

Variable responses of formyl peptide receptor haplotypes toward bacterial peptides

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Abstract The chemoattractant neutrophil formyl peptide receptor (FPR) binds bacterial and mitochondrial *N*-formylated peptides, which allows the neutrophils to find the bacterial source and/or site of tissue damage. Certain inflammatory disorders may be due in part to an impaired innate immune system that does not respond to acute bacterial damage in a timely fashion. Because the human FPR is encoded by a large number of different haplotypes arising from ten single-nucleotide polymorphisms, we examined the possibility that some of these haplotypes are functionally distinct. We analyzed the response of three common FPR haplotypes to peptides from *Escherichia coli*, *Mycobacterium avium* ssp. *paratuberculosis*, and human mitochondria. All three haplotypes responded similarly to the *E. coli* and mitochondrial peptides, whereas one required a higher concentration of the *M. avium* peptide fMFEDAVAWF for receptor downregulation, receptor signaling, and chemotaxis. This raises the possibility of additional bacterial species differences in functional responses among FPR variants and establishes a precedent with potentially important implications for our innate immune response against bacterial infections. We also investigated whether certain FPR haplotypes are associated with rheumatoid arthritis (RA) by sequencing *FPR1* from

148 Caucasian individuals. The results suggested that FPR haplotypes do not significantly contribute toward RA.

Keywords Chemoattractant receptor · Inflammation · Chemotaxis · Cell signaling · Haplotype

Introduction

The human formyl peptide receptor (FPR), expressed mainly by myeloid cells (neutrophils and monocytes), binds a large number of bacterial *N*-formylated peptides. The prototypic ligand for FPR, *N*-formyl-methionine-leucine-phenylalanine (fMLF), was purified from *Escherichia coli* cultures (Marasco et al. 1984; Schiffmann et al. 1975). A recent study examined the activation of human promyelocytic leukemia (HL-60) cells expressing FPR by formylated peptides from *Listeria monocytogenes* and found cellular responses at nanomolar concentrations (Rabiet et al. 2005). In addition, formylated proteins synthesized in mitochondria were found to bind FPR, suggesting that peptides released from necrotic tissue and sites of inflammation may attract neutrophils and monocytes (Carp 1982; Rabiet et al. 2005). It has also been observed that certain nonformylated peptides can bind to neutrophils through FPR. These include urea- and carbamate-modified peptides, viral peptides derived from human immunodeficiency virus (HIV)-1, HIV-2, Ebola, and Corona virus, annexin I, and cathepsin G (Derian et al. 1996; Hartt et al. 2000; Higgins et al. 1996; Mills 2006; Su et al. 1999; Sun et al. 2004; Walther et al. 2000). Thus, FPR binds a large number of peptides that can be of endogenous, bacterial, or viral origin.

The coding sequence of *FPR1* contains ten single-nucleotide polymorphisms (SNPs); six are nonsynonymous,

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resulting in amino acid changes, and four are synonymous (Sahagun-Ruiz et al. 2001; Zhang et al. 2003; GenBank, www.ncbi.nlm.nih.gov/SNP/; current study). Based on haplotyping by Sahagun-Ruiz et al. and us (this study), haplotypes 1, 2, and 3 are among the most common in Caucasians. In the present work, we examined the effect of two bacterial peptides, fMLF from *E. coli* and fMFEDA-VAWF from *M. avium* ssp. *paratuberculosis*, and one human mitochondrial peptide from cytochrome c oxidase subunit I, fMFADRW, on receptor signaling and chemotaxis. These studies were carried out in Chinese hamster ovary (CHO) cells stably expressing FPR haplotypes H1, H2, and H3. Our results suggest that these haplotypes may be differentially activated by the mycobacterial peptide. This may have important implications in the initial stages of for example inflammatory bowel disease, based on the finding that infection with *M. avium* ssp. *paratuberculosis* can be correlated with the disorder in a subset of patients (Subramanian et al. 2006).

Another chronic inflammatory disorder, rheumatoid arthritis (RA), has not been linked to bacterial peptides but could theoretically be exacerbated by mitochondrial peptides from necrotic tissue. To establish whether different FPR haplotypes may have a role in the pathogenesis of RA, we carried out genotype and haplotype analysis of 74 Caucasian patients with RA and 74 controls. We observed only minor differences in haplotype distributions and, thus, it is unlikely that FPR plays a major role in the pathogenesis of RA.

