Interleukin (IL)-1F6, IL-1F8, and IL-1F9 Signal through IL-1Rrp2 and IL-1RAcP to Activate the Pathway Leading to NF-κB and MAPKs*

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Interleukin 1 (IL-1) plays a prominent role in immune and inflammatory reactions. Our understanding of the IL-1 family has recently expanded to include six novel members named IL-1F5 to IL-1F10. Recently, it was reported that IL-1F9 activated NF-κB through the orphan receptor IL-1 receptor (IL-1R)-related protein 2 (IL-1Rrp2) in Jurkat cells (Debets, R., Timans, J. C., Homey, B., Zurawski, S., Sana, T. R., Lo, S., Wagner, J., Edwards, G., Clifford, T., Menon, S., Bazan, J. F., and Kastelein, R. A. (2001) J. Immunol. 167, 1440–1446). In this study, we demonstrate that IL-1F6 and IL-1F8, in addition to IL-1F9, activate the pathway leading to NF-κB in an IL-1Rrp2-dependent manner in Jurkat cells as well as in multiple other human and mouse cell lines. Activation of the pathway leading to NF-κB by IL-1F6 and IL-1F8 follows a similar time course to activation by IL-1β, suggesting that signaling by the novel family members occurs through a direct mechanism. In a mammary epithelial cell line, NCI/ADR-RES, which naturally expresses IL-1Rrp2, all three cytokines signal without further receptor transfection. IL-1Rrp2 antibodies block activation of the pathway leading to NF-κB by IL-1F6, IL-1F8, and IL-1F9 in both Jurkat and NCI/ADR-RES cells. In NCI/ADR-RES cells, the three IL-1 homologs activated the MAPKs, JNK and ERK1/2, and activated downstream targets as well, including an IL-8 promoter reporter and the secretion of IL-6. We also provide evidence that IL-1RAcP, in addition to IL-1Rrp2, is required for signaling by all three cytokines. Antibodies directed against IL-1RAcP and transfection of cytoplasmically deleted IL-1RAcP both blocked activation of the pathway leading to NF-κB by the three cytokines. We conclude that IL-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP.

Interleukin-1 (IL-1)3 is a pleiotropic cytokine that is involved in the initiation of immune and inflammatory responses in virtually every tissue in the body. IL-1 exerts its effects through activation of a set of transcription factors including NF-κB and AP-1, as well as MAPKs such as JNK and p38, leading to the production of numerous cytokines, chemokines, adhesion molecules, and enzymes (e.g. cyclooxygenase and nitric-oxide synthetase) (2). The activity of IL-1 resides in two separate cytokines, IL-1α and IL-1β, which bind to the same receptor and initiate identical responses. Binding of IL-1α and IL-1β to the type I IL-1 receptor (IL-1R) results in recruitment of the IL-1R homolog, IL-1R accessory protein (IL-1RAcP or AcP), which does not directly bind the ligands but is required for signal transduction (3). A third IL-1 family member, IL-1 receptor antagonist (IL-1ra), negatively regulates IL-1 activity by competing with IL-1 for binding to the receptor. Binding of IL-1ra to the IL-1R does not result in the recruitment of AcP and therefore does not generate a signal (4). An additional level of regulation of IL-1α activity is imposed by the type II IL-1R, which binds to and sequesters IL-1 but does not signal. Based on sequence similarity, gene structure, and predicted tertiary structure, IL-18 is also part of the IL-1 family of ligands. IL-18 is a cytokine acting on Th1 and NK cells, which induces interferon-γ production, especially in combination with IL-12 (5–7). Signaling by IL-18 is very similar to signaling by IL-1α/β although IL-18 utilizes a completely different receptor. IL-18 binds to an IL-1R family member, IL-1R-related protein (rp1 or IL-18R), and as with IL-1, IL-18 signaling is dependent upon association of a second IL-1R family member, IL-1RACPL (or AcPL for AcP-like) (8). A soluble IL-18-binding protein acts as a negative regulator of IL-18 activity by binding to and sequestering IL-18 (9).

Recently, six new members of the IL-1 family have been identified primarily through use of DNA data base searches for homologs to IL-1. These proteins are termed IL-1F5 to IL-1F10 and are classified as IL-1 family members based on amino acid sequence similarity, identity of gene structure, and predicted or known three-dimensional structure (3, 10–12). All of the new genes map to human chromosome 2 as do IL-1α, IL-1β, and IL-1ra. The genes for IL-1F5 to IL-1F10 and IL-1ra loci (11, 13, 14), suggesting that they arose from a common ancestral gene that later became duplicated. Little is known about the receptors, signaling, or function of these new IL-1 family members. IL-1F7 (F7) has been reported to bind to IL-18R with low affinity but does not result in the formation of a ternary complex with AcP and does not produce any IL-18-like signaling activities (15, 16). F7 also does not antagonize IL-18 through binding to the IL-18R; therefore, the significance of this interaction is unknown (16). Others have reported that F7 binds to IL-18-binding protein and enhances the ability of IL-18-binding protein to inhibit IL-18 activities (17). Recently, F7 was shown to have anti-tumor effects following adenoviral gene transfer of F7 into murine...
fibrosarcomas (18). Even less is known about the activities of the other new IL-1 family members. IL-1F10 has been reported to bind to soluble IL-1R, but the significance of this interaction is unknown (19). No binding of IL-1F5, IL-1F6, IL-1F8, or IL-1F9 to any IL-1R homolog has been demonstrated to date. However, IL-1F9 (F9) was reported to activate NF-κB through the orphan receptor, IL-1R-related protein 2 (IL-1Rrp2 or rp2), and IL-1F5 potently antagonized this response (1). No binding, interactions, or signaling have been reported for IL-1F6 (F6) or IL-1F8 (F8).

Similar to the IL-1 family of ligands, there are multiple IL-1R homologs, many of which are orphans. The IL-1R family members are characterized by the presence of three extracellular immunoglobulin domains and an intracellular Toll-IL-1R (TIR) domain, which they share with the 10 mammalian Toll-like receptors, Drosophila Toll and 18-Wheeler, and several recently identified adaptor molecules involved in IL-1R/Toll-like receptor signaling (2). Other than IL-1R, AcP, and the IL-1R receptors, IL-15R and ACPl, the IL-1R family contains the orphan receptors IL-1Rrp2, T1/ST2, TIGIRR, APL, and single Ig IL-1R-related molecule (SIGIRR) (3). There is no known ligand for T1/ST2; however, it is expressed on type 2 T helper cells and appears to play a role in the regulation of type 2 T helper cell function (20–22). TIGIRR and APL are located on the X-chromosome and share a high degree of sequence similarity (23). There is no known ligand for TIGIRR or APL. APL is expressed in the brain, and mutations in APL are associated with a nonsyndromic X-linked mental retardation (24). SIGIRR differs from the other IL-1R family members in that it contains only one immunoglobulin domain in its extracellular region (25). In addition, SIGIRR along with TIGIRR and APL have unusually long cytoplasmic domains with roughly 100 additional amino acids at their carboxyl termini, reminiscent of the structure of Drosophila Toll. There is no known ligand for SIGIRR, but recent evidence suggests that SIGIRR plays an inhibitory role in IL-1 signaling (26). Chimeric molecules of the IL-1R family members containing the extracellular and transmembrane domain of either IL-1R or AcP and the cytoplasmic domain of each IL-1R family member have been constructed (23). These molecules were expressed in every possible combination, and the ability of each receptor chimera combination to activate NF-κB following IL-1 stimulation was assessed. Through these experiments, the IL-1R family member cytoplasmic domains were characterized as IL-1R-like (primary receptor) or AcP-like (accessory receptor). IL-1R, IL-1R, IL-1Rrp2, and T1/ST2 were classified as IL-1-like, whereas only AcP and ACPl were characterized as AcP-like. TIGIRR, APL, and SIGIRR did not fall into either category.

