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*J Immunol* 2007; 179:7478-7487; doi: 10.4049/jimmunol.179.11.7478

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Dose-Dependent Differential Regulation of Cytokine Secretion from Macrophages by Fractalkine

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Although expression of the fractalkine (CX3CL1, FKN) is enhanced in inflamed tissues, it is detected at steady state in various organs such as the intestine, and its receptor CX3CR1 is highly expressed in resident-type dendritic cells and macrophages. We hypothesized that FKN might regulate the inflammatory responses of these cells. Therefore, murine macrophages were pretreated with FKN and then stimulated with LPS. We found that macrophages pretreated with 0.03 nM FKN but not with 3 nM FKN secreted 50% less TNF-α than did cells treated with LPS alone. Cells treated with 0.03 nM FKN and LPS also showed reduced phosphorylation of ERK1/2 and reduced NF-κB p50 subunit. Interestingly, the p65 subunit of NF-κB was translocated to the nucleus but redistributed to the cytoplasm in the early phase by forming a complex with peroxisome proliferator-activated receptor (PPAR) γ. Exogenous 15-deoxy-Δ(12,14)-prostaglandin J2, a natural ligand for PPAR-γ, also induced redistribution of p65 with decreased TNF-α secretion after LPS challenge. Pretreatment with 0.03 nM but not 3 nM FKN increased the cellular levels of 15-deoxy-Δ(12,14)-prostaglandin J2 as well as mRNA of PPAR-γ. Requirement of PPAR-γ for the effect of 0.03 nM FKN was confirmed by small interfering RNA of PPAR-γ. In contrast, pretreatment with 3 nM FKN induced higher levels of IL-23 compared with cells pretreated with 0.03 nM FKN and produced TNF-α in a CX3CR1-dependent manner. These dose-dependent differential effects of FKN establish its novel role in immune homeostasis and inflammation. The Journal of Immunology, 2007, 179: 7478–7487.

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Received for publication January 9, 2007. Accepted for publication September 25, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by grants and contracts from the Ministry of Health, Labour and Welfare, the Ministry of Education, Culture, Sports, Science and Technology, the Japan Health Sciences Foundation, Novartis Foundation (Japan) for the Promotion of Science, and the Mitsukoshi Health and Welfare Foundation.

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3 Abbreviations used in this paper: FKN, fractalkine (CX3CL1); BM, bone marrow-derived macrophage; 15d-PGL2, 15-deoxy-Δ(12,14)-prostaglandin J2; PPAR-γ, peroxisome proliferator-activated receptor γ; siRNA, small interfering RNA.

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produce large amounts of proinflammatory cytokines in response to bacterial components. The inflammatory anergy of intestinal macrophages is thought to be important to maintain intestinal homeostasis. However, the mechanism by which macrophages acquire this feature is not yet fully understood. One important working hypothesis is that intestinal epithelial cells and stromal cells provide a particular microenvironment to promote inflammatory anergy, along with a variety of cytokines and chemokines as their products. Indeed, intestinal stromal cell-derived products downregulate both monocyte receptor expression and cytokine production (32). It is also likely that FKN participates in forming this microenvironment of the intestine to render macrophages immunologically hyporesponsive. Such anti-inflammatory activity of FKN has been reported previously. For example, FKN attenuated LPS-induced production of NO, IL-6, and TNF-α by rat (34) and mouse (17) microglia, which are phagocytic cells and are responsible for cytokine production in the CNS. Pretreatment of rats with an anti-FKN Ab enhanced LPS-induced TNF-α levels in hippocampus and cerebrospinal fluid (35). However, the anti-inflammatory effect of FKN in bone marrow-derived or blood macrophages has not been documented.

In this study, we compared the effects of different concentrations of FKN on macrophages and found that relatively low concentrations of FKN suppressed LPS-induced TNF-α secretion by both bone marrow-derived macrophages (BMD) and the mouse macrophage cell line RAW264.7. We investigated the underlying mechanism and found for the first time that FKN induced the expression of both peroxisome proliferator-activated receptor (PPAR) γ and its ligand and altered the subunit usage of NF-κB after stimulation with LPS in macrophages, eventually decreasing the secretion of TNF-α. In contrast, higher concentrations of FKN, which may represent a local inflammatory condition, did not show such an immunosuppressive effect; instead, an up-regulation of IL-23 was seen.

