Pharmacologic Inhibition of Tpl2 Blocks Inflammatory Responses in Primary Human Monocytes, Synoviocytes, and Blood*

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Tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine that controls the initiation and progression of inflammatory diseases such as rheumatoid arthritis. Tpl2 is a MAP3K in the MAPK (i.e. ERK) pathway, and the Tpl2-MEK-ERK signaling pathway is activated by the pro-inflammatory mediators TNFα, interleukin (IL)-1β, and bacterial endotoxin (lipopolysaccharide (LPS)). Moreover, Tpl2 is required for TNFα expression. Thus, pharmacologic inhibition of Tpl2 should be a valid approach to therapeutic intervention in the pathogenesis of rheumatoid arthritis and other inflammatory diseases in humans. We have developed a series of highly selective and potent Tpl2 inhibitors, and in the present study we have used these inhibitors to demonstrate that the catalytic activity of Tpl2 is required for the LPS-induced activation of MEK and ERK in primary human monocytes. These inhibitors selectively target Tpl2 in these cells, and they block LPS- and IL-1β-induced TNFα production in both primary human monocytes and human blood. In rheumatoid arthritis fibroblast-like synoviocytes these inhibitors block ERK activation, cyclooxygenase-2 expression, and the production of IL-6, IL-8, and prostaglandin E2, and the matrix metalloproteinases MMP-1 and MMP-3. Taken together, our results show that inhibition of Tpl2 in primary human cell types can decrease the production of TNFα and other pro-inflammatory mediators during inflammatory events, and they further support the notion that Tpl2 is an appropriate therapeutic target for rheumatoid arthritis and other human inflammatory diseases.

Tpl2 is a member of the MAP3K family of serine/threonine kinases. It resides upstream of the MAP3Ks MEK1 and MEK2, and, in turn, the MAP3Ks ERK1 and ERK2. This Tpl2-MEK-ERK signaling module is activated in response to various pro-inflammatory stimuli, and it regulates the expression of several pro-inflammatory mediators (1–8). Notably, Tpl2 regulates the expression of TNFα protein, which is critical to the initiation and progression of many inflammatory disorders including rheumatoid arthritis (9–13). Rheumatoid arthritis occurs in nearly 1% of the adult population in most western countries, with an annual incidence of ~0.04%, and it can develop into a chronic, debilitating condition characterized by joint pain/swelling/stiffness, restricted mobility, and the erosion of bone and cartilage in affected joints (14). Protein therapeutics such as ENBREL® (etanercept) (soluble TNFRII-Fc), and REMICADE® (infliximab) and HUMIRA® (adalimumab) (anti-tumor necrosis factor α antibodies) (TNFα)3 that bind and neutralize TNFα have proven clinically efficacious, and they have provided tremendous medical benefit. The success of these drugs has validated the use of anti-TNFα therapies for treating arthritic and inflammatory diseases, and they have necessitated the search for small molecule inhibitors with similar or related mechanisms of action. Due to its physiological roles in cytokine signaling networks and its key role in the production of TNFα, a highly selective, small molecule inhibitor of Tpl2 should constitute an effective therapy for TNFα-driven inflammatory disorders such as rheumatoid arthritis.

Genetic studies with tpl2−/− mice have shown that Tpl2 is required for the expression of TNFα in circulating plasma following the administration of LPS in vivo, and cultured macrophages from tpl2−/− mice exhibited markedly reduced TNFα production following LPS stimulation (2, 8). In addition, LPS-stimulated tpl2−/− macrophages exhibited a restricted defect in ERK activation, with signaling through the JNK and p38 MAPK pathways largely unaffected (2, 8). Tpl2-deficient mice

3 The abbreviations used are: TNF, tumor necrosis factor; CaMKII, calcium-calmodulin-dependent protein kinase II; CMH, cytokemoglobin oxidase; COX, cyclooxygenase; DFLIA, dissociation-enhanced lanthanide fluoroimmunoassay; DDEM, Dulbecco’s modified Eagle’s media; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GST, glutathione-S-transferase; IC50, drug concentration required for 50% inhibition; IκBα, inhibitor of NF-κB; IKK, inhibitor of NF-κB kinase; IL-1, interleukin; JNK, c-Jun N-terminal kinase; KC, cytokine-induced neutrophil chemoattractant; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP, monocyte chemotactic protein; MEK, mitogen-activated protein kinase/ERK kinase; MK2, MAPK-activated protein kinase-2; MKK, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PG, prostaglandin; PGE2, prostaglandin E2; PKA, protein kinase A; PKC, protein kinase C; RA-FLS, rheumatoid arthritis fibroblast-like synoviocytes; RANTES, regulated upon activation, normal T cell expressed, and secreted; rh, recombinant human; rm, recombinant murine; TEPM, thiglycollate elicited peritoneal macrophages; TNFR, tumor necrosis factor receptor; S6K, p70 ribosomal S6 kinase; LDS, lithium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid.
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exhibited a strongly attenuated progression of disease in a model of TNFα-dependent inflammatory bowel disease, and TNFα-induced ERK activation was completely ablated in tpl2−/− macrophages (1, 4, 5). These data implicate the Tpl2-MEK-ERK module as a fundamental signaling component in cells of the innate immune system. However, Tpl2 signaling also regulates adaptive immunity. MEK/ERK activation in response to CD40 stimulation is ablated in tpl2−/− B cells, and a study of tpl2−/− dendritic cells demonstrated that Tpl2 is a negative regulator of IL-12 production in that cell type (4, 8). Dendritic cell IL-12 is known to induce Th1-type T cell differentiation, and Th1-skewed immune responses in tpl2−/− mice were observed following ovalbumin immunization or Leishmania major infection in vivo.

Although the foregoing studies indicate that Tpl2 is an important regulator of both innate and adaptive immunity, all of those studies were performed with Tpl2-deficient mice and cells derived from those mice. Currently, there are almost no reports of the targeted down-regulation of Tpl2 in human cell types (for one such report, see Ref. 6). These studies are necessary to further validate Tpl2 as a therapeutic target for human inflammatory disorders.

