms of IL-1Ra in myeloid cells were generated by crossing homozygous IL-1Rafllox/flox mice (B6.129-Ilm^vecent^ [27]) backcrossed onto the C57BL/6 background for six generations using a marker-assisted selection protocol (26). Genotyping for the WT or icllm1-deleted allele was performed by a three-primer PCR combining a common forward primer (P1: 5’-CCTCTAGATGATCCACCCCTCC-3’) with reverse primers specific for the WT (P2: 5’-CCGATGATCTCTTGGGAGC-3’) or the icllm1-deleted allele (P3: 5’-CCGATGATCTCTTGGGAGC-3’) (Fig. 1C).

Mice were weighed at the age of 10 and 17 wk (Fig. 1D). The deletion of exon 1 does not interfere with the expression of other Il-1Ra isoforms (sIl-1Ra, icIl-1Ra3) (Fig. 1C). PCR transcription start site in exon 2 is located 10 kb downstream of the icl-1Ra1 allele.

Mice lacking all isoforms of IL-1Ra in myeloid cells were generated by crossing homozygous IL-1Rafllox/flox mice (B6.129-Ilm^vecent^ [27]), backcrossed onto the C57BL/6 background for six generations using a marker-assisted selection protocol (26), with mice expressing the Cre-recombinase under the transcriptional control of the endogenous lysozyme M promoters driven by the Cre-recombinase transgene in myeloid cells (29). PCR genotyping of the IL-1Ralphafllox mice was previously described (27). Genotyping for the Cre-recombinase transgene was performed by PCR following the Jackson Laboratory protocols. This mouse line was maintained by intercrossing homozygous IL-1Ralpha^fllox^ mice negative for the Cre-recombinase (control littermates) with homozygous IL-1Ralpha^fllox^ mice expressing one allele of the Cre-recombinase transgene (IL-1Ralpha^cre^). All mice were maintained under conventional conditions in the animal facility of the Geneva University School of Medicine. Water and food were provided ad libitum.

Tails of casp1/11^−/− (B6N.129S2-Casp1tm1Evj [30]) and casp11/11 × casp8× receptor-interacting protein kinase 3 (Rip3k3)^−/− (Casp8tm1Raz [31]; Rip3k3^−/−)[Casp8tm1Raz [31]; Rip3k3^−/−]) mice were kindly provided by Dr. Mohamed Lamanfi (Janssen Pharmaceutica, Beerse, Belgium) and Dr. Andy Wallaert (University of Ghent) to isolate primary keratinocytes. Genotyping was performed by PCR following The Jackson Laboratory protocols or as described previously (30–32), respectively.

Animal studies were approved by the Animal Ethics Committee of the Geneva Veterinarian Office (license numbers GE/43/15, GE/113/18, and GE/77/19) and performed according to the appropriate codes of practice.

Induction of psoriasis-like dermatitis in mice

Sex- and age-matched (8-12 wk-old) mice received a daily topical dose of 12.5 mg of Aldara Cream (MEDA Pharma, Wangen, Switzerland) containing 5% IMQ on the right ear for eight consecutive days. The equivalent volume of Vaseline cream applied on the left ear served as control. In some experiments, mice were injected i.p. on days 1, 3, 5, and 7 of the Aldara experiment with a neutralizing Ab directed against IL-1α (functional grade, hamster anti-mouse IL-1α, clone ALF-161, 200 μg/mouse; Thermo Fisher Scientific, Zug, Switzerland) or the appropriate isotype control (functional grade, hamster anti-mouse IgG1, 200 μg/mouse; Thermo Fisher Scientific (33)). Ear thickness was measured in triplicates daily using a pocket thickness gauge (Mitutoyo Europe GmbH, Düsseldorf, Germany). Mice were euthanized on day 8, and ears were collected for histological analysis and RNA and protein extraction or explant cultures. For the latter, ear skin was collected as described with slight modifications (6). In brief, three pieces of skin were excised from each control and Aldara-treated ear using a circular punch biopsy tool, generating full thickness skin samples of identical surface area. Each explant (diameter: 2 mm) was incubated in 200 μl assay medium (DMEM/5% FCS/penicillin/streptomycin) for 24 h at 37°C. Supernatant was collected and pooled from the three cultures for each ear. Cytokine levels were analyzed by ELISA. Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH; Cytotoxicity Detection Kit; Roche or Sigma-Aldrich) according to manufacturer’s instructions. Lysis of parallel control cultures with 1% Triton X-100 in assay medium was used to determine the maximum amount of releasable LDH enzyme activity.