Materials and methods

Cloning of FPR haplotypes Genomic DNA was isolated from healthy donors from 250- μ l whole blood using E.Z.N.A. blood DNA Kit II according to the manufacturer's instructions (Omega Biotek, Inc., Doraville, GA, USA). The coding sequence of *FPRI*, located in a single exon at chromosome 19q13.3, was amplified from 100-ng genomic DNA by polymerase chain reaction (PCR) essentially as described (Sahagun-Ruiz et al. 2001). Amplification utilized primer pairs FPR-41F22 and FPR+1063R22, where the first nucleotide of the primer is indicated by its position relative to the adenosine in the ATG translation initiation site, followed by F or R for forward or reverse and the number of nucleotides in the primer. Haplotypes were identified after ligation of the PCR amplicons into pGEM[®]-T Easy (Promega, Inc., Madison, WI, USA), and sequencing of the plasmid insert with T7 and SP6 primers. Haplotype 3 used in this study corresponds to the R-26 complementary DNA (cDNA; Boulay et al. 1990; GenBank accession number M60627). The haplotype nomenclature is based on a publication by Sahagun-Ruiz et al. (2001).

Plasmid construction and transfection of CHO cells The *FPR* cDNA inserts encoding haplotypes 1, 2, and 3 were ligated into the pBGSA expression vector (GenBank accession number AY6607190), which confers G418 resistance (Uthayakumar and Granger 1995), and the plasmids were transfected into CHO cells, as described previously (Gripentrog et al. 2003; Miettinen et al. 1997). Stably expressing clones were identified by immunofluorescence microscopy of 18, or in some cases 36 clones, using anti-FPR antibodies. CHO transfectants with similar expression levels of the different haplotypes were selected on the basis of western blot analysis using anti-FPR antibodies. Expression levels were also compared by FACScan analysis after binding of 20-nM fluorescent ligand, formyl-norleucine-leucine-phenylalanine-norleucine-tyrosine-lysine-fluorescein (formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein; Molecular Probes, Inc., Eugene, OR, USA), to the CHO transfectants for 45 min at 4°C (not shown).

Analysis of ligand-induced activation of ERK1/2 Cells on 35-mm dishes were incubated for 1 h in serum-free medium to reduce background caused by extracellular signal-regulated kinase (ERK) activation through serum components. Cells were then incubated for 4 min without ligand or various concentrations of fMLF (1 and 10 nM), fMFADRW (100 nM and 1 μ M), or fMFEDVAWF (100 nM and 1 μ M), washed with cold phosphate-buffered saline (PBS), and extracted in Laemmli sample buffer (Cleveland et al. 1977). The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. ERK1 and 2 were detected using a mouse monoclonal antibody against p42/44 mitogen-activated protein kinase (ERK1/2) phosphorylated on Thr-202 and Tyr-204 (Cell Signaling Technology Inc., Beverly, MA, USA), or a rabbit polyclonal antibody against total human ERK1/2 (Upstate, Lake Placid, NY, USA). Chemiluminescence was carried out using Western Lightning reagent (Perkin-Elmer Life Sciences, Boston, MA, USA) and the relative intensities of the bands from the films were quantified using Scion Image software (Scion Corporation, Frederick, MD, USA) and a Scanjet IICx scanner (Hewlett-Packard Corporation, Palo Alto, CA, USA).

FPR downregulation assay Cells were removed from tissue culture plates by scraping after incubation in 1-mM sodium ethylene diamine tetra acetate (pH 8.0) in Ca²⁺/Mg²⁺-free PBS. Cells were preincubated in GIBCO CHO-S-SFMII (Invitrogen Inc., Grand Island, NY, USA) at 37°C for 1 h and then incubated at 37°C for 1 h with 15-nM to 2.5- μ M fMLF (Sigma-Aldrich, St. Louis, MO, USA) or 2.5–80- μ M fMFEDVAWF (GenScript Corporation, Piscataway, NJ).

Dimethyl sulfoxide was 0.4% in all cases. The cells were washed two times with 10-ml 10-mM sodium phosphate, pH 7.4, 3 mM KCl, 100 mM NaCl at 4°C. The cells were resuspended in 100 mM NaCl, 3 mM KCl, 10-mM sodium phosphate, pH 7.4, 1 mM Mg⁺⁺, 1 mM Ca⁺⁺, 5% fetal bovine serum, and 15 nM formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein isothiocyanate (FITC; Molecular Probes, Inc., Eugene, OR, USA), incubated for 1 h at 4°C, and analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The geometric mean fluorescence of the cells was determined. The data were then analyzed by nonlinear least squares analysis, and the EC₅₀ was determined using Prism software (GraphPad Software Inc., San Diego, CA, USA). One hundred percent down-regulation was the amount of downregulation observed with 20 μM fNle-Leu-Phe (an oxidation resistant analog of fMLF) under the same conditions. Each data point represents the mean and SEM from three different experiments carried out in duplicates.