We have used highly selective and potent small molecule inhibitors of Tpl2 activity to validate several functions of Tpl2 in human cells. First, we demonstrate that Tpl2 is required for the LPS-induced activation of MEK and ERK in primary human monocytes. Second, we show that inhibition of Tpl2 blocks LPS- and IL-1β-induced TNFα production in primary human monocytes and human blood. Third, our Tpl2 inhibitors block ERK activation, COX-2 expression, and the production of various soluble pro-inflammatory mediators in RA-FLS. Taken together, our data confirm physiological roles for Tpl2 in human inflammatory cell types, and they provide essential insights into the value of Tpl2 as a target for therapeutic intervention in human inflammatory diseases such as rheumatoid arthritis.

**EXPERIMENTAL PROCEDURES**

**Kinase Assays**—Scintiplate assays were used for human PKA (Panvera, Madison, WI), human PKCα (Panvera), human S6K (Millipore, Billerica, MA), human JNK1 (Millipore), and human MK2 (amino acids 41–400) (produced in-house). Reactions were performed at room temperature in streptavidin-coated Scintiplates (PerkinElmer Life Sciences). Reactions were performed at room temperature in streptavidin-coated plates (Sigma), or MaxiSorp plates (Nalge Nunc International, Rochester, NY) coated with anti-GST (GE Healthcare) or with poly(Glu4-Tyr) (Sigma) for the EGFR assay. 100-µl reactions contained Tpl2 inhibitor or vehicle in 20 mM HEPES (pH 7.5), 10 mM MgCl2, 5 mM MnCl2 (EGFR only), 100 µM Na3VO4 (EGFR only), 4.5 mM dithiothreitol, 0.01% Triton X-100, 3 µM ATP (10 µM ATP for EGFR), plus substrate. Final Me3SO concentrations were 2%. For Tpl2 and MEK1, 100-µl reactions contained Tpl2 inhibitor or vehicle in 20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na3VO4, 20 mM MgCl2, 1 mM dithiothreitol, 50 µM ATP, plus substrate. Tpl2 and MEK1 reactions were performed in 96-well polystyrene plates pre-blocked in 10 mM MOPS (pH 7.5), 150 mM NaCl, 0.05% Tween 20, 0.1% gelatin, 0.02% NaN3, 1% bovine serum albumin. Final Me3SO concentrations in the Tpl2 and MEK1 assays were 1%. All reactions were terminated with 50 µl of 0.5 M EDTA. Terminated Tpl2 and MEK1 assays were transferred into anti-GST-coated plates and allowed to bind for 1 h. All assays were washed 6 times with phosphate-buffered saline, 0.05% Tween 20 before a 60-min incubation with detection antibodies in 10 mM MOPS (pH 7.5), 150 mM NaCl, 0.05% Tween 20, 0.1% gelatin, 0.02% NaN3, 1% bovine serum albumin. Then, all assays were washed before a 10-min incubation with 100 µl of DELFIA Enhancement Solution (PerkinElmer). Plates were analyzed on a Wallac Victor II plate reader (PerkinElmer). Substrates used were: 35 nM unactive GST-MEK (Millipore) for Tpl2; 30 nM GST-ERK2 (produced in-house) for MEK1; 6200 nM Biotin-KKEGPWLEEEEEAYGWMDF for Src; 200 nM Biotin-QSTKVPQTPLHTSRVL for p38; 100 nM Biotin-RTKLRQASIELPSM for CAMKII and IKKβ; 30 nM GST-p38 for MK6; and plate-bound poly(Glu4-Tyr) (see above) for EGFR. Enzyme concentrations were: Tpl2, 0.4 nM; MEK1, 0.4 nM; Src, 1.2 units/ml; p38, 5 nM; CaMKII, 2.5 ng/ml; IKKβ, 250 ng/ml; MK6, 330 pm; EGFR, 500 ng/ml. Detection antibodies were as follows: Tpl2 assay, anti-phospho-MEK (Cell Signaling Technology, Beverly, MA) at 1:1000 and DELFIA Eu-N1 goat anti-rabbit IgG (PerkinElmer) at 1:4000; MEK1 assay, anti-phospho-ERK (Sigma, M-8159) at 1:1000 and DELFIA Eu-N1 rabbit anti-mouse IgG (PerkinElmer) at 1:2000; p38 assay, anti-phospho-MK2 (produced in-house) at 1:4000 and DELFIA Eu-N1 goat anti-rabbit IgG (PerkinElmer) at 1:2000; Src assay, Tyr(P)-100 (Cell Signaling Technology) at 1:1000 and DELFIA Eu-N1 rabbit anti-phospho-IgG (PerkinElmer) at 1:1000; CaMKII and IKKβ assays, anti-phospho-LSP (produced in-house) at 1:10,000 and DELFIA Eu-N1 goat anti-rabbit IgG at 1:5000 (PerkinElmer); MK6 assay, phosphothreonine antibody (Cell Signaling Technology) at 1:1000 and DELFIA Eu-N1 goat anti-
rabbit IgG (PerkinElmer) at 1:4000; EGFR assay, Eu-anti-PY (PT66) (PerkinElmer) at 1:2000.

For human Raf-1 (Millipore), direct homogeneous time-resolved fluorescence with unactive MEK protein (Millipore) as substrate was done essentially as described previously (15). 50-μl reaction mixtures containedTpl2 inhibitor or vehicle, 2.5 nM Raf-1, 10 nM GST-MEK1, and 0.1 mM ATP in 20 mM HEPES (pH 7.5), 5 mM MgCl₂, 0.0025% Brij-35, 0.05% bovine serum albumin. Reaction time was 60 min, and reactions were terminated with 5 μl of 0.1 M EDTA. Final Me₂SO concentrations were 1%. Detection solution contained 2 nM anti-phospho-MEK-Eu (antibody from Cell Signaling Technology; Eu from PerkinElmer) and 12.5 nM anti-GST-XL (Cisbio, Bedford, MA) per well, and developing times were 60 min at room temperature. Plates were read in the Wallac Victor II plate reader.

**Immunoblot Antibodies**—All immunoblot antibodies were from Cell Signaling Technology, except for anti-Cot/Tpl2 and anti-COX-2 from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary/detection antibodies were from GE Healthcare.