IL-1F9 has recently been demonstrated to activate NF-κB in Jurkat T cells in an IL-1Rrp2-dependent manner (1). We were able to replicate these results and extend them to demonstrate that F6 and F8 were also able to activate the pathway leading to NF-κB in Jurkat cells transfected with rp2 but not with other IL-1R family members. Transfection of rp2 into a number of different human and mouse cell lines conferred responsiveness to F6, F8, and F9. We identified a cell line that naturally responds to F6, F8, and F9 in an rp2-dependent manner. In addition, F6, F8, and F9 were demonstrated to activate similar signaling responses to IL-1 including activation of MAPKs and secretion of cytokines. We also provide evidence that signaling by F6, F8, and F9 not only is dependent upon rp2 but also requires the accessory protein, AcP. Identification of the receptor subunits involved in signaling through the previously orphan ligands, F6 and F8, will direct experiments designed to assess the role of F6, F8, and F9 in physiology.

**Experimental Procedures**

**Biological Reagents and Cell Culture—**Recombinant human IL-1β and murine IL-3 were produced at Amgen Corp. (Seattle, WA). Recombinant human IL-1β was purified by Peptide Institute (Osaka, Japan). Human and murine IL-1F6, IL-1F8, and IL-1F9 were cloned into pGEX-4T-1 (Amersham Biosciences) as N-terminal glutathione S-transferase fusions. A Factor Xa recognition sequence was placed immediately upstream of the first methionine of each IL-1F gene. The resulting constructs were expressed in Escherichia coli DH10B by induction with isopropyl-1-thio-galactopyranoside. lysates were run over glutathione-Sepharose (Novagen, Madison, WI) columns to capture the glutathione S-transferase/IL-1F fusion proteins and then subjected to on-column cleavage with Factor Xa (Novagen). The cleaved IL-1F proteins were eluted with PBS, and the Factor Xa was removed using a specific affinity agarose (Novagen). The IL-1F proteins were further purified by size exclusion chromatography using a Superdex 75HR column. Purified proteins were then quantitated by amino acid analysis. The Jurkat E6.1 (American Type Culture Collection (ATCC), Manassas, VA), HepG2 (ATCC), and BA/F3 (DSMZ, Braunschweig, Germany) cell lines were maintained in RPMI 1640 supplemented with 10% FBS, glutamine, and antibiotics. BA/F3 cells were supplemented with 50 ng/ml murine IL-3, NCI/ADR-RES cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium with 10% FBS, glutamine, and antibiotics. IL-1RAcP null MEFs (AcP−/−; MEFs) are primary mouse embryonic fibroblasts generated from the IL-1RAcP knockout mice (27), which were obtained from the Jackson Laboratories induced mutant resource program. The IL-1RAcP null MEFs were cultured in Dulbecco’s modified Eagle’s medium, 10% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μM Hepes, pH 7.4, and antibiotics. Rabbit anti-phospho-ERK, anti-total ERK, anti-phospho-JNK, and anti-total JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA).

**Expression Plasmids—**Full-length human IL-1R (28), IL-1RAcP (29, 30), IL-1R rp2 (31, 32), IL-1RAcP(8), SIGIRR (25), TIGIRR (23), APL (24), T1/ST2 (33), and IL-1Rrp2 (34) were subcloned into pcDNA3 (a close relative of pCD302, described in Ref. 35. Mouse IL-1Rrp2 (23) and IL-1R rp2 (30) were also subcloned into pcDNA3. The murine IL-1RAcP cytoplasmic deletion construct contains amino acids 1–400 of murine IL-1RAcP (GenBankTM accession number NP_032390), and the human IL-1RAcP cytoplasmic deletion contains amino acids 1–386 of human IL-1RAcP (GenBankTM accession number NP_002173). Both deletion constructions were subcloned into pcDNA3. The LacZ plasmid encodes β-galactosidase driven by the cytomegalovirus promoter subcloned into the pcDNA3 vector. The IL-8 promoter reporter plasmid consists of nucleotides −130 to +44 of the human IL-8 promoter (36) fused to luciferase in the pgL2 Basic vector (Promega, Madison, WI). Mouse embryonic fibroblasts generated from the IL-1RAcP knockout mice (27), transduced with a BgIII fragment (1–106) were transiently transfected via FuGENE 6 (Roche Applied Science) as per the manufacturer’s protocol. Briefly, cells were transfected with 200 ng of reporter plasmid and 400 ng of receptor or β-galactosidase plasmids with a 1:3 DNA/FuGENE 6 ratio. NCI/ADR-RES cells (7 × 104) were transfected with 500 ng of reporter plasmid alone with a 1:3 DNA/FuGENE 6 ratio. AcP−/− MEFs (4 × 104) were transfected with 50 ng of reporter plasmid and 75 ng of each receptor or β-galactosidase plasmids with a 1:3 DNA/FuGENE 6 ratio. BA/F3 cells (2 × 105) were transfected via electroporation with 2 μg of reporter plasmid and 4 μg of receptor or β-galactosidase plasmids. Twenty hours after transfection, cells were stimulated with the indicated cytokines for 5 h (Jurkat cells) or 6 h (HepG2, BA/F3, AcP−/−; MEF, and NCI/ADR-RES cells). Cells were lysed, and luciferase activity was assessed using reporter lysis buffer (Promega) (Jurkat and BA/F3 cells) or passive lysis buffer (Promega) (HepG2, NCI/ADR-RES and AcP−/−; MEF cells) and luciferase assay reagent (Promega). All results reported represent duplicate samples in each of at least two independent experiments. In the antibody-blocking reporter assays, Jurkat or NCI/ADR-RES cells were transfected as above. Twenty hours after transfection, cells were preincubated with the anti-human IL-1Rrp2 mouse IgG1 monoclonal antibody M145 or M146 (Amen or the anti-human IL-1Rrp2 mouse IgG1 monoclonal antibody M49 (Amen) for 15 min before the addition of the indicated ligands. Anti-human IL-1R type II or anti-human CD40 ligand (CD40L) mouse IgG1 monoclonal antibodies (Amen) were used as irrelevant control antibodies in this assay.

**F6 Antibody Depletion—**Human F6 was diluted to 360 ng/ml in Jurkat cell medium. 3 ml aliquots of each dilution were incubated with 75 μg of either mouse-anti-human IL-1F6 antibody M624 (Amen), mouse-anti-FLAG antibody M2 (Amen), or PBS alone. 160 μl of 2× protein G-agarose was added to each and incubated at room tempera-
...ature for 60 min. The immune complexes were removed by centrifuga-
tion, and the supernatants were transferred to a new tube. This proce-
dure was repeated five times with the last incubation at 4 °C for 17 h.
The samples were assayed via Western blot for removal of human F6
using a specific rabbit polyclonal antiseraum (Amgen) to F6.