Materials and Methods

**Mice**

Six- to 7-wk-old male C57BL/6J mice obtained from CLEA Japan and IL-10 knockout mice (C57BL/6J background; The Jackson Laboratory) were maintained under pathogen-free conditions in a facility of the Research Institute, International Medical Center of Japan (Tokyo, Japan). All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research with the approval of the local ethics committee in the International Medical Center of Japan.

**Histological immunostaining**

Frozen sections were prepared from mouse intestine, fixed with cold acetone for 10 min, dried, and treated with Blockace (Dainippon Pharmaceu-
ticals), incubated with hamster anti-FKN (22) or PE-labeled rat anti-F4/80 (Serotec) and rabbit anti-CX3CR1 (22), followed by secondary FITC-la-
beled anti-hamster IgG Ab (Southern Biotechnology Associates) or Alexa 488-labeled anti-rabbit IgG Ab (Invitrogen Life Technologies and Molec-
ular Probes). Images were captured with a fluorescence microscope (BX50/ BXFLA; Olympus) equipped with a CCD camera. Merged images were produced using Adobe Photoshop CS2 (Adobe Systems).

**Cell culture, pretreatment with FKN, and stimulation with LPS**

To obtain BMDs, bone marrow cells were harvested and differentiated in DMEM containing 10 ng/ml M-CSF and 10% FBS for 7 days. RAW264.7 cells (American Type Culture Collection) were grown in DMEM supple-
mented with 10% FCS. Aliquots of 1 × 10^6 cells in 0.2 ml of culture medium were pretreated with the indicated concentration of recombinant mouse FKN (R&D Systems) for 12 h and then stimulated by addition of the indicated concentration of LPS (from Salmonella minnesota, L-2167; Sigma-Aldrich) to the culture. In some experiments, 15d-PGJ2 (Cayman Chemical) and mouse rIL-23 (R&D Systems) was added to the culture. To examine the effect of immobilized FKN, 96-well flat-bottom plates were coated with 0.1 ml of various concentrations of FKN in PBS for 12 h at 4°C. After washing with PBS, cells were placed and the culture was per-
formed as above. Actual density of coated FKN after washing was not measured. The hamster anti-mouse FKN Ab for blocking FKN was pre-
pared at KAN Research Institute (Kobe, Japan). To neutralize the effects of CX3CR1, purified rabbit anti-rat CX3CR1 polyclonal Ab (2 µg/ml; Torrey Pines Biolabs) was used with rabbit IgG (IBL) as a control. To neutralize the actions of IL-23, purified rat anti-mouse IL-23 p19 mAb (2 µg/ml; ebioscience) and purified rat IgG1 isotype control (BD Biosciences) were used as controls.

**Cytokine production assay by ELISA**

The concentrations of cytokines in culture supernatants were measured using a Murine TNF-α ELISA Development Kit (PeproTech), Quantikine M Mouse IL-6 Immunoassay kit (R&D Systems) and Mouse IL-10 ELISA kit (Endogen).

**Flow cytometry analysis**

Cells were incubated with mAb against mouse TLR4/MD-2 complex (SA15-21; a gift from Dr. S. Takamura-Akashi, Tokyo University, Tokyo, Japan) or isotype control IgG directly conjugated with Alexa 488 and an-
alyzed by FACS (BD Biosciences).

**Western blotting and immunoprecipitation**

Cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, 1 mM Na_2VO_4, 1 mM PMSF, 1% Nonidet P-40, complete protease inhibitor mixture (Roche Molecular Biochemicals), and 50 mM NaF (pH 8.0) for 20 min on ice. After centrifugation at 10,000 × g for 20 min, protein concentrations were determined using the Bio-Rad protein assay. After separation by SDS-PAGE under reducing conditions, lysates were transferred to membranes (Immobilon; Millipore) and subsequently immunoblotted with specific Ab before visualization by chemilumines-
cence (SuperSignal West Dura; Pierce). To analyze ERK1/2 activation, membranes were probed with anti-phospho ERK 1/2 Ab or total ERK1/2 Ab (Cell Signaling Technology) and then stripped and reprobed with Ab to actin (Santa Cruz Biotechnology). To detect the amount of NF-κB p50 protein, nuclear extracts were subjected to Western blotting with anti-
NF-κB p50 Ab (sc-7178; Santa Cruz Biotechnology).

