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J Immunol 2006; 177:7050-7058; doi: 10.4049/jimmunol.177.10.7050
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The Immunosuppressant Cyclosporin A Antagonizes Human Formyl Peptide Receptor through Inhibition of Cognate Ligand Binding

Pangke Yan,‡ Masakatsu Nanamori,‡ Meiling Sun,* Caihong Zhou,† Ni Cheng,‡ Na Li,* Wei Zheng,‡ Lihua Xiao,‡ Xin Xie,* Richard D. Ye,† and Ming-Wei Wang3*

Cyclosporin A (CsA) is a fungus-derived cyclic undecapeptide with potent immunosuppressive activity. Its analog, cyclosporin H (CsH), lacks immunosuppressive function but can act as an antagonist for the human formyl peptide receptor (FPR). More recent studies have shown that CsA also inhibits fMLF-induced degranulation in differentiated HL-60 promyelocytic leukemia cells. However, it is unclear whether CsA interferes with ligand-receptor interaction, G protein activation, or other downstream signaling events. In this study we used human neutrophils, differentiated HL-60 cells, and rat basophilic leukemia (RBL)-2H3 cells expressing human FPR (RBL-FPR) to identify the action site of CsA. In functional assays, CsA inhibited fMLF-stimulated degranulation, chemotaxis, calcium mobilization, and phosphorylation of the MAPKs ERK 1/2 and the serine/threonine protein kinase Akt. CsA also blocked Trp-Lys-Tyr-Met-Val-Ile (WKYMVm)-induced functions in RBL-FPR cells. Concentrations for half-maximal inhibition with CsA are generally 6- to 50-fold higher than that of CsH. CsA was compared with another immunosuppressant, ascomycin, relative to the inhibitory effects on FPR-mediated chemotaxis, calcium mobilization, and degranulation. In these experiments, ascomycin produced no inhibitory effects at low micromolar concentrations (1–4 μM), whereas the inhibitory effects of CsA were prominent at comparable concentrations. Finally, CsA dose-dependently inhibited the uptake of f(Leu-Phe-Nle-Tyr)-fluorescein and [3H]fMLF or [125I]WKYMVm binding to FPR. However, CsA and CsH did not show any obvious inhibitory effect on FPR-like 1-mediated cellular functions. These results demonstrate that CsA is a selective antagonist of FPR and that its inhibition of fMLF-stimulated leukocyte activation is at the level of cognate ligand binding. The Journal of Immunology, 2006, 177: 7050–7058.

The observations that human neutrophils migrate to site of bacterial infection and that the injection of live bacteria into rabbit tissues induces neutrophil infiltration suggest the presence of cell surface receptors that can detect bacterial products and transduce signals for neutrophil chemotaxis (1). The human formyl peptide receptor (FPR)4 fulfills this function through its binding of the bacterially derived chemotactic peptide fMLF and its ability to activate heterotrimeric G proteins and the downstream signaling molecules (2). Human FPR, encoded by the Fpr1 gene, is a 350-aa receptor with a seven membrane-span structure characteristic of receptors in the rhodopsin family (3–5). Binding of fMLF and other formyl or nonformyl chemotactic peptides results in the activation of neutrophil functions including adhesion, chemotaxis, degranulation, and superoxide production (6). These bactericidal activities are important for the elimination of invading pathogens. Experimental data indicate that mice lacking the Fpr1 gene are more susceptible to Listeria monocytogenes infection with an increased mortality rate (7).

Cyclosporins are cyclic undecapeptides produced by fungi. The immunosuppressive effect of cyclosporin A (CsA) was initially identified in 1972 and subsequently applied in the prevention of rejection following organ transplantation in humans (8). CsA is lipophilic, readily penetrates the plasma membrane, and exerts its immunosuppressive action through binding to the cytosolic protein cyclophilin (9). The complex of CsA and cyclophilin inhibits calcineurin, thereby disrupting transcriptional induction of IL-2 and T cell activation. A variety of structural analogues of CsA are produced either by fungi or through chemical synthesis. Among these analogues, cyclosporin H (CsH) is an optical isomer of CsA that contains the amino acid residue N-methyl-d-valine at position 11 instead of N-methyl-l-valine as in CsA (10). Unlike CsA, CsH lacks immunosuppressive activity and only weakly interferes with the MDR1 P-glycoprotein involved in multidrug resistance. However, CsH displays selective antagonistic activity for human FPR (11). Early studies demonstrated that CsH is 14-fold more potent than tertiary butoxycarbonyl analogues of fMLF such as Boc-FLFLF in FPR binding assays, and ~5-fold more potent than Boc-FLFLF in the inhibition of fMLF-stimulated calcium mobilization.

