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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,*† Emiliama 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. ic-IL-1Rα1 represents the major isoform in keratinocytes. We created iCIL-1Ra1−/− mice and investigated the role of icIL-1Rα1 in Aldara (5% imiquimod)-induced psoriasis-like skin inflammation. Naïve iCIL-1Ra1−/− mice bred habitually and exhibited a normal phenotype. icIL-1Rα1 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-1Rα1 and IL-1α. Furthermore, blocking IL-1α attenuated the clinical severity of Aldara-induced ear thickening in icIL-1Rα1−/− mice. Our data suggest that upon keratinocyte damage icIL-1Rα1 acts extracellularly as an antagonist of the alarmin IL-1α to immediately counteract its inflammatory effects. The Journal of Immunology, 2020, 204: 967–979.

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, erythematos 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 lesions (2). 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−/− deficient 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
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<EGFP>cre<>) 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

**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 primers used to identify recombinant ES cells are indicated as S1 and S2. (B) Southern blot analysis of WT (+/+) and heterozygous (+/−) mice. The WT (6.4-kb [BglII digestion] or 8.9-kb [HindIII digestion]) bands) and mutant alleles (7.4-kb [BglII digestion] or 6.2-kb [HindIII digestion] bands) were detected in BglII- or HindIII-digested ES cell genomic DNA, respectively, the 5′ or 3′ probes shown in (A). (C) PCR genotyping to distinguish WT (+/+), heterozygous (+/−) and icIL-1Ra1−/− mice. The primers used are indicated as P1, P2, and P3 in (A). The sizes of the WT and icIL-1Ra1−/− PCR products are 300 and 842 bp, respectively. (D) Male (left panel) and female (right panel) WT (male; n = 13/6 [10/17 wk]; female; n = 11/6 [10/17 wk]) and icIL-1Ra1−/− (male; n = 13/6 [10/17 wk]; female; n = 11/6 [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. Theoretical molecular mass of IL-1Ra isoforms are as follows: proSL-1Ra1, 20 kDa; mature sIL-1Ra1, between 17 and 22 kDa; icIL-1Ra1, 18 kDa; icIL-1Ra3, 16 kDa (13, 69).

NS = not significant.
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 ic-il1ra1–specific first exon. 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 12.03 kb downstream of the ic-il1ra1–specific exon 1.

Mice lacking all isoforms of IL-1Ra in myeloid cells were generated by crossing homozygous IL-1Raflkexflkex mice [B6.129-Il1rncre/-mice (27)] backcrossed onto the C57BL/6J background for six generations using a marker-assisted selection protocol (28), with mice expressing the Cre-recombinase under the transcriptional control of the endogenous lysozyme M (Lyz2tm1(cre)Ifo/J) and counterstained with hematoxylin. Slides were scanned with an Axio Scan.Z1 (Zeiss, Germany) for 1 h at room temperature. Color was developed with 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 and immunofluorescence on paraffin-embedded ear sections as previously described (36). Local expression of IL-1Ra was studied by immunohistochemistry (S.p.A., Milano, Italy). Slides were scanned with an Axio Scan.Z1 (Zeiss, Jena, Germany) and the ZEN lite software (Zeiss, Germany) for 1 h at room temperature. Color was developed with diaminobenzidine, and sections were counterstained with hematoxylin.
KERATINOYTE-DERIVED icIL-1Rα1 ANTAGONIZES IL-1α

with DAPI (Thermo Fisher Scientific). Confocal fluorescence 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 ENSEMBL 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.

RNA-Seq analysis. All reads overlapping the exons of each unique gene model were reported using featureCounts version 1.4.6-p1 (39). Gene expressions were reported as raw counts and are in parallel normalized in reads per kb million to filter out genes with low expression value (1 read per kb million) before calling for differentially expressed genes. Library size normalizations and differential gene expression calculations were performed using the package edgeR (40) designed for the R software (41). Only genes having a significant fold change (Benjamini–Hochberg corrected p value <0.05) were considered for the rest of the RNA-Seq analysis.

