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The IL-1 Family Member 7b Translocates to the Nucleus and Down-Regulates Proinflammatory Cytokines

Sheetal Sharma,* Nicole Kulk,* Marcel F. Nold,‡ Ralph Gräf,† Soo-Hyun Kim,‡§ Dietrich Reinhardt,* Charles A. Dinarello,‡ and Philip Bufler2*  

The IL-1 family member 7b (IL-1F7b) is a novel homolog of the IL-1 cytokine family discovered by computational cloning. We have reported that IL-1F7b shares critical amino acid residues with IL-18 and binds the IL-18-binding protein; in doing so, IL-1F7b augments the inhibition of IFN-γ by the IL-18-binding protein. IL-1F7b also binds IL-18Rα but neither induces signal nor acts as a receptor antagonist. Hence, the function of IL-1F7b remains unknown. In the present study, we analyzed the intracellular expression pattern of IL-1F7b. Using two variants of GFP fusion constructs of human IL-1F7b stably expressed in RAW macrophages, only the postcleavage mature form of the IL-1F7b precursor—but not the N-terminal propiece—specifically translocates to the nucleus following LPS stimulation. IL-1F7b, like IL-1β, IL-18, and IL-33, is processed by caspase-1 to generate the mature cytokines. Therefore, we tested whether caspase-1-mediated cleavage of the IL-1F7b precursor is required for mature IL-1F7b to translocate actively into the nucleus. Indeed, a specific caspase-1 inhibitor markedly reduced nuclear entry of IL-1F7b. In stable transfectants of human IL-1F7b in RAW macrophages stimulated with LPS, levels of TNF-α, IL-1α, IL-6, as well as the chemokine MIP-2, were substantially reduced (72–98%) compared with LPS-stimulated cells transfected with the empty plasmid. These results demonstrate that IL-1F7b translocates to the nucleus after caspase-1 processing and may act as a transcriptional modulator reducing the production of LPS-stimulated proinflammatory cytokines, consistent with IL-1F7b being an anti-inflammatory member of the IL-1 family. The Journal of Immunology, 2008, 180: 5477–5482.

Cytokines of the IL-1 family of ligands and receptors possess several properties modulating inflammatory and immune responses (1). Several new members of the IL-1 family have been discovered mainly by computational research of which the most recently discovered member is IL-33 (IL-1F11) (2–5). The IL-1 family members are characterized by a common β-barrel structure consisting of 12 β-strands (6–8). Of the 11 members of the IL-1 family of ligands, 7 are agonists (IL-1α, IL-1β, IL-18, IL-33, IL-1F6, IL-1F8, and IL-1F9) and 1 is the naturally occurring antagonist (IL-1R antagonist). IL-1F5 and possibly IL-1F10 are also receptor antagonists (9, 10). In addition, the IL-1 family of receptors includes the IL-18-binding protein (IL-18BP), which exists only as a soluble form and neutralizes IL-18 (11). IL-1F7b is last member of the IL-1 family without a known function.

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3 Abbreviations used in this paper: IL-18BP, IL-18-binding protein; CFP, cyan fluorescence protein; YFP, yellow fluorescence protein.

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lysate was tested for transgene expression by Western blotting.

**Materials and Methods**

**Chemicals**

All reagents were purchased from Sigma-Aldrich unless indicated. Specific caspase-1 inhibitor was obtained from Vertex Pharmaceuticals. Pan-caspase inhibitor Z-VAD-FMK was obtained from Merck Chemicals/Calbiochem.

**Cell lines**

RAW264.7 cells were grown in RPMI 1640 supplemented with 5% heat-inactivated FCS, 2 mM l-glutamine, 50 mM 2-ME, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen Life Technologies).

**Plasmid construction**

The CFP-IL-1F7b or YFP-IL-1F7b construct was generated by inserting a PCR-generated fragment of IL-1F7b including the Kozak sequence (CCACC) and ATG at the 5’ end into the XhoI and BamHI site in pEYFP-C1 or pEYFP-C1 (BD Clontech). The IL-1F7b-YFP construct was produced by ligating a PCR-generated fragment of IL-1F7b lacking the TAG stop codon into the EcoRI and BamHI site of pEYFP-N1 (BD Clontech). Propiece IL-1F7b and mature IL-1F7b in pEYFP-N1 were constructed similarly. All constructs were sequenced to exclude mutations.