Expression Analysis—For the human tissue expression analyses,
commercially available RNAs (Ambion (Austin, TX), Clontech, and
Stratagene) were utilized. RNAs were DNase-treated (Ambion) and
reverse transcribed using TaqMan reverse transcription reagents (Ap-
plied Biosystems, Foster City, CA) according to the manufacturer’s
specifications using random hexamers to prime. Samples were distrib-
uted on plates at 20 ng/well and run in triplicate. TaqMan primer/probe
sets for the control genes and F6, F8, F9, and rp2 were designed using
Primer Express software (Applied Biosystems). Forward and reverse
primer concentrations for all primer sets were optimized. 6-Carboxy-
fluorescein-labeled probe (Applied Biosystems) was used at 200 nm.
Threshold cycle values (COT) were determined using Sequence Detector
software (Applied Biosystems) and transformed to \(2^{-\Delta CT}\) for relative
expression comparison of rp2, F6, F8, or F9 with control. For reverse
transcriptase PCR analysis, RNA was isolated from cells using the
RNeasy RNA isolation kit (Qiagen, Valencia, CA) as per the manufac-
turer’s instructions. Two micrograms of RNA was subjected to reverse
transcription using the Omniscript reverse transcriptase (QIAGEN) as
per manufacturer’s instruction using random primers (Invitrogen).
cDNAs were then amplified for the IL-1Rs using primers that span
introns and HotStar Taq™ Master Mix (Qiagen). Products were analy-
lized via agarose gel electrophoresis.

FACS Analysis—NCI/ADR-RES cells (1 × 10⁶) were washed in FACS
buffer (3% normal rabbit serum, 3% FBS in PBS) and resuspended in FACS buffer containing a 10 µg/ml con-
centration of the anti-human IL-1Rrp2 antibody (M145) for 30 min on ice.
Specific binding was detected with an allophycocyanin-conjugated goat
anti-mouse IgG, also at 10 µg/ml (Jackson ImmunoResearch Laborato-
ries, West Grove, PA). After staining, cells were analyzed for rp2 ex-
pression using a FACSCalibur (BD Biosciences, Mountain View, CA).

Detection of Cell Lysates and Immunoblotting—NCI/ADR-RES
(cells 2 × 10⁶ cells/well) were plated into 6-well tissue culture dishes.
The following day, cells were stimulated with F6, F8, or F9 at 1 µg/ml
or 10 ng/ml at 5, 10, 15, 30, or 60 min. Following incubation,
cells were washed in ice-cold PBS containing 1 mM Na3VO4, scrapped,
transferred to Eppendorf tubes, and centrifuged for 5 min in the cold at
5000 rpm. Cells were resuspended in 100 µl of 1× cell lysis buffer (New
England Biolabs, Beverly, MA) containing 1 mM phenylmethylsulfonyl
fluoride and 1 mM NaF. After 15 min on ice, insoluble material was
removed by centrifugation at 10,000 rpm in the cold. Supernatants were
transferred to new tubes containing an equal volume of 2× SDS sample
buffer (Invitrogen). Proteins (20 µl) were separated on a 1-mm-thick,
8–16% Tris/glycine gels and then transferred to nitrocellulose filters.
The filters were blocked with 5% nonfat dry milk powder in TBS (10 mM
Tris, 150 mM NaCl) containing 0.1% Tween 20 (TBST) at room
temperature for 1 h. Primary antibodies were diluted 1:1000 in
TBST containing 3% bovine serum albumin and 0.1% Tween 20 and
incubated with the filters overnight at 4°C. After washing with TBST,
the filters were incubated for 1 h with horseradish peroxidase-conju-
gated goat anti-rabbit IgG (Bio-Rad) at 1:3000 in 1% nonfat milk in
TBST. Filters were washed extensively with TBST, and immunoreac-
tive bands were visualized by ECL (Amersham Biosciences).

Cytokine Assay—NCI/ADR-RES cells (5 × 10⁴) were plated into
24-well dishes. The following day, cells were stimulated with F6, F8,
and F9 at 1 or 5 µg/ml or 10 ng/ml. Supernatants were collected 48 h after
the stimulation of the cytokines and were analyzed for the presence of
IL-1β, IL-6, IL-8, IL-10, IL-12(p70), tumor necrosis factor-α, interferon-γ, and granulocyte-macrophage colony-stim-
ulating factor using the Beadlyte™ human multi cytokine detection
system 3 from Upstate Cell Signaling Solutions (Lake Placid, NY) as
per the manufacturer’s instructions. Briefly, the multicytokine 3 standard
was resuspended in assay buffer and then serially diluted from
2500 to 15.6 pg/ml. 50 µl of standard or sample was added to each well
of a 96-well plate with 25 µl of the bead solution and was incubated
overnight at 4°C. The Beadlyte™ reporter solution was added to each
well and incubated at room temperature for 1.5 h. Beadlyte™ strepta-
vidin-phycocerythrin was diluted 1:25 in assay buffer and was added to
each well and incubated at room temperature for 30 min before the
addition of the Beadlyte™ stop solution. The plate was then analyzed
on the LumineX™ LabMAPSTM system (Lumines Corp., Austin, TX)
and analyzed using Masterplex QT software (MiraBio Inc., Alameda, CA).

RESULTS

IL-1F6, IL-1F8, and IL-1F9 Signal through IL-1Rrp2 and AcP

Comparison of the IL-1F6, IL-1F8, and IL-1F9 Dose-response and Time Courses with That of IL-1β—In order to better define the
activation of the pathway leading to NF-κB by the novel family
members but did not affect activation by IL-1β, indicating that
the antibodies specifically block the F6, F8, and F9 response.
The addition of an irrelevant antibody directed against IL-1R
type II had no effect on the ability of the three novel cytokines
or IL-1β to activate the pathway leading to NF-κB (Fig. 1D and
data not shown).

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data not shown).
Fig. 1. IL-1F6, IL-1F8, and IL-1F9 activate the pathway leading to NF-κB in multiple cell types transfected with IL-1Rrp2. Jurkat (A), HepG2 (B), and BA/F3 (C) cells were transfected with IL-1Rrp2 or empty vector (pDC304) plus the NF-κB-luciferase reporter gene plasmid. Twenty hours after transfection, cells were left untreated (No stim) or were stimulated with the indicated concentrations of F6, F8, F9, F5, and IL-1β for 5 h (A and C) or 6 h (B). Human IL-1Rrp2 and human F6, F8, and F9 protein were utilized in A and B and murine IL-1Rrp2, F6, F8, and F9 were utilized in C for the mouse BA/F3 cell line (see “Experimental Procedures” for transfection details). Luciferase activities were determined and expressed as relative light units (RLU). Data are shown from one of three independent experiments with similar results. D, antibodies directed against human rp2 block the activation of the pathway leading to NF-κB by F6, F8, and F9 in Jurkat cells. Jurkat cells were transfected with IL-1Rrp2, IL-1R, or empty vector plus the NF-κB reporter gene plasmid. Twenty hours after transfection, cells were either left unstimulated or were pretreated with the indicated antibodies at 5 μg/ml for 15 min prior to stimulation with F6, F8, F9, or IL-1β at the indicated concentrations. Cells were stimulated for 5 h. Luciferase activities were determined and expressed as fold activation of stimulation over basal activity. Data are shown from one of three independent experiments with similar results. M145 is a mouse anti-human rp2 monoclonal antibody. Control Ab indicates use of an irrelevant antibody control, which was a mouse anti-human IL-1R type II monoclonal antibody.

Fig. 2A). The dose response was very similar for F6 and was similar, however with a decreased magnitude, for F9 (data not shown). IL-1β, on the other hand, significantly activated the pathway leading to NF-κB at a concentration as low as 0.02 ng/ml and reached plateau with 2 ng/ml (see Fig. 2A). Therefore, much higher doses of F6, F8, and F9 are necessary to achieve significant activation of the pathway leading to NF-κB than for IL-1β. The dose-response curve for IL-18 in Jurkat cells is higher than that for IL-1β but still much lower than that of the IL-1Fs, with the minimal dose being around 1 ng/ml and the plateau occurring around 50 ng/ml (data not shown).