**RT-PCR**

Total RNA from cells was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen Life Technologies) and amplified by PCR. The following primers were used: FKN, a forward primer (5′-CACCCTCAGGG TGACAGAATAT) and a reverse primer (5′-TTTCTCACCCGCTCTCTCTC AA-3′); MD-2, a forward primer (5′-ATGGTGCAATTTATTCCTCTT 
CGAGC) and a reverse primer (ATTGACATACGCAGGCGTGAATGATG 
TG-3′); TLR4, a forward primer (5′-AGCAGAAGAAAAACATCTATG 
ATC) and a reverse primer (GGTTTAGGCCCAAGATTTCTCC-3′);

**FIGURE 1.** Expression of FKN in the intestine and detection of CX3CR1-positive macrophages. A, Frozen intestinal sections were stained with anti-FKN Ab. B, Frozen sections were double stained with anti-F4/80 (red) and anti-CX3CR1 Ab (green). Merged images are shown.
cells were cultured for 12 h and stimulated with LPS (1 ng/ml) for 6 h. Secretion of TNF-α was measured and shown as the average plus 1 SD of the percentage of cells without FKN pretreatment using three to eight independent cultures. 

For quantitative analysis, the SYBR Green PCR Kit (Applied Biosystems) was used according to the manufacturer’s instructions using 10 μl of the nuclear extracts. Quantification of bands was performed by densitometry using ATTO Densitograph version 4.0 software. For the supershift assay, we used an identical oligonucleotide probe labeled with [32P]dCTP using a Klenow fragment. In brief, nuclear extracts (3 μg) were preincubated with 1 μg of anti-p50 or p65 Ab (sc-1190X, sc-372X; Santa Cruz Biotechnology) for 60 min at 4°C before addition of the labeled probe in

**Preparation of nuclear extracts and EMSA**

Cells were suspended in 200 μl of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT) and kept on ice for 15 min followed by addition of 12.5 μl of 10% Nonidet P-40. After mixing and centrifugation (10,000 × g) for 3 min, the nuclear pellets obtained were resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) and kept on ice for 15 min with intermittent agitation. The samples were centrifuged and the supernatants were stored at −80°C until use. EMSAs were conducted using a Digoxigenin Gel Shift Kit (Roche Diagnostics) according to the manufacturer’s instructions using 10 μg of the nuclear extracts.
FIGURE 3. Pretreatment with FKN altered TLR4 and MD-2 expression and ERK1/2 phosphorylation. A, RAW264.7 cells were pretreated with the indicated concentrations of FKN for 12 h and stained with an Ab against the TLR4-MD-2 complex. Shaded histograms indicate staining with isotype control. B, The results in A are shown as the difference in mean fluorescence intensity from that of the isotype control. Results are shown as an average plus 1 SD of three experiments. C, RAW264.7 cells were pretreated with the indicated concentrations of FKN for 12 h and subjected to quantitative RT-PCR for TLR4 and MD-2. Results are shown as an average (n = 3 cultures) plus 1 SD of relative expression compared with the level of cells without FKN pretreatment. *, Statistically significant difference from cells without FKN pretreatment (p < 0.05). D, RAW264.7 cells were pretreated with the indicated concentration of FKN for 12 h and stimulated with LPS (1 ng/ml). Cell extracts were obtained after a 12-h pretreatment with FKN (LPS−) or after additional stimulation with LPS for 6 h (LPS+). Extracts were subjected to Western blotting to detect actin, phospho-ERK1/2, MAPK, and total ERK1/2. Representative data from four separate experiments are shown.

Statistics
The results were compared by the Mann-Whitney U test using the StatView II statistical program (Abacus Concepts) adapted for Mac OS.

Results
FKN and CX3CR1 are abundantly expressed in the intestine
Since CX3CR1-positive dendritic cells are abundantly distributed in the lamina propria of normal intestine (27), we initially examined the expression of the FKN ligand in the intestine. FKN was detected in epithelial as well as mesenchymal cells, especially in the proximal colon (Fig. 1A). FKN-positive mesenchymal cells, including myofibroblasts, were identified by double staining with anti-oxsme muscle actin as well as RT-PCR of murine myofibroblast cell lines which were established from the colon (our unpublished data). CX3CR1+/FoxR+/C+ macrophages were also found more frequently in the colon compared with the small intestine (Fig. 1B).