**Generation of stable RAW264.7 transfectants**

RAW cells were transfected with different IL-1F7b-YFP and CFP fusion constructs using FuGENE HD Transfection Reagent (Roche Applied Science). Transfected cells were selected in the culture medium supplemented with 200 µg/ml neomycin. Resistant cells were subcloned and the cell lysate was tested for transgene expression by Western blotting.

**Western blot**

SDS-PAGE was performed using standard 10% SDS gels and separated proteins were blotted onto nitrocellulose (Hybond ECL; Amersham Biosciences). For detection of IL-1F7b fusion protein in the lysate of transfected cells, the mAb (1154) against IL-1F7b was used (16). The membranes were developed with ECL (SuperSignal; Pierce). Signals were analyzed by densitometry using AIDA software (Raytest Isotopenmessgeräte).

**RNA isolation and quantification**

For mRNA stabilization experiments, overnight cultures of IL-1F7b-YFP-transfected stable RAW cells were used. The next day, the cells were stimulated with LPS (100 ng/ml; *E. coli* 055:B5; Sigma-Aldrich) for the indicated time. Total RNA was purified using a RNeasy kit (Qiagen). This was followed by reverse transcription using Superscript-RT (Invitrogen Life Technologies) and analysis of IL-1F7b by semiquantitative PCR as previously described (16). An equal volume of each reaction was applied to a 1% agarose gel containing ethidium bromide and visualized under UV light. Signals were analyzed by densitometry.

**Measurement of cytokines**

The liquid-phase ECL method was used to measure murine MIP-2, MIP-1α, and TNF-α in cell culture supernatants (25). Total cell-associated IL-1α was measured following lysis with 0.5% Triton X-100 by ECL. The amount of ECL was determined using an Origen Analyzer (Igen). The data are expressed per milligram of protein. Murine IL-6 and IL-10 were measured by ELISA in the cell culture supernatant (Igen). The mean fluorescence intensity for the cytoplasm was calculated by subtracting that for nucleus from the mean of whole cell fluorescence (at least 25–30 cells were evaluated). The ratio of mean nuclear intensity in the nucleus to cytoplasm was determined for each cell.

**Image analysis**

Image analysis was done using Zeiss LSM Image examiner 3.2. The mean fluorescence intensity for the cytoplasm was calculated by subtracting that for nucleus from the mean of whole cell fluorescence (at least 25–30 cells were evaluated). The ratio of mean nuclear intensity in the nucleus to cytoplasm was determined for each cell.

**Results**

**Intracellular expression of IL-1F7b fusion proteins in transfected RAW cells is up-regulated by LPS**

To study the intracellular distribution of IL-1F7b, fusion proteins of IL-1F7b CFP at the N terminus (CFP-IL-1F7b) and with YFP at the C terminus (IL-1F7b-YFP) were generated (Fig. 1A). We observed low levels of intracellular expression of both IL-1F7b fusion proteins in RAW cells after stable transfection (Fig. 1, B and C). However, overnight stimulation with LPS (100 ng/ml) induced marked expression of both IL-1F7b fusion proteins despite a constitutively active CMV promoter of the expression plasmid as detected by digital confocal microscopy and Western blot (Fig. 1, D–G).

We reported that LPS induces up-regulation of IL-1F7b protein in transfected cells by stabilization of instability elements within the coding region (16). It is possible that the instability region within the coding region of IL-1F7b transfers instability to the adjoining YFP- or CFP-specific mRNA. We therefore tested whether up-regulation of the IL-1F7b-YFP fusion protein after LPS stimulation was also due to an increase of mRNA levels. Indeed, semiquantitative PCR analysis revealed a transient increase of steady-state levels of IL-1F7b-YFP-specific mRNA in transfected RAW cells after LPS stimulation. Up-regulation of IL-1F7b-YFP-specific mRNA was rapid and readily observed after 20 min. Maximum levels of IL-1F7b-YFP-specific mRNA were detected 40 min after LPS treatment (Fig. 2).