It is not yet known why such high ligand concentrations are necessary to obtain significant activation of the pathway leading to NF-κB. However, we are confident that the activity seen with F6, F8, and F9 is due to the IL-1F proteins and not due to a contaminant in the protein preparations. Mammalian produced FLAG-tagged F6, F8, and F9 were purified from COS-7 cells and used for the ligands produced in E. coli and purified by glutathione S-transferase affinity chromatography (data not shown). IL-1F5 and IL-1F10, produced in the same manner as F6, F8, and F9, do not activate the pathway leading to NF-κB in rp2-transfected Jurkat cells (Fig. 1A and data not shown). In addition, the activity of the ligands is heat-inactivatable, and our Jurkat cells are not responsive to lipopolysaccharide, demonstrating that activation of the pathway leading to NF-κB is not due to endotoxin contamination. Finally, we used antibodies directed against human rp2 to specifically immunodeplete F6 from the protein preparations. When the E. coli-produced F6 was depleted with the anti-F6 monoclonal antibody, the remaining material was no longer capable of activating the pathway leading to NF-κB (see Fig. 2B). However, when the depletion was carried out with an irrelevant antibody (the anti-FLAG antibody, M2) or with PBS alone, the F6 preparation remained capable of activating the pathway leading to NF-κB. In addition, depletion of F8 with the anti-F6 antibody had no effect on the activation of the pathway leading to NF-κB by the F8 preparation. Therefore, the activity seen with the IL-1F preparations is likely to be due to the proteins themselves and not to contaminating molecules.

Activation of the pathway leading to NF-κB by IL-1β occurs via direct binding of IL-1β to the IL-1R, which recruits IL-1RαcP and initiates signaling (3). We reasoned that if activa-
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A. dose response of F8 and IL-1 in rp2-transfected Jurkat cells. Jurkat cells were transfected with IL-1Rrp2, IL-1R, or empty vector (pDC304) plus the NF-κB-luciferase reporter gene plasmid. Twenty hours after transfection, cells were left untreated or were stimulated with F8 or IL-1β at the indicated concentrations for 5 h. Luciferase activities were determined and expressed as RLU. Data are shown from one of three independent experiments with similar results.

B. the F6 activity can be specifically immunodepleted using an anti-F6 antibody. F6 and F8 proteins underwent several rounds of immunoprecipitation with the anti-F6 antibody, M624, with the anti-FLAG antibody, M2, or with PBS alone. Depleted material was incubated with Jurkat cells transfected with IL-1Rrp2 plus the NF-κB-luciferase reporter gene plasmid for 5 h. Luciferase activities were determined and expressed as relative light units. F6 and F8 depleted material was used at a final concentration of 125 ng/ml. C. kinetics of activation of the pathway leading to NF-κB by F8 and IL-1. Jurkat cells were transfected as in A and were left untreated or were stimulated with F8 at 250 ng/ml or IL-1β at 0.1 ng/ml. Cells were collected 1, 2, 4, 5, 6, 7, or 24 h after stimulation, and luciferase activity was measured. Data are shown from one of two independent experiments with similar results.

Fig. 2. F8 and IL-1 dose response and kinetics of activation of the pathway leading to NF-κB in Jurkat cells. A, dose response of F8 and IL-1 in rp2-transfected Jurkat cells. Jurkat cells were transfected with IL-1Rrp2, IL-1R, or empty vector (pDC304) plus the NF-κB-luciferase reporter gene plasmid. Twenty hours after transfection, cells were left untreated or were stimulated with F8 or IL-1β at the indicated concentrations for 5 h. Luciferase activities were determined and expressed as RLU. Data are shown from one of three independent experiments with similar results. B, the F6 activity can be specifically immunodepleted using an anti-F6 antibody. F6 and F8 proteins underwent several rounds of immunoprecipitation with the anti-F6 antibody, M624, with the anti-FLAG antibody, M2, or with PBS alone. Depleted material was incubated with Jurkat cells transfected with IL-1Rrp2 plus the NF-κB-luciferase reporter gene plasmid for 5 h. Luciferase activities were determined and expressed as relative light units. F6 and F8 depleted material was used at a final concentration of 125 ng/ml. C, kinetics of activation of the pathway leading to NF-κB by F8 and IL-1. Jurkat cells were transfected as in A and were left untreated or were stimulated with F8 at 250 ng/ml or IL-1β at 0.1 ng/ml. Cells were collected 1, 2, 4, 5, 6, 7, or 24 h after stimulation, and luciferase activity was measured. Data are shown from one of two independent experiments with similar results.
the paw, seminal vesicle, uterus, and prostate (Fig. 3B). IL-1Rrp2 is either not present or expressed at very low levels in human and mouse testis, heart, spleen, and small intestine (see Fig. 3). Expression of the IL-1Fs is more restricted. IL-1F6, IL-1F8, and IL-1F9 are all expressed in human skin and in mouse paw at least to some extent; both tissues contain high levels of rp2 (data not shown). Murine F6, F8, and F9 are all highly expressed in the esophagus (data not shown). F6 is also expressed in human trachea, thymus, fetal colon, and spleen and in mouse stomach (data not shown). Human F8 is expressed highest in skin but also in fetal colon and skeletal muscle. Human F9 is expressed highest in skin and at lower levels in uterus and trachea (data not shown), whereas murine F9 has a broader expression pattern with high expression in paw, esophagus, stomach, bone marrow, and peripheral blood lymphocytes (data not shown).

IL-1F6, IL-1F8, and IL-1F9 Endogenously Activate the Pathway Leading to NF-κB in an rp2-dependent Manner in NCI/ADR-RES Cells—In order to identify a cell line that may naturally respond to F6, F8, and F9, we looked for an established cell line expressing high levels of rp2. The only transformed cell lines found to express rp2 mRNA were Colo205, SW48, HT29, HBT75, HaCAT, and the mammary epithelial cell line, NCI/ADR-RES, which had the highest expression (data not shown). In order to determine whether rp2 protein was indeed expressed on the surface of the cell, NCI/ADR-RES cells were analyzed by flow cytometry after staining with an anti-human rp2 antibody. A peak shift was seen by FACS analysis on NCI/ADR-RES cells with the anti-rp2 antibody (M145) but not when only the secondary antibody was present (see Fig. 4A). When the M145 antibody was used to stain HeLa cells, which express very low levels of rp2 by reverse transcriptase-PCR, no shift was observed (data not shown). Therefore, NCI/ADR-RES cells do express rp2 on the surface of the cell.

We then sought to determine whether NCI/ADR-RES cells could respond to F6, F8, and F9 without transfection of additional rp2. When NCI/ADR-RES cells were transfected with the NF-κB reporter plasmid alone and stimulated with F6, F8, and F9 for 6 h, significant activation of the pathway leading to NF-κB was induced by all three ligands. Dose-response studies...
demonstrated that higher concentrations of ligands were necessary to achieve significant activation of the pathway leading to NF-κB than were typically seen in cells transfected with rp2 (see Fig. 4B). This was probably due to lower receptor levels on these cells than obtained through transfection of rp2. In addition, the stimulation of basal activity (fold activation) was lower in NCI/ADR-RES cells compared with all other cell lines tested. Activation of the pathway leading to NF-κB by IL-1β was similarly low, in that 100 ng/ml of IL-1β resulted in around 5–10-fold activation as opposed to the greater than 30-fold activation typically seen with this concentration of IL-1β in transfected Jurkat cells. NCI/ADR-RES cells are mammary epithelial cells. Human breast tumor cell lines, especially those negative for the estrogen receptor such as the MCF-7 cells from which NCI/ADR-RES cells are derived, are known to be characterized by high basal NF-κB activation (37, 38). The basal luciferase levels were much higher in NCI/ADR-RES cells than in all other cells tested (Jurkat, BA/F3, HeLa, etc.), which contributes to the lower fold-activation of stimulation and probably the need for higher ligand concentrations.

