

Supplemental Information

Intracellular Interleukin-1 Receptor 2 Binding

Prevents Cleavage and Activity of Interleukin-1 α ,

Controlling Necrosis-Induced Sterile Inflammation

Yue Zheng, Melanie Humphry, Janet J. Maguire, Martin R. Bennett, and Murray C.H. Clarke

Inventory of Supplemental Information

Figure S1, related to Figure 1 – Assessment of IL-1 α activity in membrane fractions of necrotic cells, and cells made necrotic by hypoxia; Western blot demonstrating cleavage of p33 to p17 IL-1 α in the presence of calpeptin at 37°C.

Figure S2, related to Figure 3 – Dose response curve confirming GST has no effect on p33 IL-1 α activity.

Figure S3, related to Figure 4 – Western blots showing lack of IL-1 α processing and IL-1R2 expression in primary T-cells and macrophages; Calpain activity in necrotic lysates; IL-1 α processing in Jurkat lysates treated with an excess of IL-1RA or inflammatory proteases; dose response of IL-1R2-mediated p33 IL-1 α protection and antagonism; proximity ligation assays showing *in situ* binding.

Figure S4, related to Figure 5 – Western blots showing lack of IL-1 α processing in HeLa necrotic lysates, and Jurkat and primary T-cell and macrophage lysates treated with a metalloprotease inhibitor; Immunofluorescence showing intracellular IL-1R2 in HeLa cells and in other cell-types by FACs; Co-localization of native p33 IL-1 α /IL-1R2 by epifluorescence and confocal microscopy; HeLa subcellular fractionation and protease K protection assays showing p33 IL-1 α /IL-1R2 to be a cytosolic complex.

Figure S5, related to Figure 6 – Sequence data showing predicted caspase cleavage sites; Histogram showing no effect of sham caspase cleavage reactions on EL4 cell response to IL-1 α .