**LPS induces nuclear translocation of IL-1F7b-YFP**

Confocal laser scanning microscopy showed equal intracellular distribution of CFP-IL-1F7b and IL-1F7b-YFP fusion proteins in the cytoplasm and nucleus of resting transfected RAW cells. The fluorescence signal for IL-1F7b in nonstimulated cells (see Fig. 1, B and C) is almost not visible on the photograph, although it is measurable and nuclear expression can be analyzed. After LPS stimulation, enhanced nuclear translocation of IL-1F7b-YFP was seen (Fig. 3, A and B). In contrast, the CFP-IL-1F7b fusion protein remained equally distributed in the cytoplasm and nucleus after LPS stimulation (Fig. 3C). Nuclear translocation of IL-1F7b without fusion protein is shown in Fig. 3D after cellular fractionation and Western blotting.

**IL-1F7b is processed by caspases in transfected RAW cells**

IL-1F7b was reported to be processed by caspase-1 in vitro (17). As shown in Fig. 4 by Western blot analysis, 27% (12–44%) of CFP-IL-1F7b from the lysate of transfected RAW cells is degraded after LPS stimulation. The breakdown product of CFP-IL-1F7b fusion protein has a molecular mass of ≈23 kDa corresponding to...
mature IL-1F7b. The pretreatment with a specific caspase-1 inhibitor reduced the processing of CFP-IL-1F7b fusion protein following exposure to LPS to 16%. The addition of a pan-caspase inhibitor nearly completely inhibited processing of CFP-IL-1F7b to 7% (Fig. 4, right panel).

Specific blockade of caspase-1 inhibits nuclear translocation of IL-1F7b-YFP fusion protein

The differential intracellular distribution pattern of IL-1F7b-YFP and CFP-IL-1F7b after LPS stimulation suggested that caspase-1 cleavage directs IL-1F7b to the nucleus. We therefore treated IL-1F7b-YFP-transfected RAW cells with caspase-1 inhibitor 2 h before LPS-stimulation. We observed a faint, granular expression pattern of IL-1F7b-YFP in the cytoplasm but complete nuclear exclusion in nonstimulated cells treated with caspase-1 inhibitor (Fig. 5A). After LPS stimulation, an enhanced cytoplasmic expression of the fusion protein was observed but nuclear staining was significantly reduced as compared with cells treated with LPS in the absence of caspase-1 inhibitor (Fig. 5B and C).

Only mature IL-1F7b is actively translocated to the nucleus

To study the intracellular expression pattern of isolated propiece and mature IL-1F7b, we separately expressed both proteins using the pEYFP-N1 expression plasmid. Propiece IL-1F7b-YFP showed a similar expression and intracellular distribution pattern before and after LPS stimulation (Fig. 6A, B and C). Mature IL-1F7b-YFP showed a weak and homogeneous expression pattern in resting cells (Fig. 6D). After LPS stimulation, expression of mature IL-1F7b-YFP was markedly increased and mature IL-1F7b-YFP translocated to the nucleus as was observed for full-length IL-1F7b-YFP (Fig. 6D and E).

Down-regulation of proinflammatory cytokines in IL-1F7b-transfected RAW cells

Because nuclear translocation indicates a possible role of IL-1F7b in transcriptional control, we hypothesized that overexpression of

FIGURE 1. IL-1F7b fusion protein is up-regulated after LPS stimulation. Fusion constructs of IL-1F7b-YFP and CFP-IL-1F7b used to generate stable transfectants of RAW cells; arrow shows caspase cleavage site (A). Stable clones of CFP-IL-1F7b- and IL-1F7b-YFP-transfected RAW cells were stimulated with LPS overnight. CFP-IL-1F7b- (B and D) and IL-1F7b-YFP- (C and E) transfected cells before (B and C) and after (D and E) LPS stimulation (100 ng/ml, overnight) were analyzed by confocal digital microscopy. Nuclear staining for CFP-IL-1F7b- and IL-1F7b-YFP-transfected RAW cells was done using TOPRO-3 and Bis-benzimide. Mean fluorescence intensity (± SEM) was measured for single cells by analyzing at least 30 individual cells (F). Statistical difference was calculated by unpaired t test; ***, p < 0.0001. Total cell lysates of CFP-IL-1F7b- and IL-1F7b-YFP-transfected RAW cells were analyzed by Western blot using a mAb against GFP (G).