FKN attenuated LPS-induced secretion of TNF-α
To investigate the effect of FKN, we chose BMφ and the mouse macrophage cell line RAW264.7 because both express the FKN receptor CX3CR1 (92 and 90%, respectively) and produce large amounts of TNF-α, an indicator of inflammation, in response to LPS stimulation for 6 h (Fig. 2). These cells were pretreated with FKN for 12 h and then stimulated with LPS. Although FKN itself did not induce secretion of TNF-α, pretreatment with FKN reduced the secretion of LPS-induced TNF-α in both BMφ and RAW264.7 cells (Fig. 2, A and B). Of interest, ~0.003–0.03 nM FKN was optimal for this effect, and a higher concentration (3 nM) of FKN failed to attenuate TNF-α secretion (Fig. 2, A and B). Addition of an anti-FKN Ab during FKN pretreatment (0.03 nM) abolished its inhibitory effect, which indicated that the effect was due to FKN rather than any minor contaminant (data not shown). In the time course assay, less secretion of TNF-α in cells pretreated with 0.03 nM FKN became obvious 6 h after LPS challenge (Fig. 2C). Since FKN is expressed as a membrane-bound molecule as well as a soluble protein, we immobilized FKN on a plate and cultured RAW264.7 cells before LPS challenge. Immobilized FKN at a concentration of 0.03 nM also showed decreased TNF-α production by macrophages in response to LPS, whereas higher...
concentrations did not show this effect (Fig. 2D). Washing the cells before LPS challenge reduced, at least in part, the inhibitory effect of FKN on TNF-α (data not shown), which indicated that both intracellular events and soluble factors released during pretreatment were involved in inhibition. To confirm that these dose-dependent, differential effects were mediated by a single FKN receptor, we added anti-CX3CR1 Ab when cells were pretreated with FKN. Reduction of TNF-α secretion in cells pretreated with 0.03 nM FKN was negated in the presence of anti-CX3CR1 Ab (Fig. 2E). This result indicated that the TNF-α suppressive effect was mediated by CX3CR1.

Pretreatment with 0.03 nM FKN also suppressed secretion of IL-6 from BMφ induced by LPS, although the inhibitory effect was not as evident as that observed with levels of TNF-α in a 6-h culture (Fig. 2F). IL-12 production also was induced by LPS in BMφ, but pretreatment with FKN did not affect its secretion significantly (data not shown). BMφ constitutively produced IL-10 and pretreatment with FKN did not alter the levels of IL-10 in the culture supernatant, either without or with LPS (Fig. 2F). Similar effects of FKN pretreatment on cytokine production were seen in RAW264.7 cells. To examine the possible involvement of IL-10, BMφ prepared from IL-10−/− mice were tested. Levels of TNF-α secretion after LPS stimulation in IL-10−/− BMφ were 2-fold higher than in wild-type mice; however, pretreatment with FKN significantly decreased the level of TNF-α, although it did not reach 50% inhibition as seen in wild-type mice (Fig. 2G). Overall results showed that the LPS-induced TNF-α response was suppressed by pretreatment with 0.03 nM FKN and involvement of IL-10 in TNF-α suppression was only partial, if at all.

Pretreatment with FKN-modulated expression of TLR4

We next investigated whether FKN pretreatment might alter the expression of TLR4 and MD-2, molecules necessary for signal transduction from LPS. Although cell surface expression of the TLR4-MD-2 complex was not high in RAW264.7 cells, it significantly decreased in cells pretreated with 0.03 nM FKN as determined by flow cytometry (Fig. 3, A and B). Levels of TLR4 and MD-2 mRNA also decreased after pretreatment with 0.03 nM FKN (Fig. 3C). However, the mean fluorescence intensity of TLR4-MD-2 and mRNA levels of MD-2 in the cells treated with 3 nM FKN was still significantly lower than that of untreated control cells. Since TNF-α production by cells treated with 3 nM FKN was comparable to that of untreated cells, we assume that the surface expression level of TLR4-MD-2 was not the major factor that directly caused suppression of TNF-α secretion, although FKN does affect TLR4-MD-2 expression.

FKN attenuated ERK1/2 activation

Since MAPK phosphorylation occurs downstream of TLR4 signaling, the effect of FKN pretreatment was assessed. FKN itself induced ERK1/2 phosphorylation within 30 min; however, after 12 h (the time point of LPS addition), there was no significant activation of ERK1/2 in cells treated with either concentration of FKN (Fig. 3D). Phosphorylation of ERK1/2 was detected 30 min after stimulation with LPS irrespective of pretreatment with FKN (data not shown); however, 6 h after LPS challenge, MAPK phosphorylation was dramatically suppressed when cells were pretreated with 0.03 nM FKN. A higher concentration of FKN (3 nM) did not inhibit ERK phosphorylation (Fig. 3D), coinciding with the failure to suppress TNF-α secretion at this concentration. Phosphorylation of p38 was seen after stimulation with LPS; however, there is no difference between cells with or without FKN pretreatment (data not shown).