Supplemental Experimental Procedures

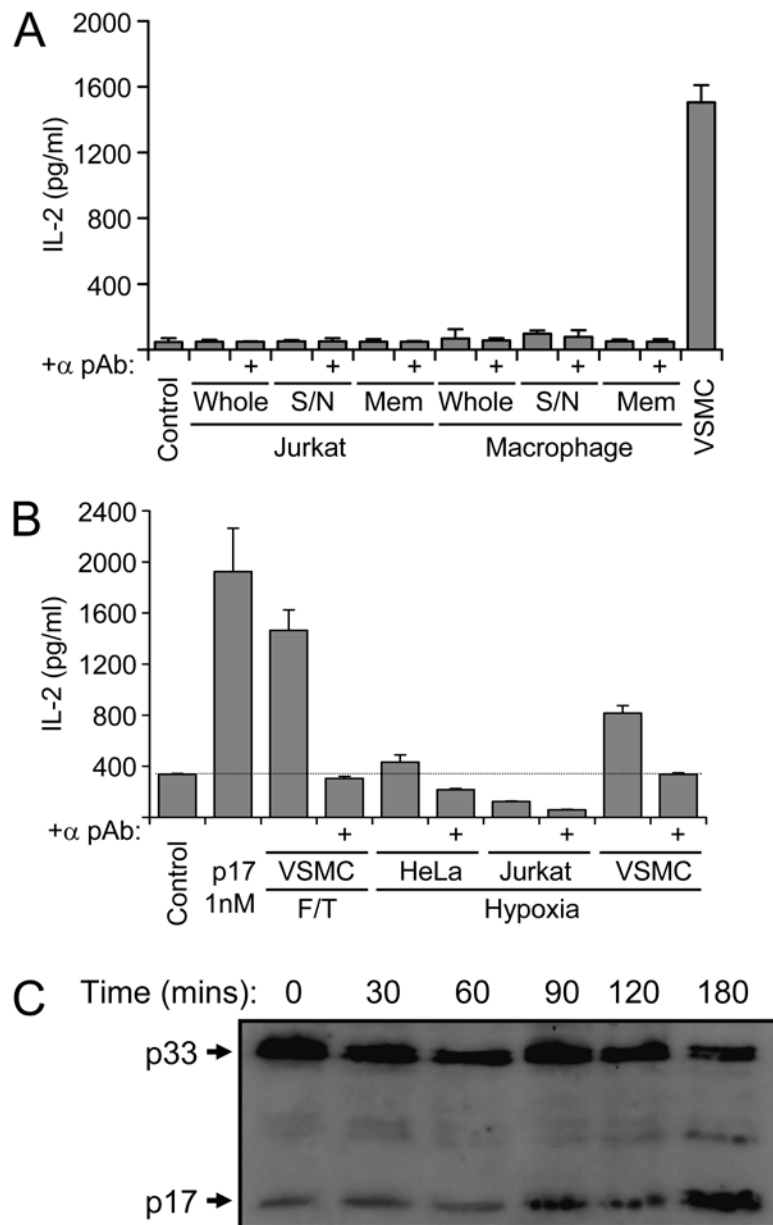


Figure S1, related to Figure 1: Necrosis-induced sterile inflammation is cell-type specific. (A,B) IL-2 concentrations in conditioned media of EL4 cells incubated with soluble, membrane or whole fractions of necrotic lysates **(A)**, or hypoxia-induced necrotic lysates **(B)**, \pm IL-1 α neutralizing antibody (α pAb). Necrotic lysates were normalized to the level of necrosis by PI staining. Data representative of mean \pm SD from n=2. **(C)** Western blot for IL-1 α in necrotic VSMC lysates after prolonged incubation at 37°C with calpeptin.

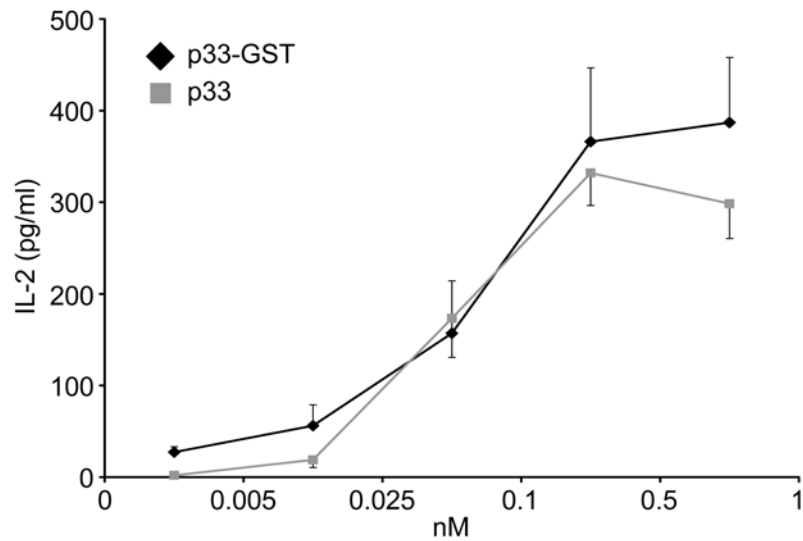


Figure S2, related to Figure 3: Cytokine response to p33 IL-1 α is not affected by a GST fusion tag. p33 IL-1 α had the GST fusion tag specifically cleaved off or was subjected to a 'sham cleavage', and was tested for ability to induce IL-2 from EL4 cells. Data represent mean \pm SEM; $p = \text{NS}$; $n = 3$.