FACScan analysis of ligand binding to FPR CHO cells were added to 3 mM KCl, 100 mM NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM Mg⁺⁺, 1 mM Ca⁺⁺ containing 5% fetal bovine serum and incubated at 4°C for 1 h with varying concentrations of peptide and 0.5 nM formyl-Nle-Leu-Phe-Nle-Tyr-Lys-FITC. The mean fluorescence of the cells was determined, and the mean fluorescence of cells with no formyl-Nle-Leu-Phe-Nle-Tyr-Lys-FITC added was subtracted (CHO cells which do not express FPR exhibited identical fluorescence in the absence or presence of 0.5 nM formyl-Nle-Leu-Phe-Nle-Tyr-Lys-FITC). Data from two different experiments with duplicate samples were analyzed as above.

Analysis of chemotaxis mediated by FPR haplotypes Chemotaxis assay with CHO transfectants was carried out essentially as described previously (Gripentrog et al. 2000; Miettinen et al. 1998). CHO cells were removed from tissue culture plates by trypsinization and allowed to equilibrate in complete medium for 1 h at 37°C. Cells were sedimented by centrifugation and resuspended in serum-free medium. Cells, 3 × 10⁵, in 300 μl were added to each upper well of Transwell™ inserts of 8-μm pore size (Corning. Inc., Corning, NY, USA). The lower wells contained serum-free medium with various concentrations of fMLF (1, 10, and 100 nM) or fMFEDVAVWF (100 nM, 1, and 10 μM). Chemotaxis was allowed to proceed for 4 h at 37°C. The optimal time for chemotaxis through the pores and spreading on the underside of the filter was previously determined by allowing the cells to migrate for 0.5–5 h toward 1-nM fMLF. Cells on the upper face of the insert were removed with cotton swabs; the cells that adhered to the underside of the filter were fixed with 2.5% parafor-

maldehyde in PBS, stained with hematoxylin Gill's stain, and the cell surface area from six random sites was quantified using an image analyzer (M4 True Color Image Analysis System, Imaging Research, St. Catherines, Ontario, Canada). Chemotaxis of CHO FPR H2 cells and CHO FPR H3 cells was always carried out simultaneously and the migration of the two cell lines was determined so that maximal chemotaxis of either H2 or H3 was set at 100% and the rest of the values were calculated accordingly.

Human subjects A total of 74 Caucasian patients with RA (80% women) and 74 controls (61% women) from the Bozeman Deaconess Hospital and the Montana State University Blood Donor Program participated in the study. The study was approved by the Institutional Review Board of Montana State University and the blood donors gave their informed consent to the study. All RA patients tested positive for rheumatoid factor and satisfied the American College of Rheumatology revised criteria for diagnosis of RA (Arnett et al. 1988). Patients ranged in age from 16 to 92 years (mean 63 years); controls ranged in age from 21 to 64 years (mean 38 years).

Genotyping and haplotyping of FPR1 DNA isolation and amplification has been described above. PCR products and plasmids were sequenced at Nevada Genomics Center (University of Nevada-Reno) using ABI BigDye chemistry and ABI 3730 DNA Analyzer. The data were analyzed using DNAssist version 2.2 and all chromatograms were visually inspected using Chromas version 2.0 to detect SNPs.

Statistical analysis Data on CHO transfectants were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA), as indicated in the text. The RA haplotype and control haplotype frequencies were compared using chi-square analysis of contingency tables.

Results

We cloned three FPR haplotypes commonly found in the Caucasian population and expressed them stably in CHO cells to examine their function in response to various formylated peptides. The peptides included the prototypic FPR ligand, fMLF, from *E. coli* (Schiffmann et al. 1975), a previously described human mitochondrial peptide from cytochrome c oxidase subunit I, fMFADRW, that causes FPR-induced intracellular calcium release with an EC₅₀ of 160 nM (Rabiet et al. 2005), and a previously described FPR ligand from *M. avium* ssp. *paratuberculosis*, fMFE-DAVAVWF (Mills 2007). The sequence for the most

common haplotype, H1, is shown in Fig. 1. The differences in amino acid sequence between the three haplotypes are shown in Table 1. CHO clones with similar receptor expression levels were selected by western blot analysis and FACScan analysis (data not shown). Most experiments were carried out using two different clones of each haplotype with similar results.