Effect of FKN pretreatment on NF-κB activation and induction of PPAR-γ

Inflammatory responses are closely linked to the activation of NF-κB, and LPS-induced transcription of TNF-α in macrophages is highly dependent on nuclear translocation of NF-κB. When RAW264.7 cells were stimulated with LPS, NF-κB activation was seen (Figs. 4A and 5A). Without LPS, very low levels of nuclear NF-κB were detected in RAW264.7 cells and treatment with either 0.03 nM or 3 nM FKN alone for 12 h did not show a significant effect on the status of NF-κB (data not shown). Unexpectedly, pretreatment with 0.03 nM FKN did not alter the amount of nuclear-translocated κB-binding protein for 1 h after LPS challenge.
Total RNA was isolated and the levels of PPAR-γ were determined in each condition. A, RAW264.7 cells were transfected with or without the indicated concentrations of either FKN or 15d-PGJ2 for 12 h and stimulated with 1 ng/ml LPS for either 1 or 2 h. Cells were then stained with anti-p65 (red) and anti-PPAR-γ (green) Ab; the merged images are shown. One representative result from four independent cultures in each condition is shown. B, RAW264.7 cells were treated with the indicated concentrations of 15d-PGJ2 for 12 h and stimulated with 1 ng/ml LPS for 6 h. Culture supernatants were subjected to TNF-α ELISA. Results are shown as the average plus 1 SD of four independent preparations. C, RAW264.7 cells were transfected with the indicated concentrations of 15d-PGJ2 for 12 h and RNA extracts were subjected to quantitative RT-PCR for PPAR-γ. Results are shown as the mean plus 1 SD of the relative expression of the levels of nonpretreated cells from three independent preparations. * Statistical significant differences from control cells without pretreatment. D, RAW264.7 cells were transfected with either PPAR-γ siRNA or control siRNA. Thirty-six hours after siRNA transfection, cells were treated with 0.03 nM FKN for 12 h. Total RNA was isolated and the levels of PPAR-γ mRNA were determined by quantitative RT-PCR. The fold induction was shown as the mRNA level relative to that of cells without siRNA transfection. Results are shown as the mean plus 1 SD of relative expression of the nonpretreated cells from six independent preparations. * Statistical significant differences from cells without transfection or transfected with control siRNA. E, RAW264.7 cells were transfected with either PPAR-γ or control siRNA. Thirty-six hours after transfection, cells were preincubated with 0.03 nM FKN or without FKN (0 nM) for 12 h and then stimulated with 1 ng/ml LPS for 2 h. Cells were stained with anti-p65 (red) and anti-PPAR-γ (green) Ab. Representative results from four independent cultures in each condition are shown.

(Fig. 4A). Therefore, to find the mechanism of decreased TNF-α production, we performed supershift analysis focusing on the effect of pretreatment with 0.03 nM FKN. Without pretreatment, supershift was seen with both anti-p65 and anti-p50 Ab. In contrast, when cells were pretreated with 0.03 nM FKN, clear supershift was not seen with the anti-p50 Ab, while the NF-κB complex was supershifted with the anti-p65 Ab (Fig. 4B).

Decreased nuclear translocation of p50 protein in FKN-pretreated cells was confirmed by Western blotting (Fig. 4C). Furthermore, pretreatment with 0.03 nM FKN did not reduce but rather slightly increased the levels of p65 protein in the nucleus compared with those of untreated cells at the time point of 1 h after stimulation with LPS (Figs. 4D and 5A). Importantly, in these FKN-pretreated cells, p65 was rapidly eliminated from the nuclei 2 h after addition of LPS (Figs. 4D and 5A). In contrast, p65 remained in the nuclei at this time point in cells either without pretreatment or pretreated with 3 nM FKN (Figs. 4D and 5A).