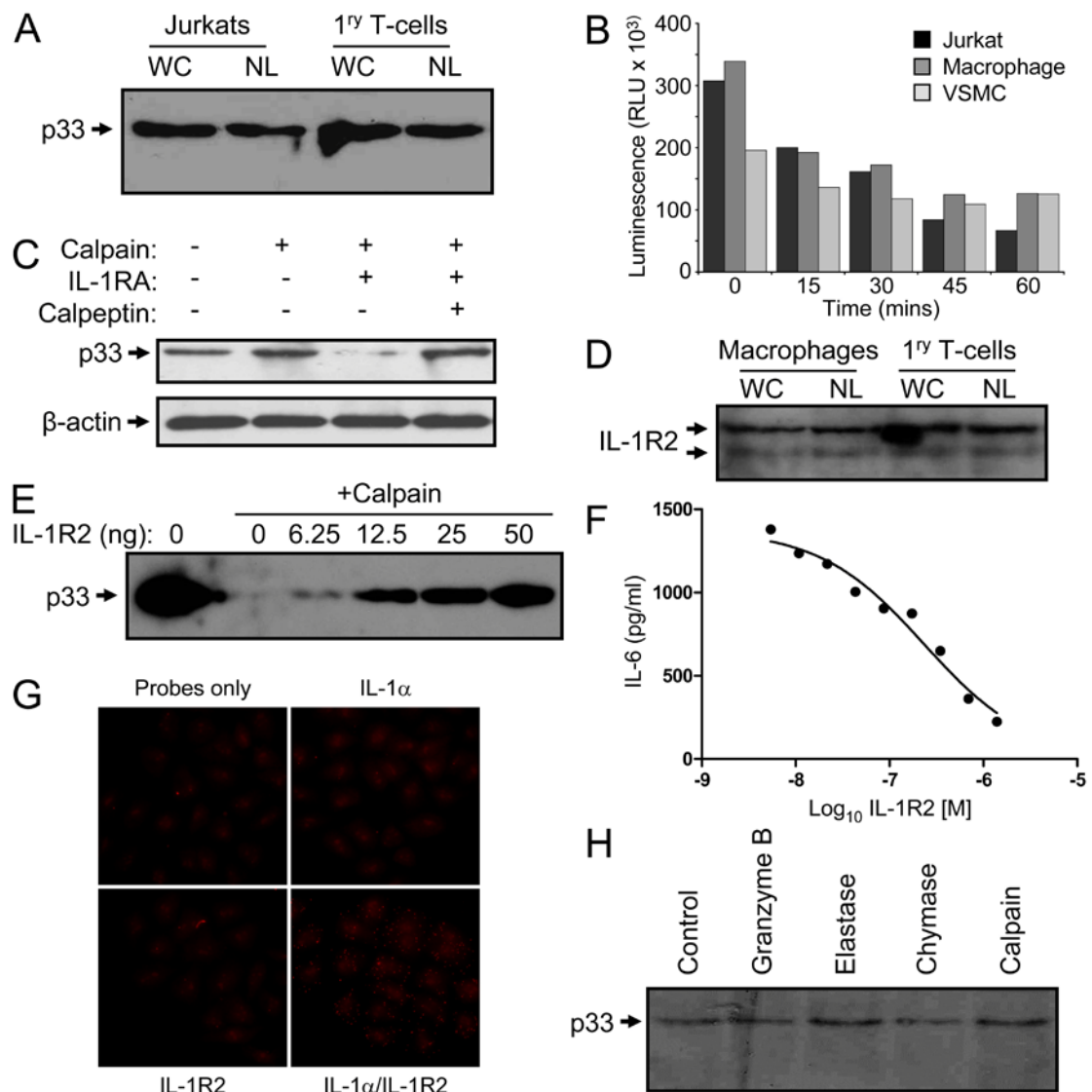


Figure S3, related to Figure 4: Intracellular IL-1R2 protects IL-1 α from calpain processing. (A) Western blot for IL-1 α in whole cell (WC) or necrotic lysates (NL) of Jurkats and primary human T-cells. (B) Enzymatic assay for calpain activity within necrotic lysates from Jurkats, primary human macrophages and VSMCs. Data representative of n=3. (C-E) Western blot for IL-1 α in Jurkat necrotic lysates following incubation with purified calpain, excess IL-1RA or calpeptin (C), IL-1R2 in whole cell or necrotic lysates from primary human macrophages and T-cells (D), IL-1 α (30ng) processing after incubation with increasing amounts of IL-1R2 (E). (F) p33-induced (6.25ng/ml) IL-6 release in HeLa cells is dose-dependently antagonized by IL-1R2. (G) Proximity ligation assay, with antibodies as indicated, shows p33/IL-1R2 association *in situ* in HeLa cells. (H) IL-1 α in Jurkat necrotic lysates \pm addition of proteases as indicated.