**HeLa Assays**—HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and passaged in DMEM supplemented with 10% FBS (Sigma). For transfection assays (Figs. 1 and 2), human TPL2 and TPL2-K167R cDNAs (16) were subcloned into a replication-defective Type 5 adenoviral vector (“pAdori,” Genetics Institute, Andover, MA) (17) under the control of the CMV promoter and all constructs were confirmed by sequencing. Cells were transfected for ~4 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions, and were then washed with DMEM and allowed to incubate in DMEM supplemented with 0.5% FBS, or, for inhibitor studies, in DMEM, 0.5% FBS containing Tpl2 inhibitors or vehicle (Me₂SO). Final Me₂SO concentrations were 0.25%. In some experiments HeLa transfectants were trypsinized and re-plated before the addition of compounds. 16–24 h later media were harvested and IL-8 levels were measured by ELISA (BioSource International, Camarillo, CA). Compound toxicity was assessed using the WST-1 Cell Proliferation Reagent as described by the manufacturer (Roche Applied Science). Cells were lysed with the addition of 1× LDS sample buffer (Invitrogen), briefly sonicated and boiled, and analyzed by standard immunoblot analysis. Immunoreactive band intensities were quantitated on an Image Station 2000MM (Eastman Kodak Co.).

For inhibitor studies with TNFα-stimulated, non-transfected HeLa cells (Fig. 7), the cells were plated in DMEM, 0.5% FBS 1 day prior to testing. Inhibitors were added 30–45 min before stimulation with 100 ng/ml rhTNFα (R&D Systems). Cell lysates were then prepared in 1× LDS buffer and immunoblotted. Media aliquots from 4-h stimulations were analyzed for IL-8 by electrochemiluminescence detection on a Sector6000 plate reader according to the manufacturer’s instructions (Meso Scale Discovery, Gaithersburg, MD). Toxicity was assessed using the WST-1 Reagent.

**Primary Human Monocytes**—Human blood buffy coats were purchased from the Blood Transfusion Service at Massachusetts General Hospital (Boston, MA). Monocytes were prepared by negative selection. For each preparation, EDTA was added to theuffy coat to a final concentration of 1 mM, and the

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**FIGURE 1.** The catalytic activity of overexpressed Tpl2 is required for TPL2-dependent IL-8 production. HeLa cells were transfected with human TPL2 or TPL2-K167R expression plasmids, or vector alone. 22 h later, MEK and ERK phosphorylation and total Tpl2 protein (B) were measured as described under “Experimental Procedures.” No cytotoxicity was observed under these conditions. Results in A and B are representative of at least three independent experiments. Arrowheads indicate the phosphorylated and unphosphorylated forms of ERK1 and ERK2 (i.e. p44 and p42).

The buffering was incubated with the RosetteSep Monocyte Enrichment antibody mixture from Stem Cell Technologies (Vancouver, Canada) for 20 min (6 ml of mixture per 50 ml of
lysates were prepared in 3–4 h with LPS or rhIL-1 as described previously (18). Blood samples were stimulated for 30–45 min. Final Me2SO concentrations were 0.25%.

Cytokine determinations, monocytes were stimulated for 3–4 h and media aliquots were analyzed by electrochemiluminescence detection. Toxicity was assessed using the WST-1 Reagent.

Peritoneal Macrophages—All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Mice were provided food and water ad libitum. tpl2−/− mice (backcrossed onto C57BL/6 for >10 generations) were obtained from Dr. Philip Tsichlis (Thomas Jefferson University). These mice were crossed with 129S5 mice to produce F1 heterozygotes. The F1 mice were inter-crossed to produce F2s. Female TPL2+/+, TPL2+/−, and TPL2−/− F2 littermates (8–12 weeks of age) were injected intra-peritoneally with 1 ml of sterile 4% Brewer thiglycollate broth (BD Biosciences), and 72 h later peritoneal exudate cells were isolated by lavage with RPMI 1640. The cells were washed and plated in RPMI 1640, 5% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin at ~3 × 106 cells per 35-mm dish. After ~5 h, non-adherent cells were removed by repeated washing, and the adherent macrophages were subjected to various treatments. Total macrophage protein levels were quantitated by Bradford assay (Bio-Rad). S. typhimurium LPS was from Sigma, and rmIL-1β and rmTNFα were from BioSource International. Cell lysates were prepared in 1× LDS buffer and immunoblotted above. cytokine levels in media supernatants were measured by electrochemiluminescence detection.

RESULTS

A Chemical Series of SelectiveTpl2 Inhibitors—We have developed a series of compounds that inhibit Tpl2, and the synthesis and structure-activity relationships of the early prototypes in this series have been described (18, 19). Subsequent, more selective, and more potent entries in this series are described herein and elsewhere (20). Compounds 1, 2, and 3 in this article (structures shown in Figs. 2A and 3A) are representative of our chemical series of Tpl2 inhibitors inasmuch as they are highly selective for Tpl2 inhibition when tested against a panel of 13 kinases. This panel includes kinases known to be sensitive of our chemical series of Tpl2 inhibitors inasmuch as they are highly selective for Tpl2 inhibition when tested against a panel of 13 kinases. This panel includes kinases known to be sensitive to Tpl2 inhibition when tested against a panel of 13 kinases. This panel includes kinases known to be sensitive to Tpl2 inhibition when tested against a panel of 13 kinases.
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Figure 3. 

The high degree of selectivity of our compounds forTpl2 inhibition (see Table 1) was also observed in LPS-treated primary human monocytes. As shown in Fig. 4A, Compound 1 potently inhibited LPS-induced MEK and ERK phosphorylation in human monocytes with IC_{50} values of 0.5 μM for LPS-induced MEK phosphorylation and 0.1 μM for ERK phosphorylation. However, Compound 1 did not inhibit phosphorylation of p38, MK2, c-Jun, or c-Jun kinase (JNK) at concentrations as high as 5 μM. These data demonstrate that Compound 1 selectively inhibitsTpl2 in LPS-stimulated human monocytes, and they are consistent with the restricted defect in ERK activation in LPS-stimulated peritoneal macrophages fromTpl2 knockout (KO) mice (2, 8). The failure to block NF-κB signaling in monocytes was corroborated with assays that employed an NF-κB-luciferase reporter plasmid stably integrated into HEK293 cells: Compound 1 did not inhibit TNF-α or IL-1β-induced luciferase activity in those cells at concentrations as high as 10 μM (data not shown).