The mycobacterial peptide shows significant differences in activation of ERK1/2 by the different haplotypes

A common method to show G protein-coupled receptor-mediated signaling is by following the activation of the mitogen-activated protein kinases, ERK1/2. Antibodies that specifically recognize phosphorylated, activated ERK1/2 can be used to determine concentration and time dependence of activation. We have previously shown with a number of FPR mutants that FPR H3 activates ERK1/2 primarily through G protein, with only minor or no β -arrestin1/2-mediated activation (Gripentrog and Miettinen 2005). Thus, we decided to analyze whether the concentration dependence of H1, H2, and H3 differ from each other in response to the mitochondrial cytochrome c oxidase

peptide fMFADRW, *E. coli* peptide fMLF, or mycobacterial peptide fMFEDAVAWF. Cells were incubated for 4 min with various concentrations of ligand and phosphorylated and total ERK1/2 was detected by western blot analysis. The activation of ERK1/2 using various concentrations of fMFADRW and fMLF was similar between H1, H2, and H3 (Fig. 2a,b). More significant differences were detected when fMFEDAVAWF was used as ligand (Fig. 2a,b). The most notable differences could be detected using 100-nM fMFEDAVAWF resulting in about 13–15-fold lower activation of ERK1/2 by H2 than by H1 and H3 ($P=0.008$, one-way ANOVA). The differences in ERK1/2 phosphorylation became smaller at 1- μ M fMFEDAVAWF but remained significant ($P=0.022$).

Haplotype 2 requires significantly higher concentration of the mycobacterial peptide fMFEDAVAWF for half maximal downregulation of the receptor

We decided to examine the cellular responses of the different haplotypes to fMFEDAVAWF in more detail. First, we analyzed the concentration-dependent downregulation of the receptor haplotypes (Fig. 3a,b.). The *E. coli*

Fig. 1 Secondary structure model of FPR haplotype 1 showing the sites of single-nucleotide polymorphisms. Amino acids that change as a result of the nonsynonymous SNP are shown as white letters with the alternative amino acids shown outside the circles. Amino acids that do not change (synonymous SNPs) are shown in thick circles

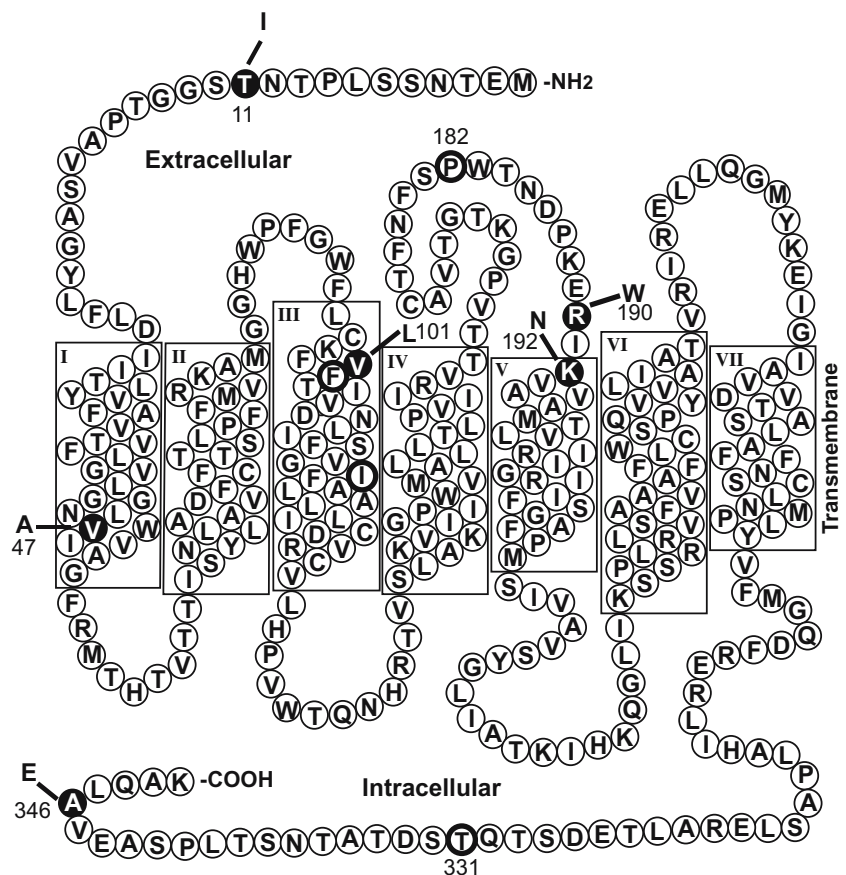


Table 1 Amino acid variations in FPR haplotypes 1, 2, and 3

Haplotype	11	47	101	190	192	346
H1	T	V	V	R	K	A
H2	T	V	V	W	N	A
H3	I	V	V	R	N	E

The numbers indicate the position of the amino acid, as shown in Fig. 1

peptide fMLF downregulated the H1, H2, and H3 haplotypes with similar EC₅₀ concentrations, although H1 required a somewhat higher concentration, 50±3 nM than either H2 (33±2 nM) or H3 (29±2 nM). The mycobacterial peptide fMFEDAVAWF exhibited selective downregulation for H1 (EC₅₀=6.0±0.4 μM) and H3 (EC₅₀=4.8±0.4 μM) compared to H2 (EC₅₀=23±2 μM; *P*<0.0001 vs. either H1 or H3), indicating that the mycobacterial peptide exhibits a fourfold preference for H1 and H3 vs. H2.