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and enzyme release (12). A subsequent study identified CsH as an inverse agonist (negative antagonist) that suppresses the constitutive activity of FPR in a GTPase activity assay (13). Both Boc-FLFLF and CsH competitively displace \(^{[1]}\)HfMLF bound to FPR, indicating that its antagonistic activity is mediated through inhibition of fMLF binding to the receptor.

Studies conducted more recently have led to the finding that CsA, although less potent than CsH, also inhibits fMLF-induced degranulation in differentiated HL-60 promyeloid leukemia cells (14). These findings are of potential significance because CsA is a therapeutic agent and its antagonistic effect may contribute to the weakened innate immunity as seen in some patients taking this immunosuppressant. However, CsA has been previously shown to suppress IgE-mediated degranulation through FcεRI cross-linking, suggesting that an inherent property, rather than an FPR-specific mechanism, may contribute to this action (15). Because CsA can readily enter into cells, its inhibition of fMLF-stimulated degranulation may result from an interruption of signaling events mediated by FPR. The current study was conducted in an attempt to further characterize the potential FPR antagonistic activity of CsA and determine the site of its action. Using combined approaches including functional assays (e.g., chemotaxis, calcium mobilization, and MAPK activation) and binding assays with radiolabeled peptide ligands, we found that CsA dose-dependently suppresses fMLF- and Trp-Lys-Tyr-Met-Val-d-Met (WKYMVM)-induced cell activation and inhibits their binding to the receptor. The inhibitory effects could not be reproduced with ascomycin, an immunosuppressant of different structure (16). In addition, CsA and CsH did not show any obvious inhibitory effect on FPR-like 1 (FPRL1)-mediated cellular functions. These results led us to conclude that CsA inhibits fMLF- and WKYMVM-induced leukocyte activation through competitive binding to the human FPR.

Materials and Methods

Materials

Cyclosporins were produced at Fujian Institute of Microbiology (Fuzhou, China) as described (17, 18). \(^{[1]}\)HfMLF and \(^{[125]}\)IUWKYMVM were purchased from PerkinElmer. Fluoro-4-acetoxymethyl ester (Fluo-4/AM), N-dibutyryl-cAMP (dbcAMP) were products of Molecular Probes. Ascomycin was bought from Calbiochem. Other reagents including probenecid, fMLF, procured from Molecular Probes. Ascomycin was bought from Calbiochem. Fluo-4-acetoxymethyl ester (Fluo-4/AM), from Sigma-Aldrich. WKYMVm was synthesized at GL Biochem. The collagen containing a 74% Percoll cushion and centrifuged at 45,000 rpm for 1 h. Neutral proteases including collagenase and enzyme release (12). A subsequent study identified CsH as an inverse agonist (negative antagonist) that suppresses the constitutive activity of FPR in a GTPase activity assay (13). Both Boc-FLFLF and CsH competitively displace \(^{[1]}\)HfMLF bound to FPR, indicating that its antagonistic activity is mediated through inhibition of fMLF binding to the receptor.

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**Ligand binding assay**

Ligand binding assay was performed as previously described (19). Briefly, RBL-FPR cells were seeded in 24-well cell culture plates at a density of 1.0 × 10\(^5\) per well for 48 h. After reaching confluency, cells were washed twice with blocking buffer (RPMI 1640 supplemented with 25 mM HEPES (pH 7.5) and 0.1% BSA) twice with 0.1% BSA, incubated in blocking buffer for 10 min at 37°C. After removing the blocking buffer, different concentrations of test compounds were added together with either 30 nM \(^{[1]}\)HfMLF or 0.3 nM \(^{[125]}\)IUWKYMVM in a final volume of 200 μl in blocking buffer (PBS with 10% BSA). In a parallel experiment, unlabeled fMLF or WKYMVM of different concentrations was added at the same time. After incubation on ice for 2 h, the buffer with unbound radioligand were removed and the cells were rinsed five times with binding buffer and lysed with lysis buffer (20 mM Tris-HCl (pH 7.5) and 1% Triton X-100). Binding assay for RBL-FPRL1 was conducted similarly except that 0.16 nM \(^{[125]}\)IUWKYMVM was used. Samples were collected and measured in a Microbeta scintillation counter (PerkinElmer).