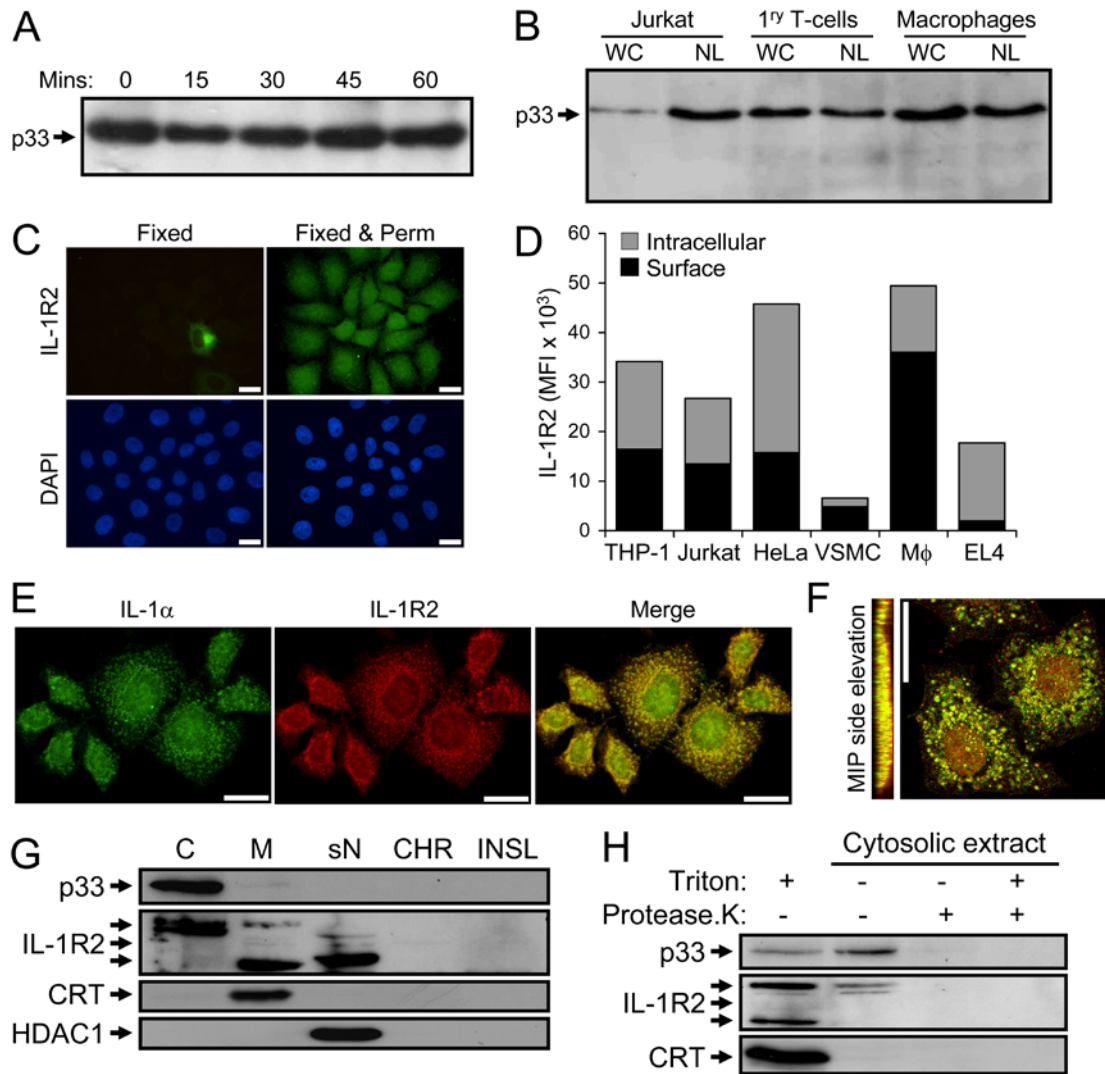


Figure S4, related to Figure 5: IL-1R2 silencing enables calpain cleavage of IL-1 α and restores inflammatory response to necrotic cells. (A,B) Western blot for IL-1 α in necrotic HeLa lysates (A), or whole cell (WC) or necrotic lysates (NL) from Jurkat cells, and primary human T-cells and macrophages treated with the metalloprotease inhibitor BB-94 (B). (C,D) Immunofluorescence reveals IL-1R2 to be intracellular by microscopy in HeLa cells (C), or by FACs in multiple cell types (D). MFI=mean fluorescence intensity. (E,F) Dual immunolabeling of IL-1R2 and p33 IL-1 α shows intracellular colocalization by fluorescent (E) and confocal (F) microscopy. MIP=maximum intensity projection. Scale bars = 25 μ m. (G,H) Western blot for IL-1 α , IL-1R2, calreticulin and HDAC1 in HeLa lysates after subcellular fractionation (G), or after a protease K protection assay (H). C = cytosolic, M = membrane, sN = soluble nuclear, CHR = chromatin associated, INSL = insoluble pellet.

A