From these results, we assumed that when cells were pretreated with 0.03 nM FKN, NF-κB p65 did not form a complex with p50 but with other molecules, which facilitated transport of p65 protein out of the nucleus. Since PPAR-γ was previously reported to have such a function (37), we performed immunoprecipitation analysis. We found that p65 protein and PPAR-γ were coprecipitated from cytoplasmic fractions 2 h after stimulation with LPS in cells that had been pretreated with 0.03 nM FKN (Fig. 4E). However, when cells were pretreated with 3 nM FKN, the p65-PPAR-γ complex was not detected (Fig. 4E). Immunofluorescence analysis also showed that nuclear p65 was efficiently moved to the cytoplasm with PPAR-γ in the cells pretreated with 0.03 nM FKN, while p65 remained in the nuclei in the cells without treatment or pretreated with 3 nM FKN 2 h after LPS stimulus (Fig. 5A). Since PPAR-γ is known to be a negative regulator of the inflammatory cytokine responses of macrophages (38, 39), we postulated that pretreatment with 0.03 nM FKN would induce and activate PPAR-γ and modulate NF-κB translocation, and finally attenuate the secretion of TNF-α. To examine this possibility, we determined the mRNA levels of PPAR-γ and found that the levels were enhanced after...
a 12-h treatment with 0.03 nM FKN when compared with untreated cells or those treated with 3 nM FKN (Fig. 4F).

Exogenous PPAR-γ ligand mimicked the effect of FKN
Since our results indicated the role of PPAR-γ activation in the anti-inflammatory effect of FKN, we pretreated cells with a natural PPAR-γ ligand and agonist, 15d-PGJ2, instead of FKN. Pretreatment of RAW264.7 cells with 10 μM 15d-PGJ2 resulted in enhanced expression of PPAR-γ and nuclear translocation of p65 1 h after LPS stimulation, and then the p65 was rapidly cleared from nuclei at 2 h, as was observed in cells pretreated with 0.03 nM FKN (Fig. 5A). As a result, secretion of TNF-α decreased to 50% of untreated cells when cells were pretreated with 10 μM 15d-PGJ2 (Fig. 5B). The overall effect seen in the cells pretreated with 10 μM 15d-PGJ2 was similar to the effect of pretreatment with 0.03 nM FKN. Furthermore, we found that exogenous 15d-PGJ2 up-regulated PPAR-γ mRNA expression (Fig. 5C).

Effect of FKN on p65 redistribution depends on PPAR-γ
To confirm the key role of PPAR-γ in the effect of FKN on p65 redistribution, we used RNA interference. The introduction of siRNA for mouse PPAR-γ resulted in NF-κB redistribution, we used RNA interference. The introduction of siRNA for mouse PPAR-γ mRNA expression and similarly altered NF-κB activation. Therefore, we investigated whether FKN itself could induce 15d-PGJ2. After a 2-h treatment with 0.03 nM FKN, 15d-PGJ2 was up-regulated and the enhanced expression was maintained for 12 h (Fig. 6, A and B). In contrast, expression levels of 15d-PGJ2 in cells treated with 3 nM FKN were not significantly different from untreated control cells.

Induction of IL-23 by high concentrations of FKN
Since both 0.03 and 3 nM FKN showed a distinct effect in macrophages, we compared the mRNA levels of cytokines in cells pretreated with these low and high concentrations of FKN. Although there was no difference in expression of anti-inflammatory cytokines such as TGF-β or IL-10, we found that IL-23 p19 mRNA expression was significantly elevated in cells pretreated with 3 nM FKN and stimulated with LPS in comparison to those in cells not pretreated or pretreated with 0.03 nM FKN (Fig. 7A). Since induction of IL-23 p19 by high concentrations of FKN was blocked by anti-CX3CR1-neutralizing Ab, it was assumed to be mediated by CX3CR1 (Fig. 7A). Since 0.03 nM FKN as well as 3 nM FKN fully induced chemotaxis (data not shown), we assumed that signal transduction via CX3CR1 was sufficient at this relatively low concentration of FKN, and additional signals that promote IL-23 p19 expression might be induced at a higher concentration of FKN. This hypothesis was supported by the observation that addition of IL-23 after pretreatment with 0.03 nM FKN abolished the suppressive effect of 0.03 nM FKN (Fig. 7, B and C). Furthermore, early export of NF-κB p65 from the nuclei in the cells pretreated with 0.03 nM FKN was prevented in the presence of IL-23 (Fig. 7C). The significance of IL-23 in the action of 3 nM FKN was also supported by experiments using anti-IL-23 Ab. Both secretion of TNF-α and retention of NF-κB p65 in the nuclei after LPS stimulation in cells pretreated with 3 nM FKN were abolished in the presence of anti-IL-23 p19 Ab (Fig. 7, D and E).