Generation and stimulation of bone marrow–derived dendritic cells and bone marrow–derived macrophages

Bone marrow–derived dendritic cells (BMDM) and bone marrow–derived macrophages (BMDM) were generated as previously described (34). A total of 1 × 10^6 BM cells per well were seeded in medium containing GM-CSF or L929 cell-derived M-CSF, respectively, into a 24-well plate and left to adhere overnight. The medium was discarded, the cells were rinsed with PBS, and fresh medium without growth factors was added to the cells. Cells were stimulated with 100 ng/ml LPS (Escherichia coli purified by phenol extraction [serotype O55:B5]; Sigma-Aldrich) for 24 h. Supernatant was collected, and cytokine production was analyzed by ELISA or Western blot. Cells were lysed for analysis by quantitative real-time PCR or Western blot.

Culture and stimulation of primary mouse keratinocytes

Primary keratinocytes were isolated from tails of adult mice. Tails were incubated in keratinocyte serum-free medium (K-SFM) without CaCl2 (Thermo Fisher Scientific) with penicillin/streptomycin/10 mg/ml Dispase II (Thermo Fisher Scientific) at 4°C overnight. The epidermis was detached from the dermis and gently mixed three times for 1 min in 0.05% Trypsin/0.02% EDTA (PAN Biotech) at 37°C, and single keratinocytes were enriched by filtration of inflammatory cells were evaluated as previously described (28) with slight modifications. In brief, tissue sections were incubated with 7.2 mg/ml 3,3’-diaminobenzidine, and sections were counterstained with hematoxylin. Slides were scanned with an Axio Scan.Z1, and pictures were taken using the ZEN lite software. For immunofluorescence, the sections were rinsed, incubated with donkey anti-goat IgG (Jackson Immunoresearch, Cambridgeshire, U.K.) and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Slides were scanned with an Axioscan.Z1, and pictures were taken using the ZEN lite software.
KERATINOCYTE-DERIVED icIL-1Ra1 ANTAGONIZES IL-1α

with DAPI (Thermo Fisher Scientific). Confocal fluorescent images were acquired on a Zeiss LSM 700 microscope (Zeiss), and pictures were taken using ZEN lite software.

**RNA extraction and quantitative RT-PCR**

Total RNA was extracted from BMDC, BMDM, mouse primary keratinocytes, or mouse ears using TRizol reagent, followed by a cleanup of the last two using RNeasy Micro Kit columns (QIAGEN). RNA was reverse transcribed, and mRNA levels were examined as previously described (37). The primer sequences (Eurofins Scientific, Ebersberg, Germany) are shown in Table 1. mRNA expression levels were determined by quantitative RT-PCR (qRT-PCR) using IQ SYBR Green Supermix (Bio-Rad Laboratories, Cressier, Switzerland) on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). Gene expression levels were calculated using the comparative method (2^ΔΔCt) for relative quantification by normalization to Mrpl32 gene expression. Non–reverse-transcribed RNA samples were included as negative controls.

**RNA-Seq analysis**

Library preparation, sequencing, and read mapping to the reference genome. Total RNA was isolated from mouse primary keratinocytes using TRizol reagent, followed by a cleanup using RNeasy Micro Kit columns (QIAGEN). cDNA libraries were constructed by the Genomics Platform of the University of Geneva, using the Illumina TruSeq Stranded RNA Library Preparation Kit according to the manufacturer’s protocol. Libraries were sequenced using single-end (50 nt-long) reads on Illumina HiSeq 4000. FastQ reads were mapped to the ENSEMBLE reference genome (GRCm38.89) using STAR version 2.4.0j (38) with standard settings, except that any reads mapping to more than one location in the genome (ambiguous reads) were discarded (m = 1).

Unique gene model construction and gene coverage reporting. A unique gene model was used to quantify reads per gene. Briefly, the model considers all annotated exons of all annotated protein-coding isoforms of a gene to create a unique gene, in which the genomic region of all exons is considered coming from the same RNA molecule and merged together.

**Subcellular fractionation**

All steps were performed on ice. Mouse primary keratinocytes were washed twice with ice-cold PBS, and the pellet was lysed in ice-cold hypotonic buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mm EDTA, 0.1 mm EGTA, 1 mm Na3VO4, 1 mm EDTA-free protease, and phosphatase inhibitor mixture) 15 min on ice. 0.125% s/v NP40 were added, and the lysate was vortexed 10 s. After centrifugation for 5 min at maximal speed, the supernatant containing the cytosolic fraction was collected. The pellet was washed once with ice-cold hypotonic buffer supplemented with 0.1% NP40 and lysed in ice-cold nuclear protein extraction buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mm EDTA, 1 mm EGTA, 1 mm Na3VO4, 1 mm EDTA-free protease, and phosphatase inhibitor mixture), vortexed 10 s and incubated 10 min on ice. This procedure was repeated for a total of five times. After centrifugation for 5 min at maximal speed, the supernatant containing the soluble nuclear protein fraction was collected. Protein concentration in cytosolic and nuclear fractions was determined using the DC Protein Assay Kit (Bio-Rad Laboratories AG).