>sp|P27930|IL1R2_HUMAN Interleukin-1 receptor type 2 OS=Homo sapiens

MLRLYLVMGVSAFTLQPAAHTGAARSCRFRGRHYKREFRLEGEVALRCPQVPYW
LWASVSPRINLTW **HK** **ND** SARTVPGEEETRM **WA** **QD** GALWLLPAL **Q** **ED** SGTYVCTTRN
A **SY** **CD** KMSIELRVF **ENTD** AFLPFISYPQILTLSTSGVLVCPDLSEFTR **DK** **TD** VKIQWYKD
S **LL** **LD** KDNEKFLSVRGTT **HL** **LV** **HD** VALEDAGYYRCVLTFAHEGQQYNITRSIELRIKKKK
EETIPVIISPLKTISASLGSRLTIPCKVFLGTGTPLTTMLWWTANDTHIESAYPGGRVTEG
PRQEYSENNENYIEVPLIFDPVTREDLHMDFKCVVHNTLSFQTLRRTTVKE - 343

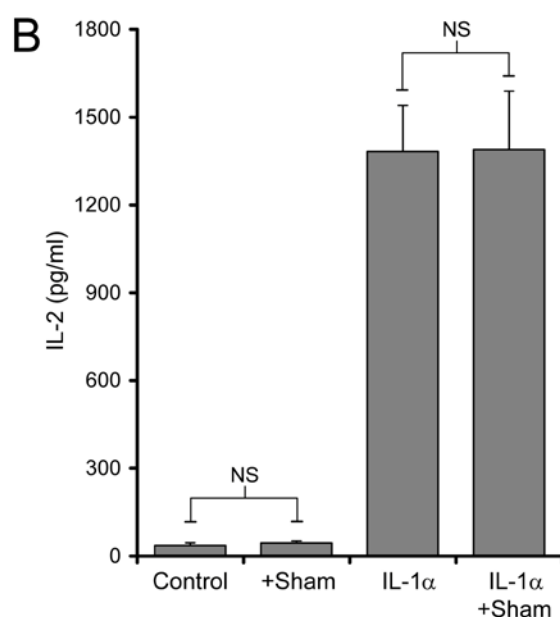


Figure S5, related to Figure 6: Caspase-1 specifically cleaves IL-1R2 which restores IL-1 α -dependent inflammation post-necrosis. (A) The human IL-1R2 protein was analysed for caspase cleavage sites with the Cascleave (Red), Pripper (Green), and CASVM (Yellow) algorithms. **(B)** IL-2 concentrations in conditioned media of EL4 cells incubated alone or with IL-1 α , \pm sham caspase-1 cleavage reactions (+Sham). Data represents mean \pm SD; $p = \text{NS}$, $n = 2$.