**Calcium mobilization assay**

A calcium mobilization assay was performed as previously described (22). Briefly, RBL-FPR cells were detached and HL-60 cells collected by centrifugation. Cells were loaded with 5 μM Fluo-4/AM in HBSS supplemented with 2.5 mM probenecid for 45 min and then washed twice with HBSS. Cell suspensions were plated onto 96-well plates at a density of 60,000 cells in 100 μl of medium per well. Cells were reattached by centrifugation, incubated with or without test compounds for 15 min before the addition of fMLF or WKYMVM (10 nM each for RBL-FPR cells and 2 nM fMLF for HL-60 cells), and then analyzed for calcium mobilization using FlexStation (Molecular Devices) with the excitation wavelength at 485 nm and the emission wavelength at 525 nm. Calcium mobilization assay for RBL-FPRL1 was conducted similarly except that 2 nM WKYMVM was used. For calcium mobilization in chemokine-stimulated cells, freshly prepared human neutrophils or THP-1 monocytic cells were loaded with Indo-1 and stimulated with 10 nM of IL-8 or MCP-1, respectively. Calcium influx was determined using a spectrofluorometer, as described previously (19).

**Degranulation assay**

The amount of β-hexosaminidase released from RBL-FPR cells was measured as reported previously (23). In brief, cells (2 × 10\(^5\)/well) were cultured overnight in 24-well tissue culture plates. They were then washed twice with HBSS and incubated for 5–8 min with or without test compounds at different concentrations before the addition of fMLF (100 nM) or WKYMVM (200 nM). The reaction was terminated 10–15 min after stimulation by placing the plate on ice. The amount of hexosaminidase secreted into the medium was determined by incubating 20 μl of supernatant with 10 μl of 1 M p-nitrophenyl-N-acetyl-β-D-glucosaminide in sodium citrate buffer (0.1 M, pH 4.5) at 37°C for 1 h. At the end of the incubation, 200 μl of 0.1 M Na\(_2\)CO\(_3\) and 0.1 M NaHCO\(_3\) (pH 10) was added. Absorbance at 405 nm was determined in a VersaMax tunable microplate reader (Molecular Devices). Total cellular β-hexosaminidase was determined with cell lysate in 0.1% Triton X-100. Data are collected from several experiments and presented as the percentage of total β-hexosaminidase released.

For β-glucuronidase release induced by FcεRI cross-linking, RBL-2H3 cells were incubated with monoclonal anti-DNP IgE (clone SPE-7) from Sigma-Aldrich; 1/5,000 or 0.2 μg/ml for 16 h. Following a brief wash with HBBS-HB (20 mM HEPES (pH 7.4) and 0.2% BSA), cells were preincubated with 10 μM cytochalasin B for 15 min on ice and then 15 min at 37°C. The indicated amounts of inhibitors were added for 5 min at 37°C before cell stimulation with DNP-BSA (GE Healthcare Bio-Sciences) for 10 min at 37°C. The degranulation reaction was terminated and the amount of secreted β-hexosaminidase was quantified by incubating 20 μl of supernatant with 10 μl of 1 M p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate buffer as described above.

For β-glucuronidase release, human blood neutrophils were preincubated with 10 μM cytochalasin B and then with cyclosporins as described above. The cells were then stimulated with fMLF for 10 min at 37°C. The degranulation reaction was terminated by placing samples on ice. The cells were separated from the supernatant through centrifugation. The amount of released β-glucuronidase was quantified by incubating 20 μl of supernatant with 20 μl of 10 mM 4-methylumbelliferyl-β-D-glucuronide hydrate in 0.1 M sodium citrate buffer (pH 4.0) and 0.1% Triton X-100 at 37°C for 15 min. This reaction was terminated by addition of 300 μl of stop solution (50 mM glycine and 5 mM EDTA (pH 10.4)). Fluorescence was measured immediately using a spectrofluorometer (Photon Technology International), with...
an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

Chemotaxis

The chemotaxis assay was performed as previously described (24). Migration of RBL-FPR cells induced by fMLF or WKYMVm was assessed in a 48-well microchemotaxis chamber (NeuroProbe). Briefly, fMLF or WKYMVm (10 nM each; 30 μl) was placed in the lower chamber and RBL-FPR cells (50 μl at 1 x 10^6 cells/ml) were preincubated with or without test compounds for 15 min and then loaded in the upper chamber, which was separated from the lower chamber by a polycarbonate filter (pore size of 8 μm). After incubation at 37°C for 4 h, the filter was removed, fixed, and stained with Diff-Quick staining solutions (IMEB). Chemotaxis was quantified by counting migrated cells in five randomly chosen high-power fields (x400).