**Western blot analyses**

Total proteins from BMDC, BMDM, or mouse dorsal skin or mouse ears were extracted in RIPA buffer (150 mM NaCl, 50 mM Tris/ HCl [pH 8], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, and 1× EDTA-free protease inhibitor mixture) for 30 min on ice or by homogenizing in 700 μl of RIPA buffer using a POLYTRON homogenizer followed by 30-min incubation on ice, respectively. The lysates were cleared by centrifugation. Protein concentration in tissue lysates was determined using the DC Protein Assay Kit (Bio-Rad Laboratories AG). Culture supernatants of BMDC and BMDM were collected and directly used for Western blotting without further concentration. The rec. mL1Ra secreted isoform (standard of mouse IL-1Ra1 DuoSet ELISA Kit [R&D Systems]) served as positive control. Samples were separated by electrophoresis in homemade 17% SDS-PAGE and blotted onto a PVDF membrane. For Western blot analyses of IL-1Ra expression in cytosolic and nuclear fractions, 4–12% gradient gels (NuPAGE; Thermo Fisher Scientific) were used. Membranes were blocked with 5% horse serum in TBS/0.1% Triton X-100 and then incubated with polyclonal goat anti-mouse IL-1Ra (capture Ab of mouse IL-1Ra1; DuoSet ELISA Kit; R&D Systems) at 0.14 μg/ml, or with polyclonal rabbit anti-human KDM1/LSHD1 (Abcam, Cambridge, U.K.) diluted 12,000 in TBS/0.1% Triton X-100/1% horse serum. Upon incubation with a donkey anti-goat IgG HRP-conjugated Ab (Santa Cruz Biotechnology) or goat anti-rabbit IgG HRP-conjugated Ab (Santa Cruz Biotechnology) membranes were developed using Clarity Western ECL Substrate (Bio-Rad Laboratories AG) and by using the imager LAS 4000 (Fujifilm, Düsseldorf, Germany). After stripping of the membranes with ReBlot Plus Strong Antibody Stripping Solution (Merck, Millipore, Schaffhausen, Switzerland) according to the manufacturer’s instructions, the membranes were probed with polyclonal rabbit anti-human GAPDH (FL-335; Santa Cruz Biotechnology) at 0.1 μg/ml in TBS/0.1% Triton X-100/1% horse serum, followed by incubation with a goat anti-rabbit IgG HRP-conjugated Ab (Santa Cruz Biotechnology).

**Measurement of cytokine levels**

In cell culture supernatants, IL-1Ra, IL-1α, CXC1L1, or IL-1β protein levels were determined using DuoSet ELISA (R&D Systems) or ELISA Ready Set Go kits (eBioscience), respectively. The IL-1Ra DuoSet ELISA detects all isoforms of IL-1Ra.

**Statistical analysis**

Significant variations were calculated using the unpaired or paired two-tailed Student t test or two-way repeated measures ANOVA, as indicated in the figure legends. A p value <0.05 was considered significant. Results are expressed as the mean ± SEM.

**Results**

**Generation and characterization of icIL-1Ra1−/− mice**

Homologous recombination of the WT allele with the targeting vector in ES cells (Fig. 1A) was confirmed by Southern blot analysis (Fig. 1B). A correctly targeted ES cell clone was used to generate icIL-1Ra1-deficient mice, which were genotyped by PCR to distinguish WT and/or targeted alleles (Fig. 1C). icIL-1Ra1−/− mice were fertile, showed no phenotypic abnormalities (data not shown), and showed no difference in body weight (Fig. 1D), in contrast to mice deficient in all four IL-1Ra isoforms (42). The insertion of the EGFP–Neo cassette at the ATG start codon in the icIL-1Ra1–specific first exon of the mouse Il1rn gene deleted only the expression of icIL-1Ra1 but did not interfere with the expression of the isoforms icIL-1Ra3 and sIL-1Ra (shown is the immature proform of sIL-1Ra [pro–IL-1Ra]; Fig. 1E). No EGFP signal could be detected by immunofluorescence microscopy on skin sections (data not shown).