Discussion
This is the first report of an immunomodulatory effect of FKN independent of its well-investigated function as a chemokine and adhesion molecule. The mechanism of the anti-inflammatory effect of relatively low concentrations of FKN involved activation of PPAR-γ by induction of its ligand, 15d-PGJ2, and alteration of signaling via ERK1/2 and NF-κB. These novel immune regulation systems in macrophages are discussed below.

Up-regulation of FKN expression in inflammatory tissue has drawn attention to its potential as a target of anti-inflammatory treatment for various autoimmune diseases. However, FKN is constitutively produced by intestinal epithelial cells and its receptor, CX3CR1, is expressed on tissue-resident dendritic cells and macrophages in the intestine and CNS. In the case of murine intestine, we found that a considerable amount of FKN was expressed in the colon, and colonic myofibroblasts were another significant source of FKN (our unpublished data). Of note, the resident macrophages of the intestine are hyporesponsive to inflammatory stimuli with bacterial components such as LPS. It is evident, especially in the large intestine, that macrophage-like cells in the colonic lamina propria are mostly anergic in response to endotoxin, in contrast to the vigorous cytokine production by circulating monocyte via the same stimuli (33). For heavy colonization of indigenous Gram-negative bacteria in the colon, hyporesponsiveness of macrophages would be important for maintaining homeostasis of...
mucosal immunity. The possibility that FKN participates in rendering macrophages hyporesponsive to LPS was first demonstrated in this study. This effect of FKN is reminiscent of the phenomenon of endotoxin tolerance, i.e., exposure of macrophages to LPS induces a hyporesponsive state to a second challenge with LPS. Although various mechanisms are involved in endotoxin tolerance, few studies have reported the mechanism of hyporesponsiveness of intestinal macrophages at the molecular level. For example, the IκBNS is a key molecule that inhibits IL-12 and IL-6 production in murine intestinal macrophages, although this mechanism was irrelevant to TNF-α secretion (31). Lack of MD-2 expression in intestinal myeloid-type cells (33) or epithelial cells (40) also has been postulated as a mechanism for the limited responses to LPS derived from indigenous flora. However, in our study, FKN did not down-regulate expression of the TLR4-MD-2 completely, although the level of mRNA was significantly reduced. Furthermore, IL-10 was not entirely responsible for the suppression of TNF-α. We also found that Bcl3 and TNFR-associated factor 6 were not significantly involved in this system (our unpublished data). Thus, the mechanism of inhibition of LPS-triggered TNF-α secretion by FKN is distinct from those mechanisms already known for the phenomenon of endotoxin tolerance.

In the current study, we found that FKN up-regulated PPAR-γ expression with its ligand and reduced production of TNF-α. This was also associated with modulation of subunit usage of NF-κB; the p65 subunit did not form a complex with the p50 subunit as seen in the LPS-challenged cells without FKN pretreatment. Instead, PPAR-γ formed a complex with the p65 subunit, which seemed to facilitate early export of p65 from nuclei. Inhibition of NF-κB activation by cytoplasmic protein IκB, which prevents nuclear translocation of p65, is not likely to be a major factor in the FKN system because nuclear translocation of p65 took place 1 h after LPS stimulation in our experiment. Our results indicated that the p65 subunit was once translocated into the nucleus, but in complex with PPAR-γ was rapidly exported. We are not the first to describe the function of PPAR-γ in regulating inflammatory responses. When intestinal epithelial cells were treated with a strain of commensal bacteria, Bacteroides thetaiotaomicron, PPAR-γ underwent nucleocytoplasmic redistribution in complex with p65, which ultimately caused the attenuation of IL-8 expression induced by pathogenic Salmonella enteritis (37). The role of PPAR-γ as an anti-inflammatory factor is well known; activation of PPAR-γ inhibited the expression of various cytokines in monocytes and macrophages, principally by preventing the activation of NF-κB; however, its mechanism of action is not clear (38, 39). An endogenous ligand of PPAR-γ, 15d-PGJ2, a metabolite of PGD2 (36), exerts a strong anti-inflammatory effect on macrophages. In our experiment, the effect of exogenous 15d-PGJ2 on NF-κB activation and cytokine production was very similar to that of FKN, especially at 10 μM. Since induction of 15d-PGJ2 was observed after FKN treatment, it is likely that up-regulation of 15d-PGJ2 elicited the anti-inflammatory effect in our experimental system. It was initially reported that 15d-PGJ2 affected NF-κB activation in a PPAR-γ-dependent manner (39); however, a PPAR-γ-independent pathway was also reported later (42–45). Based on the results that cellular 15d-PGJ2 was up-regulated rapidly after pretreatment with FKN and remained up-regulated for 12 h and that expression of PPAR-γ mRNA was enhanced by exogenous 15d-PGJ2 in macrophages, FKN most probably increased the level of 15d-PGJ2 first, followed by up-regulation of PPAR-γ, which
resulted in the modulation of NF-κB activation. Indeed, our experiment using PPAR-γ siRNA clearly showed that the anti-inflammatory effect of FKN depends on the presence of PPAR-γ, although we think it is still possible that FKN-induced 15d-PGJ2 or other unknown anti-inflammatory signaling pathways, independent of PPAR-γ, may directly affect expression of TLR4-MD-2 and phosphorylation of ERK1/2.