Phosphorylation of MAPKs and Akt

Activation of the p44/p42 MAPKs (ERK1/2) and Akt were determined based on activation-associated phosphorylation. Cells were cultured in 12-well plates for 48 h and serum starved overnight. They were pretreated with or without test compounds for 8 min, and then loaded in the upper chamber of a microchemotaxis chamber (NeuroProbe). Briefly, fMLF (100 nM) induced the release of 18% of total cellular α-N-acetylhexosaminidase in the absence of CsA. Treatment of the cells with CsA resulted in a concentration-dependent reduction in enzyme release. The IC_{50} was determined to be 165 nM. CsH, a known antagonist of FPR (12), was tested in a parallel experiment. CsH is 7- to 8-fold more potent in the inhibition of fMLF-induced β-hexosaminidase release, with an IC_{50} of 22 nM (Fig. 1B). In addition to CsA and CsH, we also tested two dihydro (2H) analogues of these cyclosporins, and their relative potency

Ligand uptake

RBL-FPR cells (~5,000) were seeded in 96-well plates for 48 h. Serum was removed and cells were incubated with serum-free medium at 37°C for 1 h. Test compounds were mixed with the cells at either 4°C or 37°C as indicated. After incubation for 5 min before fMLF (100 nM) stimulation, the indicated amounts of cyclosporins for 5 min before fMLF (100 nM) induced the release of Tyr-Lys-fluorescein (10 nM) was added thereafter, and the cells were incubated for another 45 min at 37°C. Following the incubation, the cells were washed twice with PBS and fixed with 3.7% formalin in PBS. After a brief washing, the cells were stained with Hoechst 33342 (10 μg/ml) for 15 min before washing with PBS. The cells were then placed in PBS at 4°C before imaging analysis with an ArrayScan high content screening system (Cellomics).

Data analysis

Data were analyzed using GraphPad Prism software, version 4. Nonlinear regression analyses were performed to generate dose-response curves to calculate IC_{50} values.

Results

CsA antagonizes FPR cognate ligand-stimulated degranulation

To determine the antagonistic effect on fMLF-induced receptor activation and cellular functions, a group of 14 cyclosporins were examined for their abilities to inhibit fMLF-induced calcium mobilization in RBL-FPR cells. The results are summarized in Table I. The four compounds that exhibited most potent inhibitory effects were chosen for further analyses.

Table I. Structures of cyclosporins tested in this study

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>% Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>DihydroCsH</td>
<td>[D-MeVal^{11}]dihydroCsA</td>
<td>8.77</td>
</tr>
<tr>
<td>CsH</td>
<td>[D-MeVal^{11}]CsA</td>
<td>7.12</td>
</tr>
<tr>
<td>DihydroCsA</td>
<td>R1: CH₂CH₂CH₃CH₃; R₂=CH₃; R₃=H; R₄=CH₃</td>
<td>16.88</td>
</tr>
<tr>
<td>CsA</td>
<td>R1: (E)CH₂CH=CH(=CH)CH₃; R₂=CH₃; R₃=H; R₄=CH₃</td>
<td>15.53</td>
</tr>
<tr>
<td>CsC</td>
<td>R1: (E)CH₂CH=CH(=CH)CH₃; R₂=H; R₃=CH₃; R₄=CH₃</td>
<td>51.67</td>
</tr>
<tr>
<td>CsB</td>
<td>R1: (E)CH₂CH=CH(=CH)CH₃; R₂=CH₃; R₃=H; R₄=CH₃</td>
<td>76.71</td>
</tr>
<tr>
<td>DihydroCsC</td>
<td>R1: CH₂CH₂CH₃CH₃; R₂=CH₃; R₃=H; R₄=CH₃</td>
<td>88.09</td>
</tr>
<tr>
<td>CsD</td>
<td>R1: (E)CH₂CH=CH(=CH)CH₃; R₂=H; R₃=CH₃; R₄=CH₃</td>
<td>92.46</td>
</tr>
<tr>
<td>DihydroCsD</td>
<td>R1: CH₂CH₂CH₃CH₃; R₂=CH₃; R₃=H; R₄=CH₃</td>
<td>92.64</td>
</tr>
<tr>
<td>Diacetyl CsC</td>
<td>R1: (E)CH₂CH=CH(=CH)CH₃; R₂=COCH₃; R₃=OCOCH₃; R₄=CH₃</td>
<td>94.46</td>
</tr>
<tr>
<td>Monoaacetyl CsC</td>
<td>R1: (E)CH₂CH=CH(=CH)CH₃; R₂=COCH₃; R₃=H; R₄=CH₃</td>
<td>94.78</td>
</tr>
<tr>
<td>Acetyl CsA aldehyde</td>
<td>R₁: CH₃CHO; R₂: COCH₃; R₃: H; R₄: CH₃</td>
<td>101.61</td>
</tr>
<tr>
<td>Acetyl CsA diol</td>
<td>R₁: CH₂CH(=O)CH(=O)CH₃; R₂=COCH₃; R₃=H; R₄=CH₃</td>
<td>107.47</td>
</tr>
<tr>
<td>Acetyl CsA diol</td>
<td>R₁: CH₂CH(=O)CH(=O)CH₃; R₂=COCH₃; R₃=H; R₄=CH₃</td>
<td>115.64</td>
</tr>
</tbody>
</table>