**Characterization of BMDC and BMDM from WT and icIL-1Ra1−/− mice**

It is known that upon stimulation myeloid cells express both sIL-1Ra and the intracellular isoforms icIL-1Ra1 and icIL-1Ra3. We generated BMDC and BMDM to further investigate the deletion of icIL-1Ra1. The presence of icIL-1Ra1 mRNA was only detected in BMDC and BMDM from WT mice but was absent in BMDC and BMDM from icIL-1Ra1−/− mice, both at baseline and upon LPS treatment (Fig. 2A). Expression levels of mRNA encoding both sIL-1Ra and icIL-1Ra3 (referred to as slrn) were significantly increased in BMDC or similar in BMDM of icIL-1Ra1−/− compared with WT mice upon LPS stimulation (Fig. 2A). However, by using an ELISA that detects all IL-1Ra isoforms, no differences in IL-1Ra protein levels between both genotypes were detected in the supernatants of control and LPS-treated BMDC and BMDM (Fig. 2B). Western blot analysis revealed that the only isoform detected in the supernatants of control and LPS-treated BMDC and BMDM was sIL-1Ra (Fig. 2C). Furthermore, Western blot...
analysis of cell lysates demonstrated the presence of all IL-1Ra isoforms in WT BMDC and BMDM, whereas icIL-1Ra1 was absent in BMDC and BMDM from icIL-1Ra1−/− mice (Fig. 2C). Western blot analysis identified icIL-1Ra1 as the principal isoform in whole ear lysates of control and Aldara-treated WT mice, whereas icIL-1Ra1 was absent in ear lysates of icIL-1Ra1−/− mice. Aldara treatment also led to the presence of IL-1Ra+ cells in the dermis (Fig. 4A) as well as to production of immature prosIL-1Ra and icIL-1Ra3 in ears of both genotypes (Fig. 4B), probably originating from infiltrating inflammatory myeloid cells. Therefore, to investigate the impact of myeloid cell–derived IL-1Ra during Aldara-induced skin inflammation, we used mice with LysMCre-mediated deletion of IL-1Ra in myeloid cells (IL-1RaM). Clinical scoring showed no difference in ear thickening between IL-1RaM mice and littermate controls (IL-1Raflox/flox; Fig. 4C). Furthermore, there was no difference in mRNA expression levels for icIL-1Ra1 in ears of both genotypes. In contrast, expression of mRNA encoding sIL-1Ra and icIL-1Ra3 (sIL1rm) was almost completely abrogated upon Aldara treatment in IL-1RaM mice (Fig. 4D).

No intracellular effect of icIL-1Ra1 on gene regulation in keratinocytes

Intracellular effects of icIL-1Ra1 were previously reported in vitro using icIL-1Ra1 genetically modified cells (44–46). To examine a possible intracellular role of icIL-1Ra1 in skin inflammation, we stimulated primary keratinocytes isolated from tails of naive WT or icIL-1Ra1−/− mice with IL-1α or with IL-36β. In addition to Il1r1, only three genes were differentially expressed in control keratinocytes (Thbs2, Degr, and Cyp1A1), whereas there were no differentially expressed genes, except for Il1r1, in WT as compared with icIL-1Ra1−/− keratinocytes upon stimulation with IL-1α or IL-36β (Fig. 5A). Additionally, there was no difference in expression levels of selected mRNAs in primary keratinocytes of WT as compared with icIL-1Ra1−/− mice after in vitro stimulation with Aldara or with the equivalent amount of IMQ (Supplemental Fig. 2). Of note, transcripts for IL-1β could not be detected in primary keratinocytes (data not shown). Interestingly, RNA-Seq analysis demonstrated that IL-1α− and IL-36β− induced responses were similar in primary keratinocytes from both WT and icIL-1Ra1−/− mice (Supplemental Fig. 3A, 3B). Aldara treatment leads to keratinocyte cell death accompanied by the release of icIL-1Ra1 and IL-1α

Despite the lack of a signal peptide, the three icIL-1Ra isoforms can be passively released from dying cells or actively released by a yet
unknown leaderless pathway (16–19) and can then compete extracellularly with IL-1 for IL-1R1 binding, similar to sIL-1Ra (13, 20, 21). To investigate whether icIL-1Ra1 can be released during Aldara-induced skin inflammation, we examined the presence of IL-1Ra in conditioned media of skin explants collected from control and Aldara-treated ears of WT and icIL-1Ra1/2 mice, using an ELISA that recognizes all isoforms of IL-1Ra. Levels of IL-1Ra released from Aldara-treated explants of both WT and icIL-1Ra1/2 mice were significantly higher than from control explants (Fig. 5B). Furthermore, IL-1Ra levels were significantly higher in supernatants of ear explants from WT as compared with icIL-1Ra1/2 mice. The levels of IL-1Ra detected in supernatants of icIL-1Ra1/2 explants are likely to represent sIL-1Ra and icIL-1Ra3 isoforms expressed by infiltrating myeloid cells. IL-1α is another member of the IL-1 family expressed in keratinocytes that does not have a signal peptide and can be released upon cell damage. IL-1α levels were significantly increased in supernatants of ear explants upon Aldara treatment as compared with control but without any difference between genotypes (Fig. 5B). A modest secretion of IL-1β was detected in some explant cultures of icIL-1Ra1/2 mice after Aldara treatment (Fig. 5B). Of note, in addition to the increase of icIL-1Ra1 and
IL-1Ra, a higher percentage of cell death was detected in Aldara-treated ear explants, as assessed by LDH measurement in culture supernatants (Fig. 5B). To mimic the results observed with ear explants, we isolated primary keratinocytes from naive WT and icIL-1Ra1−/− mice and stimulated them with different concentrations of Aldara in vitro. Aldara induced the release of IL-1Ra from keratinocytes of WT mice. Only marginal amounts of IL-1Ra were measured in the supernatants of icIL-1Ra1−/− mice.
keratinocytes (Fig. 5C). Furthermore, Aldara induced the release of IL-1α and a dose-dependent cell lysis in both WT and icIL-1Ra1−/− keratinocytes (Fig. 5C). IMQ alone, the main active component of Aldara, did not have any of the above-mentioned effects. The proinflammatory cytokines IL-1α, IL-1β, and IL-36β did not induce the release of icIL-1Ra1, IL-1α, or cell lysis. A dose of CXCL1 served as positive control for cell stimulation (Fig. 5C).