Another MAPKKK known to activate MEK is the kinase Raf (see Ref. 22, and references therein). To rule out the inhibition of Raf by our compounds in monocytes, we preincubated monocytes with increasing concentrations of Compound 1 and then stimulated with PMA, which selectively activates the Raf-MEK-ERK cascade. As shown in Fig. 4B, Compound 1 did not inhibit PMA-induced MEK and ERK phosphorylation. These data indicate that Compound 1 does not inhibit Raf activity in primary human monocytes. This is consistent with the

Table 1: Kinase selectivity of Compound 1 (IC_{50} μM)

| Kinase | IC_{50} μM |
|--------|-----------|
|Tpl2    | 0.012     |
|MEK1    | >40       |
p38α    | >100      |
|Src     | >100      |
|CaMKII  | 23        |
|PKA     | >100      |
|PCKα    | >100      |
|MK2     | 48        |
|S6K     | >100      |
|JNKα    | 65        |
|MKK6    | >100      |
|IKKβ    | 75        |
|EGFR    | >40       |
|Raf-1   | >100      |

that are induced byTpl2 kinase activity. Indeed, Compound 1 inhibited IL-8 production in a dose-dependent manner with an IC_{50} of 1 μM (Fig. 2B). Compound 1 also inhibited MEK and ERK phosphorylation in these cells (data not shown).

Tpl2 Catalytic Activity Is Required for the LPS-induced Activation of MEK and ERK in Primary Human Monocytes—Next, we screened ourTpl2 inhibitors by measuring their inhibition of LPS-induced MEK phosphorylation in freshly isolated primary human monocytes. To ensure that our compounds were inhibitingTpl2 in these cells, we sought a correlation between potency in the cell-freeTpl2 enzyme assay and potency in the monocyte phospho-MEK assay. Of 140 compounds with cell-freeTpl2 IC_{50} values ≤0.2 μM, 111 (79%) had monocyte phospho-MEK IC_{50} values ≤5 μM, and 81 (58%) had monocyte phospho-MEK IC_{50} values ≤1 μM. Of 17 compounds tested that had cell-freeTpl2 IC_{50} values >0.2 μM, all were not active for inhibition of monocyte phospho-MEK. This high positive correlation of potency in the cell-freeTpl2 assay with activity in the monocyte phospho-MEK assay was further demonstrated by the experiment in Fig. 3 with the (1,7)-naphthyridine-3-carbonitrile enantiomers, named here as Compounds 2 and 3. Compound 2 poorly inhibitedTpl2 in the cell-free enzyme assay with an IC_{50} of 36 μM, and when tested in LPS-treated monocytes it exhibited no inhibition of phospho-MEK and phospho-ERK. However, Compound 3 potently inhibitedTpl2 in the cell-free enzyme assay with an IC_{50} of 16 nM, and it potently inhibited phospho-MEK and phospho-ERK in LPS-treated monocytes with IC_{50} values of ≤1 μM. Taken together, these data indicate thatTpl2 is the physiological activator of MEK and ERK in LPS-stimulated primary human monocytes.

**Selective Inhibition ofTpl2 in Primary Human Monocytes by Compound 1**—The high degree of selectivity of our compounds forTpl2 inhibition (see Table 1) was also observed in LPS-stimulated primary human monocytes. As shown in Fig. 4A, Compound 1 potently inhibited LPS-induced MEK and ERK phosphorylation in human monocytes with IC_{50} values of 0.5 μM for LPS-induced MEK phosphorylation and 0.1 μM for ERK phosphorylation. However, Compound 1 did not inhibit phosphorylation of p38, MK2, c-Jun, or Erk at concentrations as high as 5 μM. These data demonstrate that Compound 1 selectively inhibitsTpl2 in LPS-stimulated primary human monocytes, and they are consistent with the restricted defect in ERK activation in LPS-stimulated peritoneal macrophages fromTpl2 knockout (KO) mice (2, 8). The failure to block NF-κB signaling in monocytes was corroborated with assays that employed an NF-κB-luciferase reporter plasmid stably integrated into HEK293 cells: Compound 1 did not inhibit TNF-α or IL-1β-induced luciferase activity in those cells at concentrations as high as 10 μM (data not shown).


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A

|       | LPS          | Compound 1 (µM) |
|-------|--------------|-----------------|
|       |              | P-MEK          |
| −     |              | P-ERK          |
| −     | 2            | 0.4            |
|       |              | P-p38          |
|       |              | P-MK2          |
|       |              | P-cJun         |
|       |              | P-IxBα         |
|       |              | Tpl2           |

B

|       | Compound 1 (µM) |
|-------|-----------------|
| −     | 2              |
| +     | 0.4            |
|       | 0.08           |
|       | PMA            |
|       | P-MEK          |
|       | P-ERK          |

C

|       | Compound 1 (µM) |
|-------|-----------------|
| −     | 5              |
| +     | 0.5            |
|       | 0.05           |
|       | IL-1β          |
|       | P-MEK          |
|       | P-ERK          |

**Figure 4.** Compound 1 selectively targets Tpl2 signaling in primary human monocytes. A, immunoblot analysis of monocytes treated with Compound 1 at the indicated concentrations, or vehicle, and then stimulated with 10 ng/ml LPS for 30 (P-MEK, P-ERK, P-p38, P-MK2, P-IxBα) or 60 min (P-cJun). B, immunoblot analysis of monocytes treated with Compound 1 at the indicated concentrations, or vehicle, and then stimulated with 100 ng/ml PMA for 5 min. C, immunoblot analysis of monocytes treated with Compound 1 at the indicated concentrations, or vehicle, and then stimulated with 100 ng/ml PMA for 5 min. In A–C, Compound 1 was not cytotoxic. Results are representative of at least three independent experiments. Asterisk, nonspecific band. Arrowheads indicate the phosphorylated forms of ERK1/2, and the long and short forms of Tpl2.

The fact that Compound 1 did not inhibit Raf-1 in the cell-free assay (Table 1).