A general structure of cyclosporins is shown in the top panel. For each compound tested in this study (first column), substitutions at the 11th position and at R1, R2, R3, and R4 are detailed in the second column. The effect of each compound (10 μM) in fMLF (10 nM)-induced calcium mobilization is shown as change in the percentage of response relative to vehicle (100% response). The compounds in bold were further analyzed in this study.
We found that the four cyclosporins also inhibited Materials and Methods. A described in to that of CsA or CsH in degranulation assays (Fig. 1, E–H) for another 15 min. The released β–hexosaminidase was measured as described in Materials and Methods. A and E, CsA; B and F, CsH; C and G, 2HCsA; D and H, 2HCsH. The results are presented as means ± SEM from three independent experiments.

(C50 = 297 nM for 2HCsA and 36 nM for 2HCsH) is similar to that of CsA or CsH in degranulation assays (Fig. 1, C and D).

The synthetic peptide WKYMVm is a ligand for both FPR and FPRL1. We found that the four cyclosporins also inhibited β–hexosaminidase release in WKYMVm-stimulated RBL-FPR cells (Fig. 1, E–H). Consistent with the study using fMLF, CsH (C50 = 12 nM; 18 nM for 2HCsH) appeared to be more potent than CsA (C50 = 523 nM; 1.3 μM for 2HCsA) in this assay.

Neutrophils respond to fMLF with a release of enzymes contained in several different populations of granules. To determine whether CsA affects neutrophil degranulation, we measured fMLF-induced release of β-glucuronidase, which is most abundant in primary granules (25). In this study, CsA clearly displayed an inhibitory effect (Fig. 2A). The potency of CsA is ~10-fold less than that of CsH based on the data from a parallel experiment (Fig. 2B).

CsA and CsH inhibit FPR cognate ligand-induced chemotaxis

Previous studies of CsA focused on fMLF-stimulated degranulation for its inhibitory effect (14). Because published data demonstrated inhibition of FcεRI-mediated degranulation by CsA treatment (15), it was unclear whether the observed CsA inhibition of fMLF-induced degranulation is FPR dependent. We sought to examine other leukocyte functions that are induced by fMLF to further characterize the inhibitory effect of CsA on FPR-mediated cell activation. Using RBL-FPR cells, the fMLF-induced chemotaxis was monitored in samples treated with CsA, and the result was compared with those from cells treated with 2HCsA, CsH, and 2HCsH (Fig. 3, A–D). An inhibitory effect on chemotaxis was evident at CsA concentrations above 20 nM (IC50 = 2.3 μM). 2HCsA displayed a similar potency in this assay (IC50 = 3.3 μM). Both CsH and 2HCsH were 10- to 50-fold more potent (IC50 = 49 and 278 nM, respectively) than CsA and 2HCsA in the inhibition of chemotaxis. Likewise, the WKYMVm-induced chemotaxis of RBL-FPR cells was also inhibited by the four cyclosporins (Fig. 3, E–H). Although potencies of CsA (IC50 = 1.7 μM), 2HCsA (IC50 = 2.2 μM), and 2HCsH (IC50 = 282 nM) remained similar, the effect exerted by CsH was somehow reduced (IC50 = 270 nM). Thus, the inhibitory effects of these cyclosporins are not restricted to leukocyte degranulation.

CsA and its analogues inhibit FPR cognate ligand-induced calcium mobilization

Calcium mobilization in response to fMLF was assayed using both RBL-FPR and dbcAMP-differentiated HL-60 cells, which express FPR on the plasma membrane and display neutrophil-like properties (26). With RBL-FPR cells, CsA and 2HCsA exhibited similar inhibitory effects on fMLF-induced calcium mobilization with C50 values in the range of 630–761 nM (Fig. 4A). Both CsH and 2HCsH showed a more potent inhibition in this assay, giving C50 values of 88 and 104 nM, respectively. In differentiated HL-60 cells, CsH and 2HCsH produced similar inhibitory effects with C50 values of 84 and 95 nM, respectively. However, CsA and 2HCsA are less potent (C50 ~ 2–4 μM) in the HL-60 cells as compared with RBL-FPR cells (Fig. 4B). The cause for this discrepancy is unclear and will be discussed below. In addition to inhibiting fMLF-induced calcium mobilization, the four cyclosporins also reduced WKYMVm-stimulated calcium flux in RBL-FPR cells (Fig. 4C). Although the C50 values were similar for
CsA and CsH displayed a reduced suppression in this case with IC50 values ranging between 844 and 858 nM. To determine the specificity of CsA and CsH in inhibiting fMLF- or WKYMVm-induced calcium mobilization through FPR, human neutrophils were treated with either CsA or CsH before stimulation with 10 nM IL-8. No inhibition of IL-8-induced calcium mobilization was observed with up to 10 μM CsA or CsH (data not shown). In another experiment, the effect of the two cy-closporins on MCP-1-induced calcium mobilization was determined in THP-1 cells. At a concentration of 10 nM, CsA did not alter MCP-1-induced calcium mobilization in the treated cells, whereas CsH treatment caused a small (10%) decrease in the magnitude of calcium mobilization (data not shown).