FIGURE 2. LPS induces stabilization of IL-1F7b-YFP mRNA expression in transfected RAW cells. Transfected RAW cells were stimulated with LPS (100 ng/ml). IL-1F7b-YFP mRNA levels were analyzed at the indicated times along with GAPDH as housekeeping gene by semiquantitative PCR.

FIGURE 3. Enhanced nuclear expression of IL-1F7b-YFP fusion protein after LPS stimulation. CFP-IL-1F7b- (A) and IL-1F7b-YFP- (B) transfected RAW cells were stimulated with LPS overnight and analyzed by confocal laser scanning microscopic. Nuclear staining for CFP-IL-1F7b- and IL-1F7b-YFP-transfected RAW cells was done using TOPRO-3 and Bis-benzimide. The ratio of mean fluorescence intensity in the nucleus to cytoplasm was calculated based on the analysis of at least 30 individual cells (C). This observation was confirmed by the analysis of three single-cell clones of stably transfected RAW cells. Subcellular fractions of RAW cells expressing IL-1F7b without fusion proteins were separated by SDS-PAGE and visualized by Western blotting using a mAb against IL-1F7b (D).
IL-1F7b might alter the expression of proinflammatory cytokines. We next measured the production of IL-1α, MIP-1α, MIP-2, TNF-α, IL-6, and IL-10 from two IL-1F7b-overexpressing RAW cells. Following LPS stimulation, the levels of IL-1α, MIP-2, TNF-α, and IL-6 were markedly reduced in IL-1F7b-overexpressing RAW cells compared with mock-transfected RAW cells similarly stimulated with LPS (Fig. 7). This reduction was particularly 98% for IL-1α and IL-6 in clone 23. In contrast, no change of MIP-1α or IL-10 concentration was detected suggesting that the interference of IL-1F7b with cytokine production was not due to a global process or the secretion of anti-inflammatory cytokines as IL-10.

Discussion

We previously reported that extracellular IL-1F7b interacts with the IL-18BP enhancing its capacity to inhibit IL-18-induced production of IFN-γ (14). Here, we describe the intracellular expression pattern of IL-1F7b and its possible functional relevance. We generated autofluorescent fusion proteins with CFP or YFP at the N or C terminus of IL-1F7b, respectively, and observed that only IL-1F7b-YFP translocates to the nucleus in transfected murine RAW cells upon stimulation with LPS. This indicated that N-terminal processing of IL-1F7b occurs before nuclear translocation. Accordingly, nuclear translocation was markedly reduced by the presence of a specific caspase-1 inhibitor. In addition, we show that overexpression of IL-1F7b in RAW cells reduces production of proinflammatory cytokines after LPS stimulation. In fact, the suppression of cytokines such as IL-1α and TNF-α was considerably decreased by 80–98%.

Both IL-1β and IL-18 are synthesized as inactive precursors. Processing by caspase-1 generates their biologically active forms (26). The precursors of IL-1α and IL-33 are active without processing. However, IL-33 requires processing by caspase-1 for optimal biological activity (1, 5). IL-1F7b was similarly shown to be cleaved by caspase-1 and to a smaller extent by caspase-4 in vitro, but the functional relevance of IL-1F7b maturation is not yet unveiled (17).

Caspase-1 cleavage is mandatory before the release of IL-1β and IL-18 from activated cells (1, 27–29). However, IL-1F7b is rarely observed in the extracellular compartment (16). Following LPS stimulation, 80% of IL-1β is diffusely present in the cytoplasm of macrophages whereas 20% is secreted together with caspase-1 by secretory lysosomes (30). In the same cell, IL-1α translocates to the nucleus via its N-terminal nuclear localization sequence (23). In a recent publication, nuclear translocation was also shown for IL-33 which may function both as a proinflammatory cytokine and an intracellular NF (4).