The relative affinities of the peptides fMLF and fMFEDAVAWF for H1, H2, or H3 were determined by assessing their abilities to displace formyl-Nle-Leu-Phe-

Nle-Tyr-Lys-FITC in a concentration-dependent manner. H1, H2, and H3 exhibited essentially identical EC₅₀s for fMLF, 33±2, 28±2, and 31±1 nM respectively (Fig. 3c). In contrast, fMFEDAVAWF exhibited a significantly increased EC₅₀ for H2, 29±3 μM, compared with either H1 or H3, 15±1 μM and 17±1 μM, respectively (*P*<0.001, one-way ANOVA; Fig. 3d).

Haplotype 3 shows more efficient chemotaxis toward the mycobacterial peptide fMFEDAVAWF than haplotype 2

Next, we examined one of the primary functions of FPR, namely chemotaxis toward bacterial formylated peptides. Because the ERK1/2 activation, receptor downregulation, and affinity studies suggested that H1 and H3 respond in a similar fashion to the *E. coli* peptide fMLF and the *Mycobacterium* peptide fMEDAVAWF, we decided to compare the chemotaxis of CHO cells expressing FPR H2 and FPR H3 only. CHO transfectants were allowed to migrate for 4 h across a polycarbonate filter with 8-μm pores toward a lower well containing different concentrations of fMLF or fMFEDAVAWF. Most of the cells that migrated through the pores adhered to the underside of the

Fig. 2 Activation of ERK1/2 by fMFADRW, fMLF and fMFEDAVAWF in CHO cells expressing FPR H1, H2 and H3. **a** CHO transfectants were exposed for 4 min to the indicated concentrations of fMFADRW, fMFEDAVAWF, or fMLF. The relative amounts of phosphorylated ERK1/2 and total ERK1/2 were examined by western analysis. **b** The relative amounts of ERK1/2 phosphorylated in response to fMLF and fMFEDAVAWF were quantified by scanning. Due to film saturation at higher concentrations of peptide, the quantitative results should not be considered absolute, but indicative of the phosphorylation trend. The results are means±SEM from three different experiments for each haplotype. One-way ANOVA: **p*<0.05, ***p*<0.01