The release of icIL-1Ra1 and IL-1α is independent of caspase 1/11, caspase 8, and RIPK3

It has previously been shown that Aldara induces pyroptosis, a caspase 1-dependent cell death, which results in the release of IL-1β, in human keratinocytes (3). We therefore decided to determine whether the release of icIL-1Ra1 and IL-1α from mouse keratinocytes was also related to Aldara-induced caspase-1 activation and pyroptosis. We observed that levels of IL-1Ra, IL-1α, and LDH did not differ in the supernatants of WT and casp1/11−/− keratinocytes cultured in the presence of Aldara, thus ruling out the involvement of the inflammasome–caspase-1 pathway in Aldara-induced cell death (Fig. 5D). To further examine the role of other cell death pathways, we used keratinocytes from mice with additional deficiency in caspase 8, which blocks extrinsically induced apoptosis, and RIPK3, which blocks necroptosis. We observed that deficiencies in these three different cell death pathways were devoid of any impact on the release of IL-1Ra, IL-1α, and cell lysis upon incubation of keratinocytes with Aldara in vitro (Fig. 5E).

IL-1α blockade attenuates the clinical severity of Aldara-induced ear thickening in icIL-1Ra1−/− mice

We demonstrated that IL-1α is released by mouse keratinocytes cultured with Aldara. We next determined whether excessive ear inflammation was because of the unbalanced effect of IL-1α in icIL-1Ra1−/− mice. In agreement with this hypothesis, in comparison with its isotype control, the administration of a neutralizing monoclonal anti–IL-1α Ab during Aldara treatment significantly attenuated the increase in ear thickness in icIL-1Ra1−/− but not in WT mice (Fig. 6).

Discussion

Using recently generated knock-in mice, in which the expression of the icIL-1Ra1 isoform was specifically deleted, we showed for the first time, to our knowledge, that icIL-1Ra1 exerts specific
FIGURE 5. icIL-1Ra1 plays no intracellular role in keratinocytes but is released together with IL-1α upon Aldara treatment. (A) Differential gene expression analysis based on RNA-Seq data of primary keratinocytes from icIL-1Ra1−/− versus WT mice. Primary keratinocytes were isolated from WT (n = 4) and icIL-1Ra1−/− (n = 4) mouse tails and left untreated as a control (left panel) or stimulated with 100 ng/ml rec. m IL-1α (middle panel) or 100 ng/ml rec. m IL-36γ (right panel) for 6 h. Total RNA was then isolated from keratinocytes for RNA-Seq analysis. A total of 10,750, 10,709, or 10,727 genes were tested for control, IL-1α−, or IL-36γ−treated cells, respectively. The fold-change threshold and Benjamini–Hochberg corrected p value threshold were set to 2 and 0.05, respectively. (B) Ear explants taken from WT (n = 6) and icIL-1Ra1−/− (n = 6) mice at day 8 after daily treatment with Vaseline (control) or Aldara were cultured for 24 h. Levels of IL-1Ra, IL-1α, and IL-1β in the supernatant or the percentage of cytotoxicity were assessed by ELISA or LDH assay, respectively. The IL-1Ra ELISA recognizes all isoforms of IL-1Ra. Data are shown as the mean ± SEM from one (Figure legend continues)
All the mean percentage of cytotoxicity were assessed by ELISA or LDH assay, respectively. The IL-1Ra ELISA recognizes all isoforms of IL-1Ra. Data are shown as of Aldara versus DMSO or IMQ, IL-1 family cytokines versus control. n/a, not applicable.