In order to determine whether F6, F8, and F9 activation of the pathway leading to NF-κB in NCI/ADR-RES cells was dependent upon rp2, we asked whether the anti-human rp2 antibodies that were demonstrated in Fig. 1D to block the rp2-dependent activation of the pathway leading to NF-κB by the three cytokines in Jurkat cells could block activation of the pathway leading to NF-κB by these ligands in NCI/ADR-RES cells. The addition of the anti-rp2 antibodies, M145 (Fig. 4C) and M146 (data not shown), to cells 15 min before stimulation completely blocked activation of the pathway leading to NF-κB by F6 and F8 and had no effect on activation by IL-1β. The addition of an irrelevant antibody, anti-human CD40L, had no effect on the activation of the pathway leading to NF-κB by F6 or F8 (Fig. 4C). Therefore, activation of the pathway leading to NF-κB by F6, F8, and F9 in NCI/ADR-RES cells is also rp2-dependent.

**IL-1F6, IL-1F8, and IL-1F9 Activate MAPKs in NCI/ADR-RES Cells**—Since NCI/ADR-RES cells respond to F6, F8, and F9 without transfection of rp2, we used these cells to look at activation of other signaling pathways by the three cytokines. IL-1β and IL-18 are known to activate MAPKs as well as NF-κB in many cell types (2). We therefore tested the ability of F6, F8, and F9 to activate JNK and ERK1/2. After stimulating NCI/ADR-RES cells with F6, F8, F9, or IL-1β for various amounts of time, MAPK activity was assessed by immunoblotting cell lysates with antibodies specific for the phosphorylated (activated) forms of JNK and ERK. We found that F6, F8, and F9 activated JNK as early as 5 min after stimulation, with maximal activation seen by all three ligands at 15 min (Fig. 5A). By 30 min of stimulation, JNK activation was decreased, and it returned to base-line levels by 60 min. The time course for activation was slightly different for F6, F8, and F9 compared with IL-1β in that maximal activation by IL-1 was seen at 30 min, with levels returning to base line by 60 min. In addition, F6, F8, and F9 strongly activate phosphorylation of the 46-kDa form of JNK but only weakly activate the 52-kDa form, whereas IL-1β activates both equally. Whether this reflects a differential activation of JNK subunits or merely different levels of activation is unclear.

F6, F8, and F9 were also shown to activate ERK in NCI/ADR-RES cells (see Fig. 5B). Activation of ERK by the three ligands was apparent by 10 min after stimulation with maximal activation seen at 15 min and return to base-line levels at 60 min. The time course for activation by IL-1β was similar except that activation remained high after 30 min of treatment. For both JNK and ERK, the blots were reprobed with antibodies that recognize total JNK and total ERK, respectively, demonstrating equal loading. Therefore, F6, F8, and F9 signal to activate JNK and ERK in addition to activating the pathway leading to NF-κB.

**Induction of Cytokines in Response to F6, F8, and F9**—IL-1 and IL-18 stimulate multiple cell types to produce a variety of cytokines and chemokines including IL-8 and IL-6 (4). In order to determine whether F6, F8, and F9 could induce IL-8, we transfected Jurkat cells with a reporter containing the IL-8 promoter fused to luciferase. F6, F8, and F9 potently activated the IL-8 promoter in Jurkat cells transfected with rp2 but not in cells transfected with vector alone (Fig. 5C). IL-1 also acti-

**Fig. 4.** F6, F8, and F9 activate the pathway leading to NF-κB in NCI/ADR-RES cells. A, NCI/ADR-RES cells express rp2 on the cell surface. NCI/ADR-RES cells were stained with either the anti-human rp2 monoclonal antibody (M145) at 10 μg/ml or an allophycocyanin-conjugated anti-murine IgG secondary antibody at 10 μg/ml or with secondary antibody alone at 10 μg/ml. Cells were analyzed by flow cytometry. B, F6, F8, and F9 activate the pathway leading to NF-κB in NCI/ADR-RES cells in a dose-dependent manner. NCI/ADR-RES cells were transfected with the NF-κB-luciferase reporter gene plasmid alone. Twenty hours after transfection, cells were left untreated or were stimulated with F6, F8, or F9 at the indicated concentrations for 5 h. Luciferase activities were determined and expressed as relative light units (RLU). Data are shown from one of two independent experiments with similar results. C, anti-rp2 antibodies block the activation of the pathway leading to NF-κB by F6, F8, and F9 in NCI/ADR-RES cells. NCI/ADR-RES cells were transfected with the NF-κB-luciferase reporter gene plasmid. Twenty hours after transfection, cells were either left unstimulated or were pretreated with the indicated antibodies at 20 μg/ml for 15 min prior to stimulation with F6, F8, or IL-1β at the indicated concentrations. Cells were stimulated for 6 h, and luciferase activities were determined and expressed as RLU. Data are shown from one of two independent experiments with similar results. M145 is a mouse anti-human rp2 monoclonal antibody. Control Ab indicates use of an irrelevant antibody control, which was a monoclonal antibody directed against human CD40 ligand.
vated this reporter in cells transfected with the IL-1R as has been demonstrated previously (36). Therefore, F6, F8, and F9 activate the IL-8 promoter in an rp2-dependent manner.

To determine whether F6, F8, and F9 could induce production of cytokines, we tested whether treatment of NCI/ADR-RES cells with F6, F8, F9 (1 μg/ml), or IL-1β (10 ng/ml) for 5, 10, 15, 30, or 60 min before preparation of cell lysates and immunoblotting. Blots were probed with anti-phospho-JNK antibody (A) or anti-phospho-ERK1/2 (B) antibody (upper panels) and then reprobed with anti-JNK antibody (A) or anti-ERK1/2 antibody (B) (lower panels) to ensure similar total protein loading. Molecular mass standards in kDa are indicated to the right of each panel. Data are shown from one of two independent experiments with similar results. C, F6, F8, and F9 activate the IL-8 promoter in an rp2-dependent manner. Jurkat cells were transfected with IL-1Rrp2, IL-1R, or empty vector plus the IL-8-luciferase reporter gene plasmid. Twenty hours after transfection, cells were left untreated or were stimulated with F6, F8, F9 (500 ng/ml), or IL-1β (0.1 ng/ml) for 5 h. Luciferase activities were determined and expressed as relative light units (RLU). Data are shown from one of three independent experiments with similar results. D, F6, F8, and F9 induce secretion of IL-6. NCI/ADR-RES cells were treated with F6, F8, F9 (5 μg/ml), or IL-1β (10 ng/ml) for 48 h before collection of supernatants. IL-6 levels in cell supernatants were measured via Luminex analyses and are expressed in pg/ml. Results are representative of two independent experiments.