It is notable that the anti-inflammatory effect of FKN was seen when cells were pretreated with FKN at a concentration of 0.03 nM but not at 3 nM. We clearly observed a dose-dependent difference in every assay of signaling systems, such as ERK1/2 phosphorylation, complex formation of NF-κB p65 and PPAR-γ, early export of p65 from nuclei, induction of PPAR-γ mRNA, as well as up-regulation of 15d-PGJ2; all supported inhibition of TNF-α secretion by 0.03 nM but not 3 nM FKN. We could only partially clarify the mechanisms of the anti-inflammatory effect specific to this concentration of FKN. Since chemotaxis was fully triggered at 0.03 nM FKN, this concentration of FKN was sufficient to transduce classical signaling via CX3CR1. It also indicated the probability that 3 nM FKN induced additional signaling pathways, and our result demonstrated that IL-23 counters the anti-inflammatory effect of FKN. We assumed that this duality might be caused by differential rates of occupancy and dimerization of CX3CR1. It is possible that the proinflammatory action of FKN may depend partially on the induction of IL-23, which would be up-regulated in the cells exposed to a higher than physiological concentration of FKN. It has been shown that IL-23 is a potent activator of macrophages that enhances TNF-α expression (46) and transgenic expression of IL-23 p19 induces multiorgan inflammation (47); however, its regulatory role in mucosal inflammation is either proinflammatory (48) or anti-inflammatory (49) according to the disease models used. Such dose-dependent dual effects of FKN were not described in microglia, in which FKN was capable of attenuating LPS-induced TNF-α secretion in a dose-dependent manner (17). Differences in surface expression of LPS receptors, such as low CD14 in microglia (50), might cause this cell-specific effect. A detailed mechanism of interaction between IL-23 and FKN in macrophages is now under investigation in our laboratory. The reason why FKN has these specific effects at certain concentrations may lie in the unrevealed general mechanism of chemotaxis. Chemotaxis occurs when cells recognize a “concentration gradient” and move toward the area of higher concentration; however, the sensor mechanism for the concentration gradient is largely unknown. To determine the direction of chemotaxis, a single cell may have sensors for both low and high concentrations of ligands that mediate different signals in a three-dimensional intracellular mapping system. Our finding of dose-dependent, differential effects through CX3CR1 might represent the nature of chemokine receptors. With respect to physiological relevance, it is reasonable that cells respond in a distinct manner in the milieu of low and high concentrations of chemokine. For example, macrophages in the intestine at steady state exposed to a relatively low physiological level of FKN acquired hyporesponsiveness to LPS, which prevented an excessive inflammatory response to commensal flora. When inflammation occurs and higher concentrations of FKN and additional inflammatory cytokines are produced, newly recruited inflammatory macrophages have the potential to fully respond to produce TNF-α as an immune defense mechanism. According to previous reports, concentrations of FKN in the plasma of healthy humans were <0.03 nM (1 ng/ml) and approached 1 nM (30 ng/ml) in the plasma of patients with inflammatory airway disease (51). Thus, this study used both low and high concentrations of FKN, which may approximate those of steady-state and inflammatory situations, respectively.

Our results showed the anti-inflammatory effect of FKN, which is constitutively expressed in the colon where a high level of PPAR-γ is also expressed (52). The presence of soluble factors is known to suppress the inflammatory reaction of macrophages (32). We propose that the physiological concentration of FKN may be one such factor that maintains immune homeostasis in the intestine.

Acknowledgments

We thank Drs. Sachiko Akashi and Kensuke Miyake at Tokyo University for providing us with mAb15-21.

Disclosures

The authors have no financial conflict of interest.

References