A comparison between CsA and ascomycin in fMLF-induced leukocyte activation assays

Based on the above data and published reports, it cannot be ruled out that the observed inhibitory effects on fMLF-induced leukocyte functions are mediated through the immunosuppressant property of CsA. This possibility was examined in side-by-side experiments with both CsA and ascomycin (FK-520), a macrolide antibiotic that displays potent immunosuppressive effect but differs from CsA in chemical structure (16, 27) (Table I). Consistent with the previous reports, both CsA and ascomycin inhibited degranulation by FcεRI cross-linking in a dose-dependent manner (Fig. 5A). In contrast, no significant inhibition of fMLF-induced β-hexosaminidase release was observed when RBL-FPR cells were pretreated with up to 1 μM ascomycin, whereas a ~40% inhibition of degranulation was obtained in CsA-treated cells (Fig. 5B). At 10 μM
ascomycin a statistically significant inhibition was obtained in ascomycin-treated cells. This inhibition may represent a nonspecific effect, possibly related to its immunosuppressive property (15).

Next, we examined the effects of CsA and ascomycin in fMLF-induced chemotaxis using the same RBL-FPR cells. In this study, ascomycin concentration of up to 2 μM did not produce significant inhibition of directed cell migration. Between 2 and 20 μM, a small but statistically significant inhibition was observed with ascomycin. In comparison, a more potent inhibition was seen in cells treated with the same amounts of CsA (Fig. 5C).

Finally, fMLF-induced calcium mobilization was examined in differentiated HL-60 cells. Treatment with CsA caused a dose-dependent decrease in calcium mobilization. In parallel studies, ascomycin did not produce significant inhibition at concentrations up to 20 μM (Fig. 5D) under the same experimental conditions.

Taken together, these results clearly demonstrate a difference between CsA and ascomycin in modulating the fMLF-induced leukocyte activation, with CsA being more potent than ascomycin in each of the above assays. The small inhibitory effect observed with high concentrations of ascomycin may be nonspecific and independent of FPR.

CsA inhibits fMLF-induced activation of ERK 1/2 and Akt

We reasoned that if CsA blocks FPR signaling at the receptor or G protein level, then fMLF-induced activation of downstream kinases would be inhibited as well. Phosphorylations of ERK and Akt are early signaling events triggered by chemoattractants in leukocytes. In RBL-FPR cells, fMLF (100 nM) stimulated a significant increase in the phosphorylation of ERK (Fig. 6A) and Akt (Fig. 6B). Treatment of these cells with CsA or CsH before fMLF stimulation dose-dependently inhibited phosphorylation of these kinases in the drug concentration range of 0.1–1 μM. In this assay, CsH is ∼10-fold more potent than CsA based on the level of protein phosphorylation.

CsA suppresses FPR-mediated uptake of a fluorescein-conjugated formyl peptide ligand

The peptide tNle-Leu-Phe-Nle-Tyr-Lys is a potent agonist for FPR and is widely used as a fluorescein-conjugated ligand in studies of FPR (28). Incubation of RBL-FPR cells at 37°C with tNle-Leu-Phe-Nle-Tyr-Lys-fluorescein caused its cellular uptake with a punctuated pattern (Fig. 7, top left panel). Preincubation with CsA and CsH abolished this effect (Fig. 7, middle left and lower left panels). When preincubation with the cyclosporins is conducted at 37°C, the reduced uptake may result from either inhibition of formyl peptide ligand binding or internalization of the cyclosporin-bound FPR, thereby reducing the available cell surface receptors. This latter possibility was tested by preincubation of the cells at 4°C, a nonpermissive temperature for receptor internalization (Fig. 7, right column). A similar inhibitory effect was observed under this experimental condition, indicating that the cyclosporins inhibit
fNle-Leu-Phe-Nle-Tyr-Lys-fluorescein uptake through interference with FPR binding.