Supplemental Experimental Procedures

All materials from Sigma-Aldrich unless otherwise stated.

Cell culture

Human aortic VSMCs were isolated from patients with local ethics committee approval. VSMCs, EL4, HEK and HeLa cells were cultured in DMEM and Jurkat, and THP-1 cells in RPMI 1640, all supplemented with 10 U/ml penicillin, 10 mg/ml streptomycin, 5 mg/ml L-glutamine and 10% FCS. VSMCs, HEK, and HeLa cells were cultured to ~80% confluence before passaging. EL-4, Jurkat and THP-1 cells were maintained at $4\text{-}10 \times 10^5$ cells/ml. Human monocyte-derived macrophages were differentiated as described previously (Brown et al., 2000). Cells were treated as indicated with; Calpeptin (30 μ M), Lactacystin (10 μ M; both Biomol), IL-1 α pAb (1 μ g/ml), IL-1 α/β (1nM; all PeproTech), IL-1RA (Amgen), IL-1R2 (250ng; R&D), Z-YVAD, Z-VAD-fmk (both 10 μ m; Bachem), LPS (1 μ g/ml), EGTA (5mM), BB-94 (1 μ g/ml; Tocris). Cells in serum-free DMEM were disrupted by 3 rounds of freeze/thaw in liquid nitrogen in <1ml, clarified (13,000g, 5 mins) and stored at -80°C until use. Cells were also made necrotic by incubation with 7-Bromoindirubin-3'-oxime (25 μ M; Enzo), pore formation with digitonin (0.1%)(**not shown**), or overnight hypoxic exposure. To activate inflammasomes, cells were treated with LPS (1 μ g/ml) for 4 h, followed by ATP (5mM) or Nigericin (20 μ M) for 30 mins. Calpain enzyme activity was determined using Calpain-Glo according to the manufacturer's instructions (Promega). VSMCs and HEK/THP-1 cells were transfected with pcDNA3 (Invitrogen) using nucleofection (Amaxa) or FugeneHD (Promega), respectively, following the manufacturer's protocols.

Protein expression and purification

Human IL-1 α was cloned into pET15b and pET30a (Novagen) for His-tagging, or pGEX-4T-3 (GE) for GST-tagging, at residues 1-271 for p33 IL-1 α or 119-271 for p17 IL-1 α (Kobayashi et al., 1990). Soluble human IL-1R2 (1-296) was cloned into pGEX-4T-3. For His purification, log-phase bacterial cultures were induced with IPTG (1mM) for 3h at 37°C, pelleted, and lysed in BugBuster (Novagen) with benzonase (100u/ml), lysozyme (100 U/ml) and protease inhibitors (10mins, RT). Urea to 6 M was added and incubated (10 mins), before clarification and purification on a Ni²⁺ column. Briefly, supernatant was adjusted to pH 7.5, applied to the column, washed with 40 mM imidazole, and eluted with 250 mM imidazole. Following concentration (Vivaspin) protein was dialyzed against decreasing concentrations of urea (4 M, 2 M) in 10 mM Tris pH 8.0, 50 mM NaCl, at 4°C for 3 h each, and overnight (0 M). For GST purification, cultures were induced with IPTG (1 mM) for 4h at RT, pelleted, and lysed in 50 mM Na₂HPO₄ pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, with benzonase (100 U/ml), lysozyme (100 U/ml), protease inhibitors at RT for 30mins, clarified and applied to glutathione agarose (Qiagen), washed, eluted with 50 mM reduced glutathione, concentrated and dialyzed against 10 mM Tris pH 8.0, 50 mM NaCl, at 4°C overnight. The GST tag was removed with biotinylated thrombin (Novagen). Protein concentration was determined with a Pierce 660 protein assay (Thermo Scientific), checked for concentration and purity by Coomassie staining, and if necessary adjusted and rechecked. Proteins were stored in 10% glycerol at -80°C.