After establishing that Compound 1 is selective for Tpl2 inhibition in monocytes, we tested its inhibition of LPS-induced TNFα production. Compound 1 potently inhibited monocyte LPS-induced TNFα with an IC₅₀ of 0.6 µM (Table 2), which is consistent with the dramatic reduction in LPS-induced TNFα production in tpl2−/− mouse macrophages (2, 8). To exclude the possibility that reductions in LPS-induced TNFα were caused by prolonged exposure of the cells to inhibitors with cytotoxic properties, we routinely measured cell viability at the conclusion of the assay by adding the tetrazolium salt WST-1 (a 5-tetrazolino-1,3-benzene disulfonate; Roche Diagnostics) to the culture media, and then measuring spectrophotometrically its rapid cleavage to formazan by mitochondrial dehydrogenases, the activity of which is directly related to the number of viable cells in the culture. A decrease in the amount of formazan dye produced relative to control cultures was interpreted as evidence of a toxic result. In 35 of 41 tests of primary monocytes, 5 µM Compound 1 caused either no cytotoxic effects or less than a 50% reduction in the appearance of the formazan dye (relative to controls). In the remaining 6 tests, 50% cytotoxicity was observed at 4.3 ± 0.14 µM (mean ± S.E.). These data indicate that for almost all human monocyte preparations tested, cytotoxic effects of Compound 1 only manifested at a concentration that was at least 1 order of magnitude higher than the IC₅₀ for LPS-induced TNFα (≈0.6 µM), and that, therefore, its potency for TNFα inhibition in these cells does not result from cytotoxic effects.

Given that LPS-induced IL-12p40 is not decreased in tpl2−/− mouse macrophages (8), we measured IL-12p40 levels in culture supernatants from LPS-treated primary human monocytes that had been pre-treated with Compound 1. Compound 1 inhibited LPS-induced IL-12p40 in primary monocytes at least 10-fold less potently than it inhibited LPS-induced TNFα (Table 2). We conclude that the pharmacologic inhibition of Tpl2 is sufficient for both a dramatic and selective reduction in LPS-induced TNFα production in primary human monocytes.

**Table 2.** Inhibition of LPS- and IL-1β-stimulated TNFα and IL-12p40 production by Compound 1 (IC₅₀ µM)

| Monocytes | Stimulus Output |
|-----------|-----------------|
| TNFα      | LPS IL-1β       |
| IC₅₀      | 0.6 ± 0.1       |
| IL-12p40  | LPS IL-1β       |
| IC₅₀      | 0.7 ± 0.1       |

| Blood     | Stimulus Output |
|-----------|-----------------|
| TNFα      | LPS IL-1β       |
| IC₅₀      | 5.4 ± 0.7       |
| IL-12p40  | LPS IL-1β       |
| IC₅₀      | 7.2 ± 2.3       |

* IC₅₀ values: mean ± S.E. from at least three independent experiments/donors.

**Asterisk,** nonspecific band. **Arrowheads** indicate the phosphorylated forms of ERK1/2, and the long and short forms of Tpl2.

**Inhibition of Tpl2 Blocks IL-1β-induced MEK/ERK Activation and TNFα Production**—During the course of this work, several reports appeared that described the activation of Tpl2-MEK-ERK by the pro-inflammatory cytokine IL-1β (1, 6, 7). Therefore, we tested whether Compound 1 would inhibit IL-1β-stimulated Tpl2 in monocytes. Compound 1 potently inhibited IL-1β-induced MEK and ERK phosphorylation in these cells, with IC₅₀ values of 0.5 µM for IL-1β-induced MEK phosphorylation and 0.3 µM for IL-1β-induced ERK phosphorylation (Fig. 4C). Compound 1 also inhibited IL-1β-induced TNFα production in human monocytes, with an IC₅₀ of 0.7 µM (Table 2).

Rodriguez et al. (6) used Tpl2-specific small interfering RNA to knock down endogenous Tpl2 in HeLa cells. Using this technique these authors demonstrated that Tpl2 is required for IL-1β-induced ERK activation in this cell type. Our results showing that the pharmacologic inhibition of Tpl2 in human monocytes blocks IL-1β-induced ERK activation and TNFα production prompted us to use a genetic approach to confirm that Tpl2 mediates IL-1β-induced ERK activation and TNFα production in cells from the monocyte-macrophage
compartment. TEPM from C57BL/6 mice are refractory to IL-1β stimulation. However, TEPM from 129S5 mice do respond to IL-1β, and in these cells IL-1β induces ERK phosphorylation in a dose-dependent manner. To test whetherTpl2 mediates ERK activation and TNFα production in TEPM following IL-1β stimulation, we crossed the original tpl2−/− mice, which are on a C57BL/6 background (2), with 129S5 mice, and then intercrossed the heterozygous F1 progeny to produce F2s. These F2s were used to generate the TEPM in Fig. 5. As shown in Fig. 5A, LPS, IL-1β, and TNFα all induced ERK phosphorylation in the TPL2+/+ and TPL2+/− TEPM. However, none of these stimuli caused ERK phosphorylation in TEPM from tpl2−/− F2 animals. Similar results were observed for MEK phosphorylation (data not shown). As expected, LPS caused an enormous induction of TNFα production in the TPL2+/+ TEPM (≈400-fold), and this effect was curtailed in the tpl2−/− TEPM (Fig. 5B). Intermediate reductions in LPS-induced TNFα production were observed with the TPL2+/− TEPM. Importantly, IL-1β caused a 50-fold increase in TNFα production in the TPL2+/+ and TPL2+/− TEPM, and this increase was almost completely ablated in tpl2−/− TEPM. Taken together, these results prove thatTpl2 is required for IL-1β-induced MEK/ERK activation and TNFα production in mouse macrophages, and they are consistent with the pharmacologic inhibition of IL-1β-induced MEK/ERK activation (Fig. 4C) and TNFα production (Table 2) in human monocytes by our Tpl2 inhibitors. In TPL2+/+ TEPM, IL-1β induced the production of IL-6, IL-12p40, KC, and MCP-1; LPS induced the production of IL-6, RANTES, IL-12p40, KC, and MCP-1; and TNFα stimulated the production of IL-6, IL-12p40, KC, and MCP-1. Aside from slight reductions in LPS- and IL-1β-induced KC, these inductions were unchanged in the tpl2−/− TEPM (Fig. 5C).