We observed a higher icIL-1Ra1 expression in keratinocytes of the middle and outer layers of the epidermis upon Aldara treatment. The activation state of keratinocytes can alter the protein expression. Lee et al. (50) showed a stronger IL-1Ra staining intensity in irritated than in naive mouse epidermis. In line with Hammerberg et al. (51), who described a cytosolic expression of icIL-1Ra1 in normal skin of healthy individuals, our findings suggested to serve as a compensatory mechanism to counter-regulate anti-inflammatory effects in vivo. Indeed, icIL-1Ra1 deficiency led to aggravated Aldara (5%) IMQ-induced skin inflammation, a mouse model for human psoriasis. We could not detect an intracellular effect of icIL-1Ra1 using isolated keratinocytes. However, Aldara led to keratinocyte cell death, accompanied by the release of both icIL-1Ra1 and IL-1α from keratinocytes. Furthermore, blocking IL-1α attenuated the clinical severity of Aldara-induced ear thickening in icIL-1Ra1−/− mice (Fig. 7).

Patients with a deficiency in all IL-1Ra isoforms (DIRA syndrome) present sterile osteomyelitis and periostitis but also present clinical features resembling human psoriasis (22, 23, 47). In distinction from mice deficient in all four IL-1Ra isoforms, which exhibit reduced body weight as well as other specific tissue-inflammarcy responses, depending on their genetic background (42, 48, 49), icIL-1Ra1−/− mice bred normally and revealed a normal phenotype. This points to either distinct roles of the different IL-1Ra isoforms or a cumulative effect in case of the deficiency in all four isoforms.

One key question is how icIL-1Ra1 controls the severity of ear inflammation. Consistent with previous reports (13, 24, 51, 52), we demonstrated that icIL-1Ra1 is constitutively expressed in keratinocytes and the main isoform found in mouse skin. An intracellular role for icIL-1Ra1 has been reported in vitro. Human ovarian cancer cells expressing icIL-1Ra1 showed a shorter induction of GRO and IL-8 mRNA in response to IL-1α as compared with cells lacking icIL-1Ra1 (46). Furthermore, icIL-1Ra1 inhibited IL-1α-induced IL-6 and IL-8 production in icIL-1Ra1-transfected Caco-2 intestinal epithelial cells through the inhibition of p38 MAPK and NF-κB signal transduction pathways (45). Moreover, icIL-1Ra1 overexpressed in a mouse skin carcinoma cell line appeared to have antiproliferative effects in vitro and in vivo (44). In contrast to these findings, we did not find differentially expressed genes, except for Il1rn in isolated keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36γ, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Thbs2, Dcfb4, and Cypl1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice under control conditions because we aimed to find a role for icIL-1Ra1 under inflammatory, not naive conditions. Furthermore, the expression levels for β-defensin 4 (gene: Dcfb4) tested by qRT-PCR were not different in keratinocytes of WT compared with icIL-1Ra1−/− mice (Supplemental Fig. 2). It cannot be excluded that icIL-1Ra1 could have intracellular effects under different physiological or pathological conditions or in other cell types.

Our findings that keratinocyte cell death is accompanied by the release of icIL-1Ra1 and IL-1α is in line with previous reports (50, 53, 54). The release of icIL-1Ra1 from keratinocytes has been suggested to serve as a compensatory mechanism to counter-regulate experiment. The unpaired or paired two-tailed Student t test was used to test statistical significance, and p values < 0.05 were considered to be significant.