Protease cleavage

His- or GST-p33 IL-1 α (20ng - 2 μ g) was incubated in 10 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 2 mM CaCl₂ with purified human erythrocyte calpain (0.5-30 U; Calbiochem) in a final volume of 30 μ l \pm calpeptin (30 μ M) or IL-1R2 (250ng) for 30 mins at RT. 'Calpain sham' reactions were assembled with calpain but without p33 IL-1 α . IL-1R2 (250ng – 2 μ g; R&D) was incubated with active caspase-1, 3, 4, 5 (1-10 U; Promokine) in 50 mM HEPES pH 7.2, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% Glycerol, and 10 mM DTT for 2 – 16 h at 37°C. Cleaved IL-1R2 was dialyzed against DMEM before cell treatment. Jurkat necrotic lysates were also incubated with granzyme B (100 nM; Cambridge Bioscience), chymase or elastase (100 nM; both Enzo).

Cytokine release assay

VSMCs were plated at 1.5x10⁴/well in 24-well plates, allowed to adhere overnight and incubated in serum free (S/F) DMEM for ~24 h. Fresh S/F DMEM was added to a final volume of 500 μ l, including treatments and incubated for 6 h. EL4 cells were washed and plated at 1x10⁵/well in S/F DMEM to a final volume of 500 μ l including treatments and incubated for 24 h. The equivalent of 17.5x10³ necrotic VSMCs or macrophages, or 5.25x10⁴ necrotic Jurkat or HeLa cells were used per 500 μ l in 24-well plates. For IL-1R2 antagonism, 5x10³ HeLa cells were plated per well of a 96-well plate, adhered overnight and treated with a sub-saturating concentration of p33 (6.25ng/ml) \pm IL-1R2 (0-50 μ g/ml) in 100 μ l for 8 h. Supernatants were clarified by centrifugation and cytokine level assayed by ELISA (IL-6, MCP-1, IL-1 α ; PeproTech) or Cytomix (IL-2; eBioscience).

Western blotting, Edman degradation and Co-IP

Whole cell (directly lysed in Laemmli buffer) or necrotic lysates from 1×10^5 VSMCs or macrophages, or 3×10^5 Jurkat, HeLa, or primary T-cells were loaded per lane, which gave equivalent loading. Antibodies used were: anti-IL-1 α (1:500; PeproTech), anti-His (1:12000; GE), anti- α -spectrin (1:1000; Millipore), anti- β -actin (1:100,000; Sigma), anti-calreticulin (1:1000; Cell Signalling), anti-HDAC1 (1:200; Santa Cruz) and anti-IL-1R2 (1:250; R&D). ~10 pmol of caspase-1 cleaved c-terminal IL-1R2 was separated on a gradient gel, electroblotted to PVDF, Coomassie stained and bands excised for Edman degradation (ABI Procise 494HT). For Co-IP, 4×10^6 HEK cells, transfected as indicated with p33 IL-1 α -GST or IL-1R2-HIS, were incubated on ice for 10 mins (50mM Tris pH 8, 150mM NaCl, 1% Triton X-100, protease inhibitor cocktail), freeze/thawed 3x, clarified (13,000 g, 15 mins), and incubated with 2 μ g of anti-His or 2 μ g of anti-GST (GE) antibody overnight at 4°C. Immunocomplexes were precipitated with magnetic protein-G beads (Dyna), supernatants recovered, and beads washed 3x (PBS) before elution with Laemmli buffer. Half the Co-IP was loaded, whilst ~1/50th of the supernatant was loaded.