IL-1RAcP Is Required for Induction of the Pathway Leading to NF-κB by F6, F8, and F9—IL-1 and IL-18 require the association of two IL-1R family members in order to generate a signal (2, 3). Since F6, F8, and F9 have many of the same structural features as IL-1 and IL-18 and signal through one member of the IL-1R family, rp2, we reasoned that signaling by the novel cytokines is probably dependent upon association of a second IL-1R family member. Through chimeric receptor experiments, rp2 was classified as an IL-1R-like receptor in that a chimera consisting of IL-1R extracellular and transmembrane domains fused to the intracellular domain of rp2 was able to cooperate with AcP to generate an IL-1 response (23). Those experiments suggested that signaling by rp2 would require a member of the accessory receptor group, namely AcP or AcPL. Expression of IL-1R family members was analyzed in all cell types in which activation of the pathway leading to NF-κB by F6, F8, and F9 in an rp2-dependent manner had been demonstrated. IL-1RAcPL was not expressed in all of the cell types analyzed.
**A.**

| Cell Type   | IL-1R | AcP | RP1 | AcPL | SIGIRR | TIGIRR | RP2 | APL | ST2 |
|-------------|-------|-----|-----|------|--------|--------|-----|-----|-----|
| Jurkat      |       |     |     |      |        |        |     |     |     |
| COS7        | +     |     |     |      |        |        |     |     |     |
| HepG2       | ++    | ++  | +   |      |        |        |     |     |     |
| HeLa        |       |     |     |      |        |        |     |     |     |
| NCI/ADR-RES | +     |     |     |      |        |        |     |     |     |
| BAF         |       |     |     |      |        |        |     |     |     |
| NIH3T3      | +     | +   |     |      |        |        |     |     |     |

**B.**

- **pDC304**
- **AcP**
- **IL-1Rrp2**
- **IL-1Rrp2 + AcP**

**C.**

- **IL-1Rrp2**
- **IL-1R**

**D.**

- **pDC304 + lacZ**
- **IL-1R + lacZ**
- **IL-1R + AcPDN**
- **IL-1Rrp2 + lacZ**
- **IL-1Rrp2 + AcPDN**
- **IL-18R + AcPL + lacZ**
- **IL-18R + AcPL + AcPDN**

*Fig. 6. AcP is required for signaling through F6, F8, and F9.*

A. AcP is the only IL-1R family member besides SIGIRR that is expressed in all cell lines that are responsive to F6, F8, and F9 upon transfection with rp2. RNAs were isolated from cell lines and analyzed for expression of the IL-1R homologs via reverse transcriptase-PCR analyses with gene-specific primers. PCR products were analyzed by agarose gel electrophoresis and are represented in tabular form. +, the RNA is expressed in that cell line; ++, high expression of the RNA; +/-, expression is barely detectable; –, the RNA is not expressed in that cell line.

B. F8 does not activate the pathway leading to NF-κB in rp2-transfected AcP−/− MEF cells unless AcP is transfected. MEF cells isolated from AcP null mice (AcP−/− MEF) were transfected with murine IL-1Rrp2, AcP, IL-1Rrp2 + AcP, or empty vector plus the NF-κB-luciferase reporter gene plasmid. Twenty hours after transfection, cells were left unstimulated or were stimulated with mouse F8 at 500 ng/ml or IL-1β at 10 ng/ml for 6 h. Luciferase activities were determined and expressed as relative light units (RLU). Data are shown from one of two independent experiments with similar results.

C. Antibodies directed at AcP block activation of the pathway leading to NF-κB by F6, F8, and F9. Jurkat cells were transfected with rp2 or IL-1R plus the NF-κB-luciferase reporter gene plasmid. Twenty hours after transfection, cells were left unstimulated or were stimulated with F6, F8, F9, IL-1β, M49, or the irrelevant control antibody alone at the indicated concentrations or with F6, F8 (both at 150 ng/ml), F9 (250 ng/ml), or IL-1 (0.01 ng/ml) plus 10 μg/ml concentration of the indicated antibodies. Transfected cells were preincubated with the antibodies for 15 min prior to the stimulation. Cells were stimulated for 5 h, and luciferase activities were determined and expressed as relative light units. Data are shown from one of two independent experiments with similar results. M49 is a mouse anti-human AcP monoclonal antibody. *Control Ab* indicates use of an irrelevant antibody control, which was a monoclonal antibody directed against human CD40 ligand.

D. Cytoplasmically deleted AcP acts as a dominant negative mutant and blocks activation of the pathway leading to NF-κB by F6, F8, and IL-1β but not IL-18. Jurkat cells were transfected with IL-1Rrp2 + lacZ, IL-1Rrp2 + AcPDN, IL-1R + lacZ, IL-1R + AcPDN, IL-18R + AcPL + lacZ, or IL-18R + AcPL + AcPDN all plus the NF-κB-luciferase reporter gene plasmid. Twenty hours after transfection, cells were left unstimulated or were stimulated with F6, F8, F9, IL-1, or IL-18 at the indicated concentrations for 5 h. Luciferase activities were determined and expressed as RLU. Data are shown from one of three independent experiments with similar results. AcP dominant negative (AcPDN) represents AcP extracellular and transmembrane domains minus the cytoplasmic region. Both human and murine cytoplasmically deleted AcP constructs were generated and transfected along with rp2 with identical results.
lines that were responsive to the three ligands (Fig. 6A), demonstrating that AcPL is not required. AcP was the only IL-1R family member, other than SIGIRR, that was expressed in every cell line in which F6, F8, and F9 responses were generated (see Fig. 6A). SIGIRR is not a likely candidate in that overexpression of SIGIRR decreases the ability of IL-1 to activate the pathway leading to NF-κB and JNK (26). Therefore, AcP was the most likely candidate.

Transfection of cells with rp2 plus AcP leads to a high basal activation of the pathway leading to NF-κB, similar to that seen upon transfection of IL-1R plus AcP (data not shown), suggesting that they may form a signaling pair. In order to determine whether AcP is required for signaling by F6, F8, and F9, we analyzed activation of the pathway leading to NF-κB in cells lacking AcP. Mouse embryonic fibroblasts were isolated from AcP knockout mice (AcP+/− MEFs) and were analyzed for their ability to respond to F8 and IL-1β. AcP+/− MEFs were transfected with the NF-κB reporter plus rp2, with and without AcP. F8 did not activate the pathway leading to NF-κB in cells transfected with rp2 alone. However, when AcP+/− MEFs were transfected with rp2 plus AcP, F8 significantly activated the pathway leading to NF-κB (Fig. 6B). This is the only cell line tested in which transfection of rp2 alone did not confer responsiveness to the three ligands, suggesting that AcP is required. High basal activation was seen with transfection of rp2 plus AcP, resulting in only about a 2-fold activation of the pathway leading to NF-κB following F8 stimulation.

Antibodies directed against human AcP were used to determine whether they could block the activation of the pathway leading to NF-κB by F6, F8, and F9. Jurkat cells transfected with rp2 or IL-1R were preincubated with the anti-AcP monoclonal antibody, M49, or an irrelevant control antibody. The addition of the anti-AcP antibody, but not the irrelevant control antibody, blocked activation of the pathway leading to NF-κB by F6, F8, and F9 (Fig. 6C). Activation of the pathway leading to NF-κB by IL-18 (data not shown) was not affected by the addition of either the AcP antibody or the irrelevant control. Preincubation with M49 had a lesser effect on the activation of the pathway leading to NF-κB by IL-1β (see Fig. 6C). It is not clear why the anti-AcP antibody blocks the F6, F8, and F9 responses more effectively than the IL-1 response.