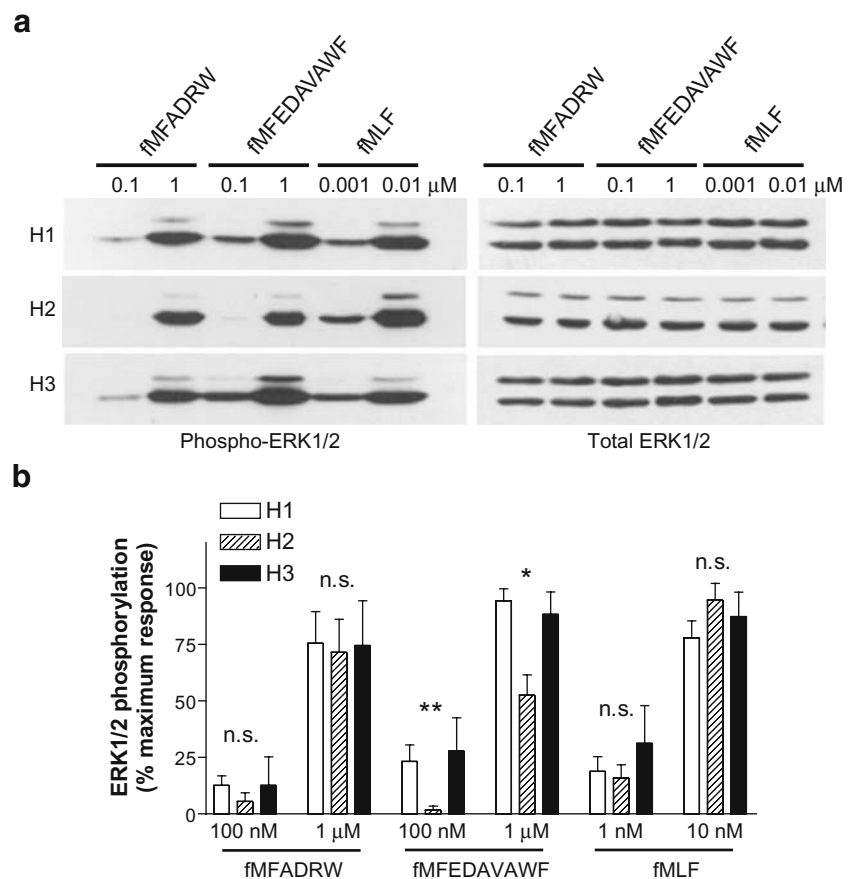
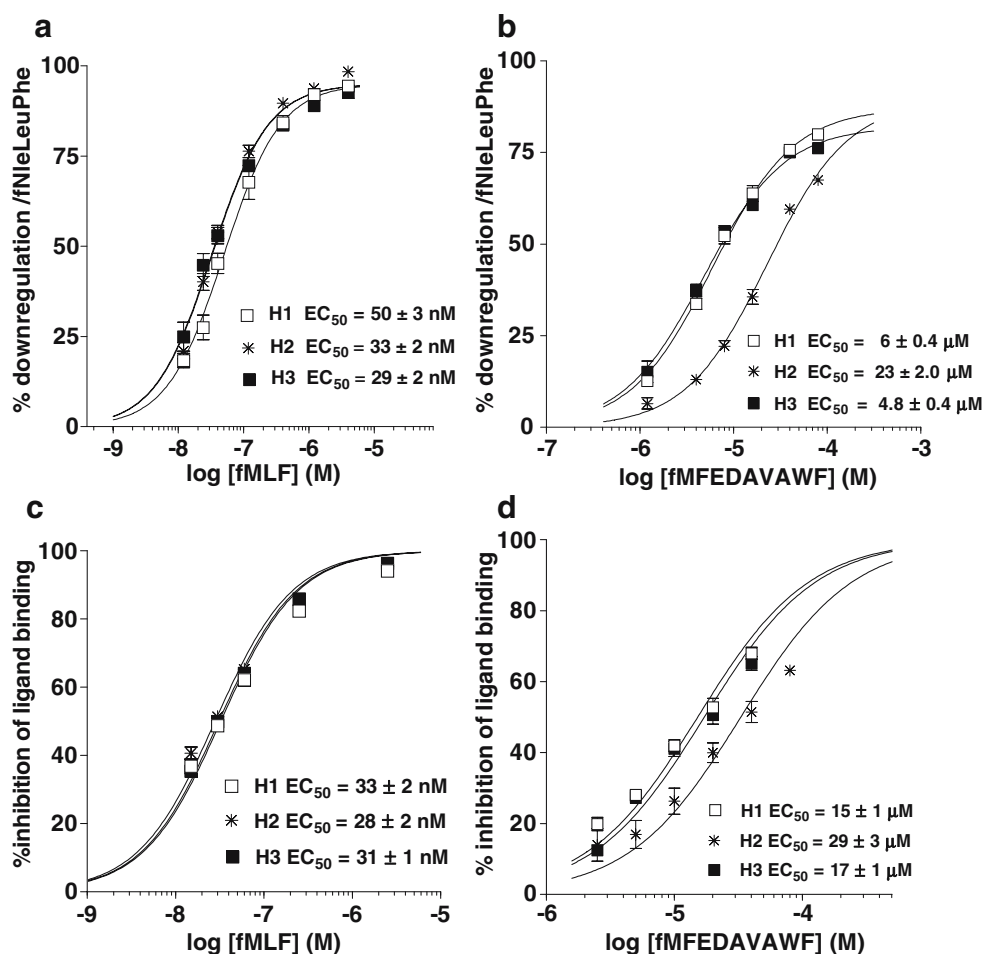


Fig. 3 Haplotype-specific receptor downregulation and peptide binding. CHO cells expressing FPR H1, FPR H2, and FPR H3 were incubated for 1 h with 15 nM–2.5 μ M fMLF (a) or 2.5–80 μ M fMFEDAVAWF (b). The amount of receptor remaining on the cell surface was quantified by incubation with formyl-Nle-Leu-Phe-Nle-Tyr-Lys-FITC. Each data point represent the mean and SEM from three different determinations with duplicate samples. The EC_{50} for peptide-mediated downregulation was calculated using Prism software (GraphPad Software, Inc., San Diego, CA, USA). EC_{50} s for binding of fMLF (c) or fMFEDAWF (d) were calculated from competitive inhibition binding with formyl-Nle-Leu-Phe-Nle-Tyr-Lys-FITC. Each data point represents the mean \pm SEM from two different determinations with duplicate samples



filter and were stained for quantification by image analysis. As shown in Fig. 4a,b, cells expressing FPR H2 migrated with a fMLF concentration dependence similar to that of cells expressing FPR H3 but migrated poorly toward 100-nM fMFEDAVAWF, compared to cells expressing FPR H3 ($P=0.014$). Based on the typical bell-shaped concentration curves (Fig. 4b), maximal migration of cells expressing FPR H3 toward fMFEDAVAWF occurred at 100 nM–1 μ M, whereas cells expressing FPR H2 migrated maximally toward 1–10- μ M fMFEDAVAWF. In chemotaxis toward the *E. coli* peptide, both FPR H2 and FPR H3 transfectants showed maximal migration toward 1–10-nM fMLF, with FPR H3 transfectants exhibiting somewhat higher chemotaxis at lower concentration (1 nM) although the results were not statistically different from each other (Fig. 4b).