n.d., not detected. (C-E) Primary keratinocytes isolated from tails of WT [(C): n = 5; (D): n = 5; (E): n = 4], icIL-1Ra1−/− [(C): n = 5], casp1/11−/− [(D): n = 5], and casp1/11 × casp8 × RIPK3−/− [(E): n = 4] mice were left untreated or stimulated with DMSO as control or with different dilutions of Aldara, IMQ, 100 ng/ml rec. m IL-1α, 100 ng/ml rec. m IL-1β, or 100 ng/ml rec. m IL-36γ for 6 h. Levels of IL-1α, IL-1β, and CXCL1 in the supernatant or the percentage of cytotoxicity were assessed by ELISA or LDH assay, respectively. The IL-1Ra ELISA recognizes all isoforms of IL-1Ra. Data are shown as the mean ± SEM from three (C and D) or two (E) independent experiments. The unpaired two-tailed Student t test was used to test statistical significance. All p values < 0.05 were considered to be significant. *p < 0.05, **p = 0.034, and NS with line spanning the different conditions used for stimulation: WT versus icIL-1Ra1−/− (C), WT versus casp1/11−/− (D), WT versus casp1/11 × casp8 × RIPK3−/−. #p < 0.05, ##p < 0.01, ###p < 0.001 dilutions of Aldara versus DMSO or IMQ. IL-1 family cytokines versus control. n/a, not applicable.
FIGURE 7. Balance between the antagonist icIL-1Ra1 and the alarmin IL-1α. icIL-1Ra1 and IL-1α are constitutively expressed in mouse keratinocytes. Topical application of Aldara induces release of icIL-1Ra1 and IL-1α independent of pyroptosis, apoptosis, or necroptosis. LDH is found in the supernatant of treated keratinocytes, which is indicative of cellular damage because of loss of plasma membrane integrity. The biological activity of IL-1α is mediated by IL-1R1 expressed on target cells in the skin, such as keratinocytes in the epidermis or fibroblasts in the dermis. IL-1α binding induces a conformational change in the extracellular domain of IL-1R1, enabling its interaction with IL-1R accessory protein (IL-1RACP), which is required for MyD88-mediated intracellular signaling and induction of an inflammatory response. In WT mice, icIL-1Ra1 controls the IL-1α–mediated inflammation by competing with IL-1α for binding to IL-1R1. In contrast to IL-1α, binding of icIL-1Ra1 does not recruit IL-1RACP and thus no inflammatory response is induced. Deficiency of icIL-1Ra1 leads to enhanced binding of IL-1α to IL-1R1 and excessive inflammation. Neutralizing anti–IL-1α Abs attenuate this imbalance in icIL-1Ra1–deficient mice. Taken together, our results suggest that icIL-1Ra1 is stored in keratinocytes and coreleased with the alarmin IL-1α upon cell death, thereby counteracting its inflammatory potential and acting as a natural anti-alarmin.
inflammation in the skin provoked by keratinocyte-derived IL-1α (50, 55). Because of the lack of a signal peptide, icIL-1Ra1 and IL-1α are believed to require a cell death program with loss of membrane integrity to get released, as shown for IL-1β (30, 56). Furthermore, previous data (13) and our finding in BMDC and BMDM showing that LPS stimulation led to the release of sIL-1Ra but not of the two intracellular isoforms suggest that an inflammatory response is not sufficient to release icIL-1Ra1. Disruption of the plasma membrane occurs during pyroptosis, apoptosis, necroptosis, and necrosis (see review in Ref. 57). Release of icIL-1Ra1 and IL-1α, as well as death of primary keratinocytes, were not reduced in absence of caspase 1/11, caspase 8, and RIPK3, which excludes pyroptosis, caspase 8-mediated apoptosis, and necroptosis as forms of Aldara-induced keratinocyte cell death and points to necrosis or another type of lytic cell death. However, it cannot be excluded that icIL-1Ra1 and IL-1α are actively released by an unknown mechanism also leading to loss of membrane integrity. Supporting our data, Cohen et al. (53) observed that, together with other components, such as β-actin, IL-1α was present in the supernatant of necrotic but not apoptotic mouse B16 skin melanoma cells. However, our findings differ from recent data that identified pyroptosis as the type of cell death of human keratinocytes upon Aldara treatment (3). The lack of inflammasome proteins and IL-1β in mouse but not human keratinocytes could explain these different findings (58–64). Additionally, and in divergence from the findings of Walter et al. (3), we could not detect transcripts or protein for IL-1β in isolated primary mouse keratinocytes. The modest levels of IL-1β protein, which we found in supernatant of ear explants, were probably coming from infiltrating myeloid cells, which are known to express and release IL-1β during Aldara-induced skin inflammation (2, 65).

Our in vitro data suggest that icIL-1Ra1 is passively released by dying keratinocytes. We tried to make cell death of keratinocytes visible in situ on ear sections from Aldara-treated ears by different approaches, but all these approaches failed. One possible explanation of the difficulty in detecting dying keratinocytes in situ is that they are efficiently and rapidly cleared from the tissue by phagocytic cells in vivo. It is also conceivable that Aldara induces in vivo a transient, nonlethal membrane injury of keratinocytes that allows the release of icIL-1Ra1 and IL-1α as shown for keratinocytes in response to cellular strain (66).

An alteration of the icIL-1Ra1/IL-1α ratio in human psoriasis has been documented (51, 52). Consistently, we could demonstrate a critical role for IL-1α in Aldara-induced skin inflammation because injection of neutralizing anti–IL-1α Abs attenuated the increase in ear thickness in icIL-1Ra1−/− mice. This finding supports our hypothesis of an extracellular but not intracellular role of icIL-1Ra1 in this mouse model. Our findings differ from data by Rabeoey et al. (7), who could not detect a difference in the clinical severity of the back skin between WT and IL-1α−/− mice in the Aldara mouse model. It is conceivable that a role of IL-1α can only be uncovered when its antagonist icIL-1Ra1 is not present, whereas in WT conditions the inflammatory effect of IL-1α is controlled by icIL-1Ra1. Indeed, we only detected a significant effect of neutralizing anti–IL-1α Abs on ear thickness in icIL-1Ra1−/− but not in WT mice.