In order to provide further evidence that AcP is required for signaling through F6, F8, and F9, AcP cytoplasmic deletion constructs were generated for both human and mouse AcP. The AcP cytoplasmic deletion construct contains the extracellular and transmembrane regions of AcP but lacks the TIR domain. Based on structural studies, it is likely that the extracellular immunoglobulin domains are involved in the assembly of IL-1RI and AcP into a heterodimer (2). Regions within the TIR domain of the cytoplasmic portion of AcP have been demonstrated to be required for IL-1 signaling and are essential for recruitment of signaling molecules to the receptor complex (39). Thus, the AcP cytoplasmic deletion construct should function as a dominant negative mutant, because deleted AcP would still interact with the receptor upon ligand binding but would be unable to signal due to lack of key residues contained within the TIR domain. Transfection of Jurkat cells with rp2 or IL-1R in combination with murine or human AcP cytoplasmic deletion constructs dramatically decreased the activation of the pathway leading to NF-κB by F6, F8, F9, and IL-1 but had no effect on activation of the pathway leading to NF-κB by IL-18 (see Fig. 6D). Therefore, deletion of the cytoplasmic region of AcP does have a dominant negative effect on responses to IL-1, F6, F8, and F9, indicating that AcP is required for signaling by the three novel ligands as well as IL-1.

IL-1F6, IL-1F8, and IL-1F9 signal through rp2 and AcP to activate the pathway leading to NF-κB and MAPKs; however, multiple attempts to detect binding of these ligands to rp2 and/or AcP via BIACore analyses have been unsuccessful. When rp2 or AcP or the combination of both receptors was immobilized on a BIACore chip, no binding was detected when F6, F8, and F9 were passed over the chip (data not shown). Binding of IL-1 to IL-1R+/− AcP and IL-18 to IL-1R+/− AcPL were detected by this technique (data not shown). The inability to detect binding of F6, F8, or F9 to rp2 and AcP requires further exploration.

**DISCUSSION**

This paper identifies IL-1Rrp2 as the receptor for the previously orphan ligands, F6 and F8. We also provide evidence that AcP is a coreceptor required for signaling by F6, F8, and F9. The three novel cytokines activate similar signaling molecules with a similar time course as IL-1 including the transcription factor, NF-κB, and the MAPKs JNK and ERK. F6, F8, and F9 and the receptor, rp2, are expressed in tissues such as skin and airway, where inflammatory mediators can serve as the first line of defense against pathogens. These studies identify F6, F8, and F9 as agonistic ligands capable of activating signal transduction pathways and secretion of cytokines similar to those induced by IL-1 and identify rp2 and AcP as the receptors for these ligands.

In the search for F6 and F8 receptors, Jurkat cells were transfected with each of the IL-1R family members. Transfection of rp2, but not other IL-1R homologs, conferred responsiveness to F6, F8, and F9 in Jurkat cells as well as multiple other human and mouse cell lines (Fig. 1). Through expression analysis, several cell lines were identified which responded to rp2. NCI/ADR-RES, a mammary epithelial cell line, was found to have the highest expression of rp2 mRNA of any cell line tested and was demonstrated to express significant levels of rp2 protein on the cell surface (Fig. 4A). F6, F8, and F9 activated the pathway leading to NF-κB in NCI/ADR-RES cells without further receptor transfection (Fig. 4B). This activation was found to be dependent upon rp2 because the addition of anti-rp2 antibodies blocked activation of the pathway leading to NF-κB in NCI/ADR-RES cells (Fig. 4C). The fact that all three cytokines appear to utilize the same receptor seems improbable at first glance. However, F6, F8, and F9 are more homologous at the amino acid level (between 45 and 57% homology) than are IL-1α and IL-1β (24% homology) (19, 40). Since IL-1α and IL-1β are known to bind to the same receptor and activate identical signaling pathways despite their low degree of similarity, it seems likely that the novel ligands could do the same. At present, the only differences observed between F6, F8, and F9 are expression differences. It is unclear whether differences in signaling or co-receptor usage among the ligands may emerge or whether the ligands will have redundant roles and any functional differences will largely be based on expression pattern.

Signaling through IL-1 begins with IL-1 binding directly to the IL-1R, which induces the formation of a higher affinity binding complex containing IL-1R and AcP. Several molecules are then recruited to the receptor complex including the adaptor molecule, MyD88 and IL-1R-associated kinases 1 and 4, which then associate with tumor necrosis factor receptor-associ- ated factor-6. This association leads to activation of NF-κB as well as the MAPKs JNK, ERK, and p38, resulting in activation of multiple transcription factors including AP-1 (41). Signaling through IL-18 occurs in much the same way and results in the activation of NF-κB, MAPKs, and AP-1 (2, 41). We reasoned that since F6, F8, and F9 signal through an IL-1R homolog, they may activate similar signaling pathways as do IL-1 and IL-18. We show here that F6, F8, and F9 activate the MAPKs
JNK and ERK in addition to activation of the pathway leading to NF-κB (Fig. 5, A and B). All three ligands activate JNK and ERK within 10 min of stimulation, which is a similar time course to that seen for IL-1. In addition, the time course for activation of the pathway leading to NF-κB by F6 and F8 in several cell types was virtually identical to that for activation by IL-1 (Fig. 2C). These results demonstrate that F6, F8, and F9 activate similar signaling pathways as IL-1 and IL-18. Furthermore, the kinetics of this activation suggests that they do so directly. If the novel cytokines were acting via another receptor molecule to induce a second cytokine that then binds to and signals through rp2, the kinetics of activation of the pathway leading to NF-κB should be slower than that of activation by IL-1, which signals through direct binding to the IL-1 receptor. Also, in the absence of direct signaling via the rp2 receptor, activation of MAPKs in 10 min would be unlikely. Therefore, it is probable that F6, F8, and F9 signal directly through rp2.

In addition to activation of signaling pathways similar to those activated by IL-1, IL-1F6, F8, and F9 activated similar downstream effects as well. IL-1, as well as the three IL-1F ligands activated an IL-8 promoter reporter in rp2-transfected Jurkat cells (Fig. 5C) and induced secretion of cytokines including IL-6 and IL-8 in NCI/ADR-RES cells (Fig. 5D). Thus, F6, F8, and F9 appear to signal in a similar fashion to IL-1 and induce many of the same downstream effects. Whether signaling by the novel cytokines through rp2 and AcP involves recruitment of the same adaptor molecules and upstream kinases as IL-1 and IL-18 seems likely but requires further study.

In the search for a second subunit for the F6, F8, and F9 receptor, we concluded that virtually every cell line successfully transfected with rp2 was capable of responding to all three ligands, suggesting that if a second IL-1R homolog is necessary for signaling through these ligands, it must be ubiquitously expressed. In fact, the only cell line to date in which F8 (and probably F6 and F9) was not able to activate the pathway leading to NF-κB upon rp2 transfection was the AcP null MEF line generated from AcP knockout mice (Fig. 6D). Antibodies generated against human AcP were able to block the F6, F8, and F9 response, providing further evidence that AcP is required for signaling by these ligands (Fig. 6C). An AcP variant lacking the cytoplasmic domain functioned as a dominant negative mutant and blocked activation of the pathway leading to NF-κB by IL-1 as well as activation by F6, F8, and F9 (Fig. 6D), providing strong evidence that AcP is required for signaling through the novel ligands in addition to IL-1. Based on chimeric receptor studies, only two of the IL-1R family members were classified as accessory receptors, AcP and AcPL, whereas multiple receptors were determined to be IL-1R-like (23). Through this classification, it would stand to reason that the two members in the accessory group may be involved in signaling through multiple IL-1R homologs. AcP is very widely expressed and is present in cells and tissues that express rp2 as well as IL-1R (2, 30). AcP increases the affinity of IL-1 for the IL-1R but does not bind the ligand itself and is essential for the recruitment of key signaling molecules to the receptor complex (39). Perhaps AcP interacts with rp2 in much the same way as it does with the IL-1R and thus may function to increase the affinity of ligand binding and in the recruitment of signaling molecules.