Specific FPR haplotypes do not appear to be associated with RA

To examine whether certain FPR haplotypes or SNPs are associated with RA, we carried out genotype–haplotype

analysis of 74 RA patients and 74 control individuals. We used the haplotyping nomenclature established by Sahagun-Ruiz et al. (2001). We identified three haplotypes with a frequency of >10% in the control population and/or in the RA population (1A, 2A, 4A) and 28 haplotypes with a frequency of <10% (Table 2). In addition, we identified one novel SNP, *c.140T>C/p.V47A* (haplotype 24A). Interestingly, the *c.140C* nucleotide was linked to nucleotides found in the 19A haplotype in all six cases and was only found in the control population. Another haplotype of interest, 16A, was found only in the RA population (four haplotypes out of 148). In both cases, the low numbers prevented any conclusions regarding RA association due to lack of statistical power. Chi-square analysis of the 2×31 contingency table suggested no clear relationship between FPR haplotypes and RA ($P=0.209$, $\chi^2=36.0$, $df=30$). Upon pooling the haplotypes based on amino acid sequence rather than nucleotide sequence, the P value was 0.071 ($\chi^2=21.1$, $df=13$). This study demonstrated that a very large number of FPR haplotypes exists and only two SNPs (*c.568T/p.W190* and *c.140C/p.A47*) show strong linkage disequilibrium with the other SNPs. Therefore, FPR SNP