Subcellular Fractionation

HeLa cells were fractionated using the subcellular protein fractionation kit (Pierce) according to the manufacturer's instructions. Sequential fractions equivalent to 1.33×10^5 HeLa cells were loaded per lane. Staining for the ER protein calreticulin and soluble nuclear protein HDAC1 verified integrity of the extracts.

Protease K protection assay

Cytosolic HeLa cell extracts, made using the cytosol extraction buffer from the subcellular protein fractionation kit, were left untreated or treated with Triton X-100 (1%), Protease K (250 μ g/ml), or both, in an ice bath (30 mins). Protease inhibitor cocktail and PMSF (1mM) was added, followed by immediate boiling in Laemmli buffer.

RT-PCR

RNA was extracted using TRI-reagent, DNase treated (Ambion) and reverse transcribed (Promega) before PCR using the following primers: IL-1R1: AGGAGACGGAGGACTTGTGT & GCGTCATAGGTCTTTCCATC. Total IL-1R2: CATTACAAGCGGGAGTTCAG & TAGTGCAGACGTAGGTGCCA. Soluble IL-1R2: TGGCACCTACGTCTGCACTA & TGTCTCCAAAAGGAAGAGCGA. GAPDH: TGTTGCCATCAATGACCCCTT & CTCCACGACTGACTCAGCG.

siRNA knockdown

IL-1R2 knockdown was performed using SMARTpool siRNA and controls according to the manufacturer's instructions (Dharmacon). Briefly, HeLa cells were transfected with 20nM of siRNA using HiPerFect (Qiagen), re-transfected after 48 h, and harvested 48 h later.

Immunofluorescence

For IL-1R2 cells were fixed in 2% formaldehyde (15 mins, RT) permeabilized with 0.5% NP-40 (2 mins, RT). For IL-1R2/p33 cells were fixed in 2% formaldehyde, washed (PBS/0.05% Tween), and fixed on ice with ice cold methanol (10 mins). All

were blocked in 1% BSA, incubated overnight at 4°C with anti-IL-1R2 mAb (1:40; R&D), anti-N-terminal IL-1 α pAb (1:100; Aviva Systems Biology), or isotype controls, washed, incubated with Alexa Fluor anti-rabbit 488 or anti-mouse 568 (1:500; Molecular Probes) for 1 h at RT, and washed before imaging on a BX51 (Olympus) or a TCS SP2 AOBS (Leica) microscope. Proximity ligation assays were performed as above, but with substitution of 2^{xy} antibodies for the Duolink probes, and then conducted according to the manufacturer's protocol (Olink). For flow cytometry cells were fixed in 2% formaldehyde (10 mins, RT), washed, blocked in 1% BSA (1 h, RT), stained with anti-IL-1R2 mAb (1:40) or anti-mouse IL-1R2 for EL4 cells (1:40, R&D), washed, stained with Alexa Fluor anti-mouse 488 (1:500) or anti-goat FITC for EL4 (1:160), washed and analysed on a C6 (Accuri). For intracellular staining all steps, except fixation, had 0.5% saponin present. MFIs reported have surface or intracellular control values subtracted from the specific MFI. For intracellular-specific MFI the surface-specific value was subtracted.

Animal Protocols

Animal experiments were performed under UK Home Office licensing. Mice were injected intraperitoneally with saline or 0.29 fmol/g body weight of p17 or p33 IL-1 α , or 8.3×10^4 necrotic HeLa cells. After 6 h, the peritoneal cavity was lavaged with 5ml of PBS. GR-1 +ve cells were enumerated by FACS following anti-GR-1-FITC staining (1:200; Biolegend).

Statistics

Parametric tests were employed for analysis of continuous ELISA and peritonitis data, which was conducted using a one-way, two-tailed ANOVA (Excel).