Inhibition of LPS- and IL-1β-induced TNFα Production in Human Blood by Compound 1—Although circulating blood cells are not precisely the same as the cells found in arthritic joints, it is reasonable to assume that circulating immune cells do migrate to arthritic joints and participate in the disease process. Therefore, as one predictor of the in vivo efficacies of potential anti-arthritic drugs, we tested our Tpl2 inhibitors for inhibition of TNFα production in an in vitro human blood TNFα assay. Consist-
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A

|       | 0.5 | 0.05 | Compound 1 (μM) |
|-------|-----|------|-----------------|
| IL-1β|     |      | IL-1β           |
| P-ERK|     |      | P-ERK           |
| ERK   |     |      | ERK             |

B

|       | 2.5 | Compound 1 (μM) |
|-------|-----|-----------------|
| IL-1β|     | IL-1β           |
| COX-2|     | COX-2           |
| Tpl2  |     | Tpl2            |

FIGURE 6. Compound 1 inhibits inflammatory responses in RA-FLS. A, immunoblot analysis of RA-FLS treated with Compound 1 at the indicated concentrations, or vehicle, and then stimulated with 100 ng/ml IL-1β for 30 min. Similar results were obtained with RA-FLS from four additional donors. B, immunoblot analysis of RA-FLS treated with 2.5 μM Compound 1, or vehicle, and then stimulated with 100 ng/ml IL-1β for 4 h. Similar results were obtained with RA-FLS from three additional donors. No cytotoxicity was observed under these conditions. Arrowheads indicate the phosphorylated and unphosphorylated forms of ERK1/2, and the long and short forms of Tpl2.

TABLE 3

Inhibition of inflammatory mediators in RA-FLS by Compound 1 (IC50 μM)

| Stimulus | Output | IL-6 | IL-8 | PGE2 | MMP-1 | MMP-3 | PGE2 |
|----------|--------|------|------|------|-------|-------|------|
| IL-1β    | 6.9 ± 0.9 | 7.9 ± 1.1 | 0.4 ± 0.1 | 4.0 ± 0.8 | 2.3 ± 0.5 | 0.7 ± 0.1 |

and Table 3). In all RA-FLS isolates, incubation with Compound 1 for ≥4 h, in either the presence or absence of IL-1β, caused no cytotoxic effects at all concentrations tested (≥10 μM). The production of COX-2 and PGE2 are ablated in LPS-stimulated tpl2−/− mouse macrophages (3), so our results suggest that Tpl2 is required for IL-1β-induced COX-2 expression and PGE2 production in RA-FLS.

IL-1β also stimulated RA-FLS to produce IL-6, IL-8, and the matrix metalloproteinases MMP-1 and MMP-3. The physiological characteristics of RA-FLS explants can vary from one isolate to the next; however, Compound 1 consistently and reproducibly inhibited IL-1β-induced IL-6, IL-8, and MMP-3 in the RA-FLS isolates used here (Table 3). The inhibition of IL-1β-induced MMP-1 was somewhat less reproducible: in three of five donors the IC50 was 4.0 ± 0.8 μM, but in two of these five donors there was little or no inhibition. Compound 1 caused no cytotoxicity under these conditions. Thus, barring only a few exceptions, Compound 1 inhibited IL-1β-induced IL-6, IL-8, MMP-1, and MMP-3 in RA-FLS.

As Tpl2 is required for cellular responses to TNFα in murine macrophages and fibroblasts (1, 4), we tested whether Compound 1 could inhibit TNFα-stimulated responses in human monocytes, blood, RA-FLS, and the human fibroblastic HeLa cell line. In our hands, TNFα treatment did not induce the production of cytokines and other inflammatory mediators in human monocytes and blood, nor did it induce MEK/ERK phosphorylation in monocytes. In four RA-FLS isolates PGE2 production was induced 5–30-fold by stimulation with TNFα for 24 h, and this induction was potently inhibited by Compound 1 with an IC50 of 0.7 ± 0.1 μM (Table 3). In HeLa cells IL-8 production was induced 8-fold upon stimulation of HeLa cells for 4 h with TNFα, and Compound 1 inhibited this induction with an IC50 of 2.5 μM (Fig. 7A). Moreover, TNFα-induced ERK phosphorylation in HeLa cells was potently inhibited by Compound 1 with an IC50 of 0.2 μM (Fig. 7B). In these TNFα induction experiments with RA-FLS and HeLa cells no cytotoxicity was observed under the conditions employed. Thus, we conclude that Tpl2 mediates cellular responses to TNFα stimulation in these human fibroblastic cell types.

DISCUSSION

Recent clinical successes with protein therapeutics that bind and neutralize TNFα have confirmed a role for TNFα in the progression of rheumatoid arthritis, inflammatory bowel disease, psoriasis, psoriatic arthritis, and ankylosing spondylitis (13). Thus, a reasonable approach to the development of therapies for these diseases is to identify drugs that block the signaling pathways that induce TNFα production, and for this reason we have created a series of inhibitors of the Tpl2 MAPKKK. Many of our 8-chloro-quinoline-3-carbonitrile (e.g. Compound 1) and 1,7-naphthyridine-3-carbonitrile (e.g. Compounds 2 and 3) Tpl2 inhibitors are highly selective for Tpl2 in our cell-free kinase assay panel of 13 kinases. The unique structure of Tpl2 may have facilitated our generation of highly selective Tpl2 inhibitors. Tpl2 is the only kinase in the human kinome that has a proline instead of a conserved glycine at the first glycine in the
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GXGXXG motif of the ATP binding loop (23). This may allow Tpl2 to exclude inhibitors that cannot accommodate this unique primary structure. Indeed, Tpl2 is not inhibited by the broad-spectrum kinase inhibitor staurosporine, which binds in the ATP binding loop (24). A more complete understanding of both the selectivity and potency of our inhibitors will be possible once the Tpl2 crystal structure is obtained.

The high degree of cell-free kinase selectivity that characterizes our Tpl2 inhibitors translates into a high degree of selectivity for Tpl2 inhibition in isolated human monocytes. Our inhibitors selectively block LPS-induced MEK/ERK activation in human monocytes with IC50 values for Tpl2 inhibition in isolated human monocytes. Our inhibitors translate into a high degree of selectivity once the Tpl2 crystal structure is obtained.