Interestingly, Rabeoey et al. (7) demonstrated that Aldara-induced skin inflammation was significantly attenuated in mice deficient for both IL-1α/IL-1β but not in IL-1α or IL-1β single-deficient mice. icIL-1Ra1 can neutralize the inflammatory effects of both IL-1β, which is constitutively expressed by keratinocytes, and IL-1β, which is expressed most likely by infiltrating myeloid cells during Aldara-induced skin inflammation. However, and in line with previous findings (6), IL-1α protein levels were markedly higher in ear explants of Aldara-treated mice than those of IL-1β. Furthermore, the finding that neutralizing anti–IL-1α Abs attenuated skin inflammation in icIL-1Ra1−/− mice, whereas inflammatory IL-1β was not neutralized and thus biologically active, argues for a predominant role of IL-1α in this particular experimental setting and thus a regulatory effect of icIL-1Ra1 directed mostly against IL-1α.

Like heat shock proteins, defensins, S100 proteins, or the cytokines IL-33 and high-mobility group box 1 (HMG1), IL-1α is considered an alarmin. Alarmins are endogenous, constitutively expressed molecules that are released after necrotic cell damage to rapidly mobilize and activate immune cells (67). Because of their prominent role in immune responses, the activity of alarmins must be strictly regulated. Tetramerization of the alarmins S100A8/S100A9, for instance, is a mechanism of autoinhibition and regulation of alarmin activity (68). icIL-1Ra1 is present in keratinocytes as a reservoir of biologically active IL-1α that can immediately counteract the inflammatory potential of the alarmin IL-1α, which is released upon lytic cell death of keratinocytes. Our findings give rise to the hypothesis that icIL-1Ra1 acts as a natural inhibitor for the alarmin IL-1α in the skin.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1: Subcellular localization of icIL-1Ra1 in keratinocytes.

(A) IL-1Ra isoforms were examined in cytoplasmic (C) and nuclear (N) extracts of keratinocytes isolated from tails of naïve WT and icIL-1Ra1–/– (KO) mice using a polyclonal goat anti-mouse IL-1Ra antibody recognizing all IL-1Ra isoforms. Probing with GAPDH and LSD1 antibodies was used as qualitative control of the cytoplasmic and nuclear preparations, respectively. 1 ng of recombinant sIL-1Ra served as control. Arrows indicate different IL-1Ra isoforms. One representative experiment out of three is shown.

(B) Representative confocal microscopy images showing expression of icIL-1Ra1 (red) on ear sections of WT mice on day 8 after daily application of vaseline (control) or Aldara. Sections were taken at 2.5 μm, 5.0 μm and 7.5 μm depth for control, and at 2.2 μm, 4.4 μm and 6.6 μm depth for Aldara-treated ears. Nuclei are stained with DAPI (blue). Dashed line on sections of control ears indicate border epidermis/dermis. Only the epidermis is shown on sections of Aldara-treated ears. Scale bars: 20 μm.
**Supplemental Figure 2:** No intracellular role of icIL-1Ra1 in keratinocytes.

Primary keratinocytes isolated from tails of WT and icIL-1Ra1-/- mice were left untreated or stimulated with DMSO as a control, or with different dilutions of Aldara, imiquimod (IMQ), 100 ng/ml rec. mIL-1α, 100 ng/ml rec. mIL-1β or 100 ng/ml ec. mIL-36β for 6 h. Determination of mRNA levels for icIL-1Ra1, IL-1α, CXCL1, CXCL2, G-CSF, IL-23p19, S1009, Involucrin and beta-defensin 4 was performed on total RNA isolated from keratinocytes of WT (n=5) and icIL-1Ra1-/- (n=5) mice by qRT-PCR. Results represent icIl1rn1, Il1a, Cxcl1, Cxcl2, Csf3, Il23a, S100a9, Iv1 and Defb4 mRNA levels relative to Mrpl32 mRNA levels. Data are shown as the mean ± SEM from three independent experiments. The unpaired two-tailed Student’s t-test was used to test statistical significance. WT vs. icIL-1Ra1-/-. P values of < 0.05 were considered to be significant. NS = statistically not significant.
Supplemental Figure 3: Heat map of RPKM data in primary keratinocytes of WT and icIL-1Ra1−/− mice.

Primary keratinocytes were isolated from the tails of WT (n=4) and icIL-1Ra1−/− (n=4) mice and left untreated or stimulated with rec. mIL-1α or rec. mIL-36β for 6 h. Total RNA was isolated from keratinocytes for RNA-Seq analyses. (A) Heat map of RPKM data. (B) Differential expression analysis of RNA-Seq data in primary keratinocytes stimulated with IL-36β versus IL-1α. 10710 or 10717 genes in keratinocytes of WT or icIL-1Ra1−/− mice were tested, respectively. The fold-change threshold and Benjamini-Hochberg corrected p-value threshold were set to 2 and 0.05, respectively.