Although the evidence is compelling that the receptor for F6, F8, and F9 requires rp2 and AcP, questions remain. The dose of the three ligands required for a response is much higher than is typically seen for cytokines and is much higher than that required for IL-1 or IL-18 responses. In addition, we have been unable to detect binding of F6, F8, or F9 to rp2 with or without AcP present. The basis for these findings is unknown. It is possible that production of the ligands in E. coli as was done here generates preparations that are only partially active. We have generated mammalian produced F6, F8, and F9 in COS-7 cells, and these ligands are active, but the same high concentrations are required as for the E. coli-produced ligands. However, the mammalian produced ligands have a C-terminal tag for purification, which may decrease their activity. F6, F8, and F9 lack both a classical leader sequence and a distinct prodomain (such as found in IL-1, IL-18, and perhaps IL-1F7) (40, 42). Therefore, it is not known how the cytokines are released from cells and whether F6, F8, and F9 can be found on the surface of cells. Perhaps cells endogenously expressing F6, F8, and F9 are able to present the ligands to responding cells or perhaps only cells that naturally produce these ligands are capable of secreting fully active molecules. There is also the possibility that some additional molecule on the responding cell, either a receptor component or possibly a cofactor, is necessary to generate a high affinity binding receptor but is missing in our experiments.

In their initial report of signaling by IL-1F9 through IL-1Rrp2, Debets et al. (1) also provided data suggesting that IL-1F5 can antagonize this response. In our hands, IL-1F5 provides inconsistent and only incomplete antagonism of signaling by IL-1F6, IL-1F8, and IL-1F9. The reason for this discrepancy is unknown.

IL-1Rrp2 is expressed at low levels in many human tissues and in much higher levels in human skin. Human F8, F9, and to a lesser extent F6, are all expressed in skin. Debets et al. (1) reported that F9 and rp2 were both increased in human lesional psoriasis skin as compared with normal healthy skin. We have preliminary evidence that F6 and F8 expression is increased in psoriatic skin (data not shown). These data suggest that F6, F8, and F9 may play a role in the pathogenesis of psoriasis. In addition, F6 was previously demonstrated to be up-regulated in a mouse contact hypersensitivity model and following infection with Herpes simplex virus (42).

Expression analysis provides other hints as to the potential role of this ligand/receptor system in disease. Epithelial tissues such as trachea and esophagus express rp2 and high levels of all three ligands, suggesting F6, F8, and F9 may play a role in defense against pathogens (Fig. 3 and data not shown). In addition, epithelial cells in the lung (small airway epithelial cells) express rp2, and normal bronchial epithelial cells express all three ligands and rp2 (data not shown). Perhaps these ligands play a role in inflammatory disease in the lung. There is evidence that expression of both the ligands and the receptor are regulated in that F6, F8, and F9 expression is increased in monocytes following treatment with lipopolysaccharide, whereas monocytes basally express high levels of rp2 and lipopolysaccharide treatment actually decreases this expression (data not shown). However, rp2 expression is up-regulated in multiple cell types in response to phorbol 12-myristate 13-acetate (data not shown). Further studies are necessary to assess the conditions in which both the ligands and receptor are regulated and at what level. The biological activity of the novel ligands remains to be characterized and will probably require mouse models of disease and the generation of gene knockout or overexpression mice. However, it is now known that F6, F8, and F9 are all capable of activating responses that enhance immune responses and promote inflammation. This new ligand receptor system probably plays a role in the immune system and in the pathogenesis of inflammatory diseases.

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REFERENCES
1. Debré, R., Timans, J. C., Homey, B., Zurawski, S., Sana, T. R., Lo, S., Wagner, J., Edwards, G., Clifford, T., Menon, S., Bazar, J. F., and Kaestle, R. A. (2001) J. Immunol. 167, 1440–1446
2. Dunn, A., and O'Neill, L. A. (2003) Science’s STKE 171, re3
3. Sims, J. E. (2002) Curr. Opin. Immunol. 14, 117–122
4. Dinarello, C. A. (1998) Int. Rev. Immunol. 16, 457–499
5. Micallef, M. J., Ohtsuki, T., Kohno, K., Tanabe, F., Ushio, S., Namba, M., Tanimoto, H., Akira, S., and Nakatsuji, K. (1998) J. Immunol. 161, 3400–3407
6. Yoshimoto, T., Takada, K., Tanaka, T., Okusu, K., Kashivamura, S., Okamura, H., Akira, S., and Nakatsuji, K. (1998) J. Immunol. 161, 3467–3477
7. Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., Akita, K., Motoshi, N., Tanabe, F., Komishi, K., Fukuda, S., and Kurimoto, M. (1995) Nature 378, 88–91
8. Born, T. L., Tommassen, E., Bird, T. A., and Sims, J. E. (1998) J. Biol. Chem. 273, 29445–29450
9. Novick, D., Kim, S. H., Fantuzzi, G., Reznikov, L. L., Dinarello, C. A., and Kim, S. H. (2002) J. Immunol. 168, 13723–13728
10. Dolganov, G. M., Schnur, S., Tagari, S. S., Taguchi, H., Fujii, M., Ohta, T., Ikeda, M., Murakami, T., Sanou, O., Kojima, H., Fujii, M., Ohta, T., Ikeda, M., Ikeyami, H., and Kurimoto, M. (1997) J. Biol. Chem. 272, 25737–25742
11. Parey, P., Garka, K. E., Bonnert, T. P., Dower, S. K., and Sims, J. E. (1996) J. Biol. Chem. 271, 23877–23879
12. Li, H., Tago, K., Ito, K., Kuriwa, K., Arai, T., Iwahana, H., Tomiagina, S., and Yanagisawa, K. (2000) Genes Dev. 14, 284–290
13. Lechner, T. W., Calmes, D. T., Liu, X., Li, X., Clevenger, W., Oltersdorf, T., Do Souza, E. B., and Maki, R. A. (1996) J. Neurinmunol. 70, 113–122
14. Maksymowych, W. P., Beckmann, M. J., March, C. J., Izerdra, R. L., Gimpel, S. D., VandenBus, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J. M., Smith, C., Gallis, B., Sims, J. E., Urda, D., Widmer, M. B., Cosman, D., and Park, L. S. (1989) Cell 59, 335–348
15. Gayle, M. A., Slavik, J. L., Bonkert, T. P., Renshaw, B. R., Sonoda, G., Taguchi, T., Tuesta, J. R., Dower, S. K., and Sims, J. E. (1996) J. Biol. Chem. 271, 5784–5788
16. Smith, K. E., Bello, B. E., Kim, D. W., Zaneski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. (1997) J. Clin. Invest. 100, 2952–2960
17. Nakahara, H., Hase-Dakahara, Y., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. (1997) Mol. Cell Biol. 17, 3629–3639
18. Radic, I., Dam, A., Van den Berghe, J., Cardona, A., Van Buggenhout, G., Frints, S., Kamradt, T., and VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J. M., Smith, C., Gallis, B., Sims, J. E., Urda, D., Widmer, M. B., Cosman, D., and Park, L. S. (1989) Cell 59, 335–348.
Interleukin (IL)-1F6, IL-1F8, and IL-1F9 Signal through IL-1Rrp2 and IL-1RAcP to Activate the Pathway Leading to NF-κB and MAPKs

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