Our compounds tested that had cell-free Tpl2 IC50 values ≤0.2 μM, 111, or 79%, had monocyte-phospho-MEK IC50 values ≤5 μM. Thus, if potency in the monocyte-phospho-MEK assay is decided by chance then ~79% of all compounds should have monocyte-phospho-MEK IC50 values ≤5 μM. However, of 17 compounds tested that had cell-free Tpl2 IC50 values >0.2 μM none of them were active for inhibition of monocyte-phospho-MEK at 5 μM. As these 17 compounds do not have physical characteristics (e.g. permeability and solubility) that are significantly different from other compounds in this series, the most likely explanation for their inability to inhibit phospho-MEK in human monocytes is due to their inability to inhibit Tpl2. This high positive correlation between the potencies of our inhibitors in the cell-free Tpl2 assay with their potencies in the monocyte-phospho-MEK assay, together with their high degree of selectivity for Tpl2, indicate that Tpl2 catalytic activity is required for the LPS-induced activation of MEK and ERK in primary human monocytes.

Our Tpl2 inhibitors block TNFα production in primary human monocytes and human blood (Table 2). This is therapeutically important, because extravasated monocytes and synovial macrophages are primarily responsible for TNFα production in the inflamed joints of rheumatoid arthritis patients, and it is likely that monocyte-macrophage–derived TNFα is instrumental in the pathophysiology of other inflammatory diseases (11, 12). The inhibition of blood TNFα provides a potential biomarker for tracking the effect of these inhibitors in a clinical trial, and their IC50 values may establish initial target exposures necessary for achieving in vivo efficacy. It is important also to note that all of our compounds that inhibit monocyte-phospho-MEK in LPS-stimulated monocytes also inhibit monocyte-LPS-induced TNFα with similar potency (e.g. 0.5 and 0.6 μM, respectively, for Compound 1). This is consistent with the notion that in human monocytes Tpl2 regulates TNFα production via activation of the MEK/ERK axis.

RA-FLS secrete cytokines, eicosanoids, and MMPs that are critical components in the pathogenesis of rheumatoid arthritis (25). The expression of these factors can be induced by pro-inflammatory cytokines, such as IL-1β, which are found at increased levels in rheumatoid joints (Refs. 11, 12, and 25, and references therein). COX-2, the central enzyme in the PG biosynthetic pathway, is expressed at low levels in RA-FLS under basal conditions, but it is dramatically induced in RA-FLS in response to IL-1β (26, 27). Compound 1 inhibited IL-1β-induced COX-2 expression and PGE2 secretion in cultured RA-FLS (Fig. 6 and Table 3). The IC50 for IL-1β-induced PGE2 was 0.4 ± 0.1 μM; however, complete inhibition of IL-1β-induced PGE2 was observed at concentrations ≥3 μM, which is in close agreement with the near complete ablation of IL-1β-induced COX-2 expression at 2.5 μM (Fig. 6). Taken together with previous observations that COX-2 and PGE2 expression are almost completely ablated in tpl2−/− mouse cells (3), these data suggest that IL-1β induces COX-2 (and PGE2) in RA-FLS through a signaling mechanism that requires Tpl2.

Studies with tpl2−/− mouse fibroblasts indicate that Tpl2 is selectively required for ERK activation in that cell type following stimulation with IL-1β, and that Tpl2 is not required for JNK and NF-κB activation under those conditions (1). A similar restricted profile of Tpl2 selectivity may exist in RA-FLS: Compound 1 inhibited IL-1β-stimulated ERK activity in RA-FLS as expected (Fig. 6), but preliminary immunoblot analyses indicate that our Tpl2 inhibitors do not block JNK and IKKβ activation in RA-FLS under those conditions. It is well established that NF-κB is a critical mediator of the expression of IL-6 and IL-8 in RA-FLS following stimulation with IL-1β (28), and Compound 1 dose-dependently inhibited IL-6 and IL-8 in IL-1β-stimulated RA-FLS (Table 3). However, with each RA-FLS isolate studied the dose-response profiles tended to level off at ≥50% inhibition (see Table 3). Thus, it is possible that a selective Tpl2 inhibitor cannot completely ablate IL-1β-induced IL-6 or IL-8 production in RA-FLS because of a coordinate, Tpl2-independent role for NF-κB in the induction process. In addition, our pharmacologic inhibition of IL-1β-induced IL-6 with Compound 1 in RA-FLS suggests that in tpl2−/− mouse fibroblasts IL-1β-induced IL-6 may be curtailed relative to Tpl2+/+ controls. This possibility was not addressed in the earlier report (1). If, indeed, there is such a defect, it would help to differentiate these cells from the mouse macrophages described here in which a deficiency in TPL2 has no discernible effect on IL-1β-induced IL-6 production, and it would thus highlight another important difference between fibroblasts and macrophages.

The excess MMPs in affected joints drives the progressive bone and cartilage destruction associated with rheumatoid arthritis and other arthritides (29). In the RA-FLS cultures studied here, MMP-1, -2, and -3 were detectable, but MMP-7, -8, -9, -12, and -13 were not, and only MMP-1 and -3 were induced in response to IL-1β. Compound 1 inhibited IL-1β-induced MMP-1 and MMP-3 expression (Table 3), but it did not inhibit MMP-2. Increased serum MMP-3 has been shown to correlate
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with increased radiographic scores in rheumatoid arthritis, and MMP-1 has been found at the sites of cartilage erosions (30–32). Our results indicate that Tpl2 inhibition might slow or halt the progressive structural damage in arthritic disease by blocking the expression of these destructive enzymes. It is interesting to point out that Compound 1 was 10-fold more potent against PGE2 than MMP-1 and MMP-3 (IC50 values of 0.4 μM versus 4.0 μM and 2.3 μM, respectively). These data suggest that in RA-FLS different thresholds of Tpl2-dependent ERK activation are required for optimal induction of these downstream events. It has been shown that the expression of several inflammatory genes in LPS-stimulated mouse macrophages require different levels of Tpl2-MEK-ERK signaling (33).

In future studies our compounds will be critical tools for discovering and delineating the many aspects of Tpl2 signaling biology that are currently unknown. In the present work they have provided valuable insights into various functions of Tpl2 in human cell types, and our data lend positive support to the notion that Tpl2 is a valid target for therapeutic intervention in a range of human inflammatory diseases, including rheumatoid arthritis.