To analyze whether IL-1F7b shares similarity to IL-1α, we studied the intracellular distribution pattern before and after LPS stimulation. This was done by overexpressing fusion proteins with CFP or YFP at the N or C terminus of IL-1F7b in murine RAW cells. Both plasmids encoding CFP and YFP fluorescence proteins contain a constitutively active CMV-promoter to ensure high levels of transgene expression. The first phenomenon we observed was that, unless stimulation, only minor protein levels were detected for both fusion constructs. In our previous study, we showed that exon 5 of the IL-1F7b-coding region contains an instability element which induces constitutive down-regulation of IL-1F7b mRNA in transfected cells unless stimulated (16). Therefore, we hypothesized that the instability element within the coding region of IL-1F7b transfers instability to the adjoining fusion partner CFP or YFP. Indeed, we were able to show that steady-state levels of the fusion protein specific mRNA were rapidly increased after stimulation with LPS. A similar mechanism was described for the

**FIGURE 4.** CFP-IL-1F7b fusion protein is processed in transfected RAW cells. The lysate of transfected RAW cells treated or not with LPS and a specific caspase-1 inhibitor (ICEi, 10 μM; left panel) or pan-caspase inhibitor (10 μM; right panel) was analyzed by Western blot. For detection, a mAb against IL-1F7b was used. One of four (ICEi) or five (pan-caspase inhibitor) independent experiments is shown.

**FIGURE 5.** Presence of caspase-1 inhibitor (ICEi) reduces nuclear translocation of IL-1F7b-YFP fusion protein after LPS stimulation. A. Nonstimulated cells; B. LPS-stimulated cells. The ratio of mean fluorescence intensity in the nucleus to cytoplasm was calculated based on the analysis of ≥30 individual cells (C). Nuclear staining was done using Bis-benzimide.

**FIGURE 6.** Mature IL-1F7b translocates to the nucleus. Stable clones of RAW cells expressing propiece (A) or mature IL-1F7b-YFP (B) were stimulated with LPS (C and D). Ratio of mean fluorescence intensity in the nucleus to cytoplasm of ≥30 individual cells was analyzed by confocal microscopy (E). Nuclear staining was done using Bis-benzimide.
human plasminogen activator inhibitor type 2 and it was demonstrated that coding region instability elements confer instability to a human growth hormone reporter transcript (31).

In resting transfected RAW cells, IL-1F7b fusion proteins were equally expressed in the cytoplasm and in the nucleus. Similarly, this was described for IL-1α when expressed as fusion protein attached to CFP or YFP at its N or C terminus, respectively (23). After LPS stimulation, we observed a marked presence of nuclear IL-1F7b-YFP. In contrast, there was no increased nuclear expression of CFP-IL-1F7b after LPS treatment.

Nuclear shuttling of proteins with a molecular mass of >50 kDa is an active process and relies on a specific transport machinery. However, small proteins with a molecular mass <50 kDa easily enter and exit the nucleus through nuclear pores (32, 33). The molecular mass of IL-1F7b-YFP is ~55 kDa. This indicates that IL-1F7b-YFP needs active transport into the nucleus. Indeed, there was no passive diffusion of IL-1F7b-YFP from the nucleus of transfected RAW cells incubated at 4°C (data not shown) as reported for low molecular mass proteins (34). In addition to our experimental data, computerized sequence analysis revealed that IL-1F7b is predicted as being mainly expressed as a nuclear protein (prediction model by Reinhardt et al. (35), http://psort.nibb.ac.jp/). However, as of this writing, no nuclear localization signal has been identified.

Eukaryotic cells have evolved to regulate the movement of macromolecules between the cytoplasm and the nucleus such that the transfer of information occurs only under conditions in which a transcriptional response is required. Because CFP-IL-1F7b was not present inside the nucleus after LPS stimulation, we hypothesized that N-terminal processing of IL-1F7b takes place in transfected RAW cells before nuclear translocation.