Subcellular fractionation

All steps were performed on ice. Mouse primary keratinocytes were washed two times 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 DTT, 1× 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. m IL-1Rα secreted isoform (standard of mouse IL-1Rα 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-1Rα expression in cytosolic and nuclear fractions, 4–12% gradient gels (NuPAGE, Thermo 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-1Rα (capture Ab of mouse IL-1Rα; DuoSet ELISA Kit; R&D Systems) at 0.14 μg/ml, or with polyclonal rabbit anti-human KDM1/LSD1 (Abcam, Cambridge, U.K.) diluted 1:2000 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-1Rα, IL-1α, CXCL1, or IL-1β protein levels were determined using DuoSet ELISA (R&D Systems) or ELISA Ready Set Go kits (eBioscience), respectively. The IL-1Rα DuoSet ELISA detects all isoforms of IL-1Rα.

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-1Rα1−/− 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-1Rα1-deficient mice, which were genotyped by PCR to distinguish WT and/or targeted alleles (Fig. 1C). icIL-1Rα1−/− 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-1Rα isoforms (42). The insertion of the EGFP–Neo cassette at the ATG start codon in the icIL-1Rα1–specific first exon of the mouse Il1rn gene deleted only the expression of icIL-1Rα1 but did not interfere with the expression of the isoforms icIL-1Rα3 and sIL-1Rα (shown is the immature proform of sIL-1Rα [pro–IL-1Rα]; 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-1Rα1−/− mice

It is known that upon stimulation myeloid cells express both sIL-1Rα and the intracellular isoforms icIL-1Rα1 and icIL-1Rα3. We generated BMDC and BMDM to further investigate the deletion of icIL-1Rα1. The presence of icIL-1Rα1 mRNA was only detected in BMDC and BMDM from WT mice but was absent in BMDC and BMDM from icIL-1Rα1−/− mice, both at baseline and upon LPS treatment (Fig. 2A). Expression levels of mRNA encoding both sIL-1Rα and icIL-1Rα3 (referred to as sl1rn) were significantly increased in BMDC or similar in BMDM of icIL-1Rα1−/− compared with WT mice upon LPS stimulation (Fig. 2A). However, by using an ELISA that detects all IL-1Rα isoforms, no differences in IL-1Rα 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-1Rα (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).

**Increased severity of Aldara (5% IMQ)-induced ear thickness in icIL-1Ra1−/− mice**
icIL-1Ra1 is the major IL-1Ra isoform expressed in naive mouse dorsal skin (Fig. 3A). Therefore, we investigated the functional role of icIL-1Ra1 in Aldara (5% IMQ)-induced skin inflammation, a widely used mouse model for human psoriasis. Consistent with the clinical picture, the histopathological analysis showed a trend for higher epidermal thickness (acanthosis) (p = 0.086) and more infiltration of inflammatory cells (p = 0.086) into ears of icIL-1Ra1−/− compared with WT mice (Fig. 3B). Consistent with the clinical picture, the histopathological analysis showed a trend for higher epidermal thickness (acanthosis) (p = 0.086) and more infiltration of inflammatory cells (p = 0.086) into ears of icIL-1Ra1−/− compared with WT mice (Fig. 3C, 3D). In support of the clinical and histopathological data, mRNA expression levels for the proinflammatory cytokines IL-1α, IL-1β, IL-36β, IL-17A, IL-22, and IL-23p19, which are all implicated in Aldara-induced skin inflammation (43), were significantly increased in ears of icIL-1Ra1−/− as compared with WT mice upon Aldara treatment (Fig. 3E).

Furthermore, expression levels of mRNA encoding sIL-1Ra and icIL-1Ra3 (sI1r1m) and IL-36R antagonist (IL-36Ra; gene Il1r5) were significantly increased in ears of icIL-1Ra1−/− as compared with WT mice upon Aldara treatment, whereas there was no difference in mRNA expression levels for the decoy IL-1R2. Transcript levels for the signaling receptor IL-1R1 were downregulated upon treatment with Aldara but did not differ between the genotypes. There was no difference between genotypes in control ears for all tested mRNA expression levels except for icIL-1Ra1.

**icIL-1Ra1 is the main IL-1Ra isoform regulating Aldara-induced skin inflammation**
icIL-1Ra1 is constitutively expressed and represents the major IL-1Ra isoform in mouse keratinocytes (12, 13). Indeed, by using an anti-IL-1Ra Ab that recognizes all IL-1Ra isoforms, ear sections from control and Aldara-treated WT mice but not from icIL-1Ra1−/− mice showed IL-1Ra keratinocytes (Fig. 4A). Of note, upon Aldara treatment, icIL-1Ra1 staining was barely detectable in basal layers but rather was restricted to keratinocytes in the middle and outer layers of the epidermis. Staining with Ki67, a proliferation-associated protein, showed rare Ki67+ cells in control ears and an increased Ki67 expression restricted to the basal keratinocyte layer upon Aldara treatment (data not shown). Under

**Table I. Sequences of primers used for qRT-PCR**

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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.