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REFERENCES

1. Das, S., Cho, J., Lambertz, I., Kelliher, M. A., Eliopoulos, A. G., Du, K., and Tsichlis, P. N. (2005) J. Biol. Chem. 280, 23748–23757
2. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Stamatakis, K., Lin, J. H., Patriots, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000) Cell 103, 1071–1083
3. Eliopoulos, A. G., Dumitru, C. D., Wang, C. C., Cho, J., and Tsichlis, P. N. (2002) EMBO J. 21, 4831–4840
4. Eliopoulos, A. G., Wang, C. C., Dumitru, C. D., and Tsichlis, P. N. (2003) EMBO J. 22, 3855–3864
5. Kontoyiannis, D., Boulougouris, G., Manoloukos, M., Armakia, M., Apostolaki, M., Pizarro, T., Kotlyarov, A., Forster, I., Flavell, R., Gaestel, M., Tschilis, P., Cominelli, F., and Kollias, G. (2002) J. Exp. Med. 196, 1563–1574
6. Rodriguez, C., Pozo, M., Nieto, E., Fernandez, M., and Alemany, S. (2006) Cell Signal. 18, 1376–1385
7. Stafford, M. J., Morrice, N. A., Peggie, M. W., and Cohen, P. (2006) FEBS Lett. 580, 4010–4014
8. Sugimoto, K., Ohata, M., Miyoshi, J., Ishizaki, H., Tsuboi, N., Masuda, A., Yoshikai, Y., Takamoto, M., Sugane, K., Matsuo, S., Shimada, Y., and Matsuguchi, T. (2004) J. Clin. Investig. 114, 857–866
9. Choy, E. H., and Panayi, G. S. (2001) N. Engl. J. Med. 344, 907–916
10. Aggarwal, B. B., Samanta, A., and Feldmann, M. (2001) in Cytokine Reference: A Compendium of Cytokines and Other Mediators of Host Defense (Oppenheim, J. I., and Feldmann, M., eds) pp. 413–434, 1st Ed., Academic Press, San Diego
11. Feldmann, M., and Maini, R. N. (2001) Annu. Rev. Immunol. 19, 163–196
12. Schottelius, A. J., Moldawer, L. L., Dinarello, C. A., Asadullah, K., Sterry, W., and Edwards, C. K. (2003) Exp. Dermatol. 13, 193–222
13. Simmons, D. L. (2006) Drug Discov. Today 11, 210–219
14. Doran, M. F., Pond, G. R., Crowson, C. S., O’Fallon, W. M., and Gabriel, S. E. (2002) Arthritis Rheum. 46, 625–631
15. Jia, Y., Quinn, C. M., Clabbers, A., Talanian, R., Xu, Y., Wishart, N., and Allen, H. (2006) Anal. Biochem. 350, 268–276
16. Patriotis, C., Makris, A., Chernoff, I., and Tsichlis, P. N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9755–9759
17. Helm, G. A., Alden, T. D., Beres, E. J., Hudson, S. B., Das, S., Engh, J. A., Pittman, D. D., Kerns, K. M., and Kallmes, D. F. (2000) J. Neurosurg. 92, Suppl. 2, 191–196
18. Gavrin, L. K., Green, N., Hu, Y., Janz, K., Kaila, N., Li, H. Q., Tam, S. Y., Thomson, J. R., Gopalsamy, A., Ciszewski, G., Cuozzo, J. W., Hall, J. P., Hsu, S., Telliez, J. B., and Lin, L. L. (2005) Bioorg. Med. Chem. Lett. 15, 5288–5292
19. Hu, Y., Green, N., Gavrin, L. K., Janz, K., Kaila, N., Li, H. Q., Thomson, J. R., Cuozzo, J. W., Hall, J. P., Hsu, S., Nickerson-Nutter, C., Telliez, J. B., Lin, L. L., and Tam, S. (2006) Bioorg. Med. Chem. Lett. 16, 6067–6072
20. Green, N., Hu, Y., Janz, K., Li, H. Q., Kaila, N., Guler, S., Thomson, J., Joseph-McCarthy, D., Tam, S. Y., Hotchandani, R., Wu, J., Huang, A., Wang, Q., Leung, L., Pelker, J., Marusic, S., Hsu, S., Telliez, J. B., Hall, J. P., Cuozzo, J. W., and Lin, L. L. (2007) J. Med. Chem. 50, 4728–4745
21. Channavajhala, P. L., Wu, L., Cuozzo, J. W., Hall, J. P., Liu, W., Lin, L. L., and Zhang, Y. (2003) J. Biol. Chem. 278, 47089–47097
22. Caivano, M., Rodriguez, C., Cohen, P., and Alemany, S. (2003) J. Biol. Chem. 278, 51214–51230
23. Patriotis, C., Makris, A., Bear, S. E., and Tsichlis, P. N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2251–2255
24. Luciano, B. S., Hsu, S., Channavajhala, P. L., Lin, L. L., and Cuozzo, J. W. (2004) J. Biol. Chem. 279, 52117–52123
25. Firestein, G. S. (1996) Arthritis Rheum. 39, 1781–1790
26. Crofford, L. J., Tan, B., McCarthy, C. J., and Hla, T. (1997) Arthritis Rheum. 40, 226–236
27. Crofford, L. J., Wilder, R. L., Ristimaki, A. P., Sano, H., Emmers, F. E., Epps, H. R., and Hla, T. (1994) J. Clin. Investig. 93, 1095–1101
28. Georganas, C., Liu, H., Perlmutter, H., Hoffmann, A., Thimmappaya, B., and Pope, R. M. (2000) J. Immunol. 165, 7199–7206
29. Close, D. R. (2001) Ann. Rheum. Dis. 60, Suppl. 3, iii, 62–67
30. Posthumus, M. D., Limburg, P. C., Westra, J., van Leeuwen, M. A., and van Rijswijk, M. H. (2000) J. Rheumatol. 27, 2761–2768
31. Roux-Lombard, P., Eberhardt, K., Saxne, T., Dayer, J. M., and Wollheim, F. A. (2001) Rheumatology (Oxf.) 40, 544–551
32. Woolley, D. E., Crossley, M. J., and Evanson, J. M. (1977) Arthritis Rheum. 20, 1231–1239
33. Papoutsopoulou, S., Symons, A., Tharmalingham, T., Belich, M. P., Kaiser, F., Kioussis, D., O’Garra, A., Tybulewicz, V., and Ley, S. C. (2006) Nat. Immunol. 7, 606–615