To prove that IL-1F7b undergoes proteolytic processing before nuclear translocation, we pretreated transfected RAW cells with a specific caspase-1-inhibitor and observed a significantly reduced nuclear translocation of IL-1F7b-YFP after LPS stimulation. Second, we analyzed the lysate of transfected RAW cells expressing CFP-IL-1F7b fusion protein by Western blot and detected a ~23-kDa protein corresponding to mature IL-1F7b after LPS stimulation. The expression of mature IL-1F7b was markedly reduced by the addition of a specific caspase-1 inhibitor. Because processing of the CFP-IL-1F7b fusion protein was not completely abolished by the caspase-1 inhibitor, we speculated that caspases other than caspase-1 are involved in IL-1F7b maturation. In fact, the addition of a pan-caspase inhibitor almost completely inhibited the maturation of pro IL-1F7b following LPS exposure as shown by Western blot analysis.

Only 10–29% of total IL-1F7b is processed to mature IL-1F7b in transfected RAW cells after LPS stimulation. It is possible that part of mature IL-1F7b is secreted following LPS stimulation. Indeed, we found mature IL-1F7b in the supernatant of transfected RAW cells but the release of mature IL-1F7b was also little in comparison to the proform of IL-1F7b (data not shown) and, thus, does not explain the low detection of mature IL-1F7b in the cell lysate. In addition, we showed that specifically mature IL-1F7b-YFP, but not the N-terminal propiece of IL-1F7b, is detected in the nucleus after LPS stimulation.

Recent studies unraveled an increasing number of cytokines acting both as a secreted cytokine and transcriptional regulator independent of their cell surface receptors. In particular, this was shown for the IL-1 family members IL-1α and IL-33 (4, 23, 36) as well as high-mobility group box 1 protein (37, 38) and IL-16 (39). The expression pattern of IL-1F7b also indicates a functional dichotomy acting as an immunoregulatory cytokine as well as a transcriptional modulator. However, it is presently unclear how expression of human IL-1F7 in mouse macrophages results in such profound suppression of LPS-induced cytokines. Both clones of IL-1F7b-expressing RAW cells triggered a substantial decrease in the synthesis of TNF-α, MIP-2, IL-1α, and IL-6 after LPS stimulation. In parallel, the production of MIP-1α and IL-10 was not changed. These results support the concept that IL-1F7b acts to suppress LPS-induced cytokines but because there was no suppression of MIP-1α and IL-10, we conclude that the effect is not global or due to altered cell metabolism or the induction of anti-inflammatory cytokines. Any protein that migrates from the cytosol to the nucleus may function as a regulator of transcription (either by direct suppression or via increased expression of inhibitory factors). Alternatively, because we measured reduced protein and not gene expression, suppression of synthesis by suppressors of cytokine signaling is also a possible explanation of intranuclear IL-1F7.

Caspase-1 inhibitors inhibit the maturation of proinflammatory cytokines as IL-1β and IL-18, which play a key role in inflammatory disorders (i.e., rheumatoid arthritis). Our data indicate that caspase-1 inhibition will also impact anti-inflammatory pathways by reducing the activity of anti-inflammatory cytokines as IL-1F7b. However, the therapeutic potential of this observation has not yet been established.

In summary, here we show that IL-1F7b undergoes maturation by proteolytic processing and subsequently translocates to the nucleus modulating the expression of proinflammatory cytokines. This is in contrast to IL-1α and IL-33, in which proteolytic

**Figure 7.** Reduction of proinflammatory cytokines in stable RAW clones expressing IL-1F7b. Two stable clones of IL-1F7b (C17, C23) or Mock-transfected RAW cells were stimulated with LPS (100 ng/ml) and, after 24 h, TNF-α, MIP-2, MIP-1α, IL-10, and IL-6 were measured in the supernatant. IL-1α was measured in the lysates. The data represent the mean ± SEM of four separate experiments.
processing by calpain or caspase-1 is not a prerequisite for nuclear translocation (4, 23).

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Disclosures

The authors have no financial conflict of interest.

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