CsA dose-dependently inhibits [3H]fMLF and [125I]WKYMVm binding to FPR

To directly examine whether the inhibitory effect of CsA is at the receptor level, RBL-FPR cells were incubated with fixed amount of [3H]fMLF and increasing concentrations of either CsA or 2HCsA in a competitive binding assay (Fig. 8A). Both cyclosporins dose-dependently displaced the FPR-bound radioligand with IC$_{50}$ values of 1.8 and 1.7 µM, respectively. In comparison, CsH and 2HCsH more readily displaced the bound [3H]fMLF with IC$_{50}$ values of 102 and 124 nM, respectively. Similar results were obtained from competitive binding with [125I]WKYMVm (Fig. 8B), and estimated IC$_{50}$ values were 6.3 µM for CsA, 7.8 µM for 2HCsA, 630 nM for CsH, and 351 nM for 2HCsA. Taken together, the data indicate that CsA, like its more potent analog CsH, directly suppresses cognate ligand binding to FPR.

CsA is a selective FPR antagonist

FPRL1 is a receptor that shares 69% of amino acid sequence homology with FPR. Competitive binding assay was conducted with RBL-FPRL1 cells to examine whether the cyclosporins also can bind to this receptor. Our results, shown in Fig. 9A, indicate that CsA and CsH could not compete for [125I]WKYMVm binding to FPRL1. Similarly, WKYMVm-induced calcium mobilization was not significantly affected by CsA or CsH pretreatment (Fig. 9B). Thus, CsA and CsH are not effective ligands for FPRL1.

Discussion

Previous studies have led to the discovery and characterization of CsH as a potent antagonist of human FPR (11, 12). These studies showed inhibition of fMLF-induced superoxide production in differentiated HL-60 cells, which express FPR and the NADPH oxidase components (26). In a subsequent study that investigated the mechanism of CsH inhibition, it was found that CsH could act as a negative antagonist (inverse agonist) that reduced the constitutive activity of human FPR when assayed for its ability to mediate GTP$_{i}$ binding to the G protein (13). Because the study was conducted using FPR-reconstituted Sf9 cell membrane, the results unequivocally demonstrated that CsH acts at the receptor level. Further evidence supporting this notion was obtained from competitive binding assay in which CsH displaced receptor-bound [3H]fMLF with a ~10-fold higher potency than Boc-FLFLF (13).

In comparison, the effect of CsA in FPR-mediated cell signaling and activation has not been characterized to the same extent. CsA is much less potent than CsH in inhibiting fMLF-induced superoxide production (11). Furthermore, although more recent studies demonstrated the inhibition of fMLF-induced degranulation by CsA and its analogues, it remains unclear whether the receptor or a signaling pathway was targeted by these cyclosporins (14, 29). Several immunosuppressants have been shown to inhibit mast cell degranulation induced by FceRI cross-linking (27, 30), an effect
Competitive binding of [125I]WKYMVm with cyclosporins. Binding assays were performed as described in Materials and Methods. A fixed amount of 3H-labeled fMLF (30 nM; A) or 125I-labeled WKYMVm (0.3 nM; B) was added to each well along with variable amounts of CsA ( ), 2HcsA ( ), CsH ( ), or 2HcsH ( ). After incubation on ice for 2 h, unbound radiolabeled ligand was washed off and cell-associated radioactivity determined by scintillation counting. Data presented are means ± SEM from three independent experiments.

FIGURE 8. Competitive binding of radiolabeled peptide ligands with cyclosporins. Binding assays were performed as described in Materials and Methods. A fixed amount of 3H-labeled fMLF (30 nM; A) or 125I-labeled WKYMVm (0.3 nM; B) was added to each well along with variable amounts of CsA ( ), 2HcsA ( ), CsH ( ), or 2HcsH ( ). After incubation on ice for 2 h, unbound radiolabeled ligand was washed off and cell-associated radioactivity determined by scintillation counting. Data presented are means ± SEM from three independent experiments.

apparently mediated through a mechanism shared among these agents and related to their immunosuppressive properties (15). Therefore, the inhibition of fMLF-induced degranulation by CsA may be attributed to a mechanism irrelevant to FPR antagonism. In fact, it was reported that FK-506, a potent immunosuppressant, could inhibit several neutrophil functions induced by fMLF in a FPR-independent manner (31). A previous study conducted on human basophils showed that CsA failed to competitively displace receptor-bound [125I]fMLF (32). Taken together, these findings suggest that CsA may exert its inhibitory effect on leukocyte activation through an FPR-independent mechanism.