**FIGURE 2.** Characterization of BMDC and BMDM from WT and icIL-1Ra1/− mice. (A) Determination of mRNA levels for icIL-1Ra1 (icIl1rn1) and for sIL-1Ra and icIL-1Ra3 (sIl1rn) in BMDC (left panel) and BMDM (right panel). BMDC and BMDM from naive WT (n = 5) and icIL-1Ra1/− (n = 5) mice were left untreated (control) or stimulated with 100 ng/ml LPS for 24 h. Total RNA was isolated for qRT-PCR analysis. Results represent icIl1rn1 and sIl1rn mRNA expression levels relative to Mrpl32 mRNA levels. (B) Release of IL-1Ra protein (all isoforms) into the supernatant. BMDC (left panel) and BMDM (right panel) from naive WT (n = 5) and icIL-1Ra1/− (n = 5) mice were left untreated (control) or stimulated with LPS for 24 h. IL-1Ra in the supernatant was measured using an ELISA that recognizes all isoforms of IL-1Ra. (A and B) Data are shown as the mean ± SEM of values obtained from three (BMDC) or two (BMDM) independent experiments. The unpaired or paired two-tailed Student t test was used to test statistical significance. All p values <0.05 were considered to be significant. (C) Analysis of IL-1Ra isoforms in lysates and cell supernatants of BMDC (left panel) and BMDM (right panel) by Western blot. BMDC and BMDM from naive WT and icIL-1Ra1/− (KO) mice were left untreated (control) or stimulated with LPS for 24 h. IL-1Ra isoforms were analyzed in cell lysates or collected supernatants (30 µl) using a polyclonal goat anti-mouse IL-1Ra Ab recognizing all IL-1Ra isoforms. Protein loading was assessed by the determination of GAPDH expression in lysates. One nanogram of rsIL-1Ra served as control. Two representative samples out of n = 5 per genotype are shown. Arrows indicate different IL-1Ra isoforms. Theoretical molecular mass of IL-1Ra isoforms is as follows: prosIL-1Ra1, 20 kDa; mature sIL-1Ra1, between 17 and 22 kDa; icIL-1Ra1, 18 kDa; icIL-1Ra3, 16 kDa. n.d., not detected. SN, supernatant.
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−/−
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-1Ra1, 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-36β (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-36β–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)
anti-inflammatory effects in vivo. Indeed, icIL-1Ra 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-1Ra using isolated keratinocytes. However, Aldara led to keratinocyte cell death, accompanied by the release of both icIL-1Ra and IL-1α from keratinocytes. Furthermore, blocking IL-1α attenuated the clinical severity of Aldara-induced ear thickening in icIL-1Ra−/− 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-inhibitory responses, depending on their genetic background (42, 48, 49), icIL-1Ra−/− 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.

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 demonstrate the expression of icIL-1Ra1 only in the cytoplasm of naive keratinocytes, whereas Aldara causes expression also in the nucleus. Why icIL-1Ra1 shuttles into the nucleus upon Aldara treatment and why its expression is increased in keratinocytes of the middle and outer layers of the epidermis in inflamed ears remains unknown and is to be addressed in further studies.

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-1 as compared with cells lacking icIL-1Ra1 (46). Furthermore, icIL-1Ra1 inhibited IL-1β-induced IL-6 and IL-8 production in icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Tibh2, Defb4, and Cyp1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Tibh2, Defb4, and Cyp1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Tibh2, Defb4, and Cyp1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Tibh2, Defb4, and Cyp1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Tibh2, Defb4, and Cyp1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Tibh2, Defb4, and Cyp1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes. We did not follow up on the three differentially expressed genes (Tibh2, Defb4, and Cyp1A1), found in keratinocytes from icIL-1Ra1−/− compared with WT mice upon in vitro stimulation with either IL-1α or IL-36β, which argues against an intracellular role of icIL-1Ra1 in keratinocytes.

Our findings that keratinocyte cell death is accompanied by the release of icIL-1Ra1 and IL-1α are 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
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-1Ra1 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 approached 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 Rabeony 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, Rabeony 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-1α, 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-1Ra 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.

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