Alternatively, it is conceivable that CsA acts on the FPR due to its structural similarity to CsH, an optical isomer. In the current study we present evidence that inhibition by CsA on fMLF- or WKYMVm-induced activities is not confined to degranulation and can include chemotaxis, calcium mobilization, and MAPK and Akt activation. We also showed that ascomycin, which suppresses FceRI-dependent mediator release in basophils and RBL-2H3 cells (27) (Fig. 5A), does not inhibit degranulation, calcium mobilization, and chemotaxis unless used at concentrations >10 μM. We further demonstrate that both IL-8-induced calcium influx in human neutrophils and MCP-1-induced calcium flux in the THP-1 monocytic cells were not affected by CsA at concentrations up to 10 μM (data not shown). These observations unequivocally point to a specific inhibitory effect of CsA on FPR under the experimental conditions. Additionally, our experimental data indicate that CsA inhibits the uptake of a fluorescein-labeled formyl peptide and that this effect results from the interference of formyl peptide binding but is not due to the CsA-induced FPR internalization that can reduce the available receptor on the cell surface. Finally, data from a competitive binding assay reveal that CsA competes with radiolabeled fMLF and WKYMVm for binding of the human FPR in transfected RBL-FPR cells (Fig. 8). These results provide strong evidence that CsA targeting at FPR is a primary mechanism for its inhibition of fMLF- and WKYMVm-induced leukocyte activation.

In previous studies conducted using HL-60 cells (11, 12), the inhibitory effect of CsA was significantly less than what we have observed in this report using the transfected RBL-FPR cells and human neutrophils. Our results also indicate a less potent inhibitory effect on calcium mobilization in dbcAMP-differentiated HL-60 (Fig. 4B) as compared with the transfected RBL-FPR cells (Fig. 4A). This apparent difference may have contributed to the early conclusion that CsA is not an antagonist for FPR. Although the mechanism for the discrepancy is unclear at present, there are several possibilities. The potency of the antagonistic effect of CsA on FPR may be affected by different cellular environments. Extending this possibility, CsA may exert its inhibitory effect through both antagonizing FPR and inhibiting an intracellular signaling event that contributes to the specific leukocyte functions. Our experimental data suggest this to be the case at high CsA concentrations (e.g., >10 μM). It is also possible that FPR polymorphism may affect experimental outcome using CsA. HL-60 contains two variants of FPR: FPR-98 and FPR-26 (3). The RBL-FPR cells that we generated only express the FPR-26. In the event that these two variants of FPR bind differently to CsA but respond equally to fMLF or WKYMVm, a discrepancy in CsA antagonism may result. This possibility will be examined in a future study.

The ligand binding pocket of FPR consists of several key residues, including Lys85 in the second transmembrane helix and Asp284 in the transmembrane 7 helix (33–35). Previous studies using fluorescein-labeled formyl peptides identified the size of the FPR binding pocket to be sufficient to accommodate five, but not six, amino acids (36). In comparison, cyclosporins are 11-aa cyclic peptides. Exactly how CsH and CsA interact with FPR and what structural basis dictates the different potency between CsH and CsA in FPR antagonism remain unresolved questions and are subject to further investigations. Published data suggest that the binding sites for fMLF and WKYMVm on
FPR may be adjacent but not identical (19, 37). Yet, CsA and CsH could effectively compete for the binding of both fMLF and WKYMVm as demonstrated unequivocally in this study. One of the possibilities is that the cyclosporins could occupy an area on the FPR that covers or overlaps with the binding sites of both fMLF and WKYMVm. It is also conceivable that the cyclosporins can produce an allosteric effect that causes reduced binding of fMLF and WKYMVm to the FPR. Extensive structural modifications and characterizations of cyclosporins have been conducted recently (14, 29) and have begun to provide information regarding the structural features of the ligand relevant to FPR interaction. Studies of FPR polymorphism and site-directed mutagenesis will likely help to uncover the determinants on the receptor that form a binding pocket for these structurally distinct ligands.

The specificity of CsA and CsH as FPR antagonists is confirmed by our experimental data showing that FPR1 binding of WKYMVm is not significantly affected by these cyclosporins. Whereas FPR1 is a low affinity receptor for fMLF, it binds WKYMVm with high affinity. Despite its shared binding property with FPR, FPR1 apparently contains different structural determinants that can distinguish WKYMVm from fMLF and cyclosporins. Because neutrophils express both FPR and FPR1, the absence of cyclosporin inhibition on FPR1 indicates that FPR, but not FPR1, is targeted by CsA and CsH. As a widely used immunosuppressant, CsA exerts its therapeutic action on T cells at an effective concentration of ≥10 nM (38, 39). Based on the data from this study as well as other published results (14, 40), inhibition of fMLF- or WKYMVm-induced cellular activities becomes apparent at low micromolar concentrations. Therefore, routine clinical use of CsA as an immunosuppressant probably affects neutrophil functions only minimally.

Acknowledgments
We are indebted to Xiuyan Cheng for technical assistance and to Dr. Dale E. Ma for critical review of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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