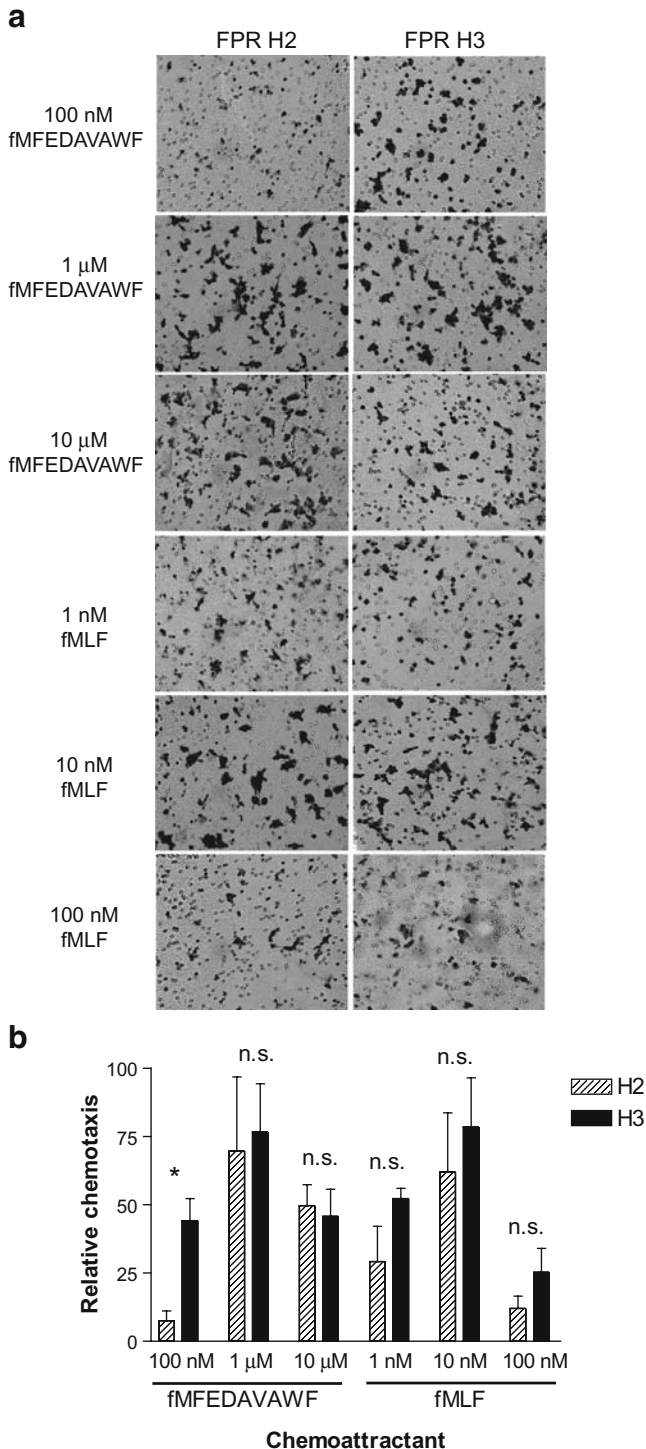


Fig. 4 Chemotaxis of CHO transfectants toward fMLF and fMFEDAWWF. CHO transfectants were allowed to migrate for 4 h toward peptide ligand, as indicated. The cells that migrated through holes and adhered to the lower surface of the filters were fixed, stained, and photographed. **a** Representative fields of the chemotaxis filters. The *small round circles* are the 8- μ m pores and the *black structures* are the cells that migrated through the pores. **b** The filters were quantified by image analysis. The graphs show mean value \pm SEM from three different experiments. Student's *t*-test, * p <0.05

typing alone without corresponding haplotype analysis may not be sufficient to uncover disease associations.

Discussion

The outcome of neutrophil-mediated defense against bacteria is partly dependent on how readily the cells become activated and respond to bacterial infection. For example, inflammatory bowel disease is thought to occur in people with a delayed or insufficient innate immune response toward bacteria that have penetrated the mucosa of the gut (for review, see Bouma and Strober 2003). The human gut contains 500–1,000 bacterial species and about 80% of these have not yet been cultured or characterized. The role of bacteria in inflammatory bowel disease remains controversial with results both favoring and discounting their role (Sartor 2006; Subramanian et al. 2006). One of the prominent bacteria described in cases of Crohn's disease is *M. avium* ssp. *paratuberculosis*. Analyses of mycobacterial DNA or antigens in Crohn's disease have produced both positive and negative results (Autschbach et al. 2005; Brunello et al. 1991; Romero et al. 2005; Suenaga et al. 1999). Similarly, antimycobacterial therapy has been successful in some but not all cases (Borody et al. 2002; Jarnerot et al. 1989; Schultz et al. 1987). Thus, individual genetic differences may determine susceptibility toward bacteria of different origins.

Studies from the past 6–7 years show that single-nucleotide polymorphisms in human receptors that recognize bacterial products can result in Crohn's disease. The best known example is the *CARD15* gene, where a frameshift and two missense variants alter the function of the *CARD15*-encoding cytoplasmic protein, nucleotide-binding oligomerization domain 2 (NOD2; Hugot et al. 2001; Ogura et al. 2001). NOD2 binds a bacterial muramyl dipeptide and activates nuclear factor (NF)- κ B, an important transcription factor in innate immune responses (Inohara et al. 2003). An estimated 8–17% of Caucasian patients with Crohn's disease have two copies of the main risk alleles, whereas none of the three *CARD15* mutations have been found in Chinese, Korean, or Japanese Crohn's patients (Croucher et al. 2003; Leong et al. 2003; Tosa et al. 2006). This provides strong evidence for the presence of genetic heterogeneity among patients from different ethnic backgrounds. Other genes linked to Crohn's disease belong to the family of toll-like receptors (TLRs) and include genes encoding TLR1, TLR2, TLR4, TLR5 and TLR6 (Franchimont et al. 2004; Gewirtz et al. 2006; Pierik et al. 2006). Both NOD2 and the TLRs belong to a class of pattern recognition receptors of the immune system that recognize conserved pathogenic motifs or pathogen-associated molecular patterns. Thus, Crohn's disease seems

Table 2 FPR haplotype frequencies in RA patients and healthy controls

	FPR amino acid number										RA	Control
	11	47	101	102	116	182	190	192	331	346	<i>n</i> =148 (%)	<i>n</i> =148 (%)
1A	T	V	V	F-t	I-c	P-c	R	K	T-c	A	35 (23.6)	26 (17.6)
1B	T	V	V	F-t	I-c	P-a	R	K	T-c	A	0	1 (0.7)
1C	T	V	V	F-t	I-c	P-c	R	K	T-t	A	0	1 (0.7)
2A	T	V	V	F-t	I-c	P-c	W	N-c	T-c	A	24 (16.2)	16 (10.8)
3A	I	V	V	F-t	I-c	P-c	R	N-t	T-c	E	3 (2)	7 (4.7)
3B	I	V	V	F-t	I-c	P-c	R	N-t	T-t	E	5 (3.4)	5 (3.4)
3C	I	V	V	F-t	I-t	P-a	R	N-t	T-t	E	0	1 (0.7)
4A	I	V	L	F-t	I-c	P-c	R	N-t	T-c	A	13 (8.8)	21 (14.2)
4B	I	V	L	F-t	I-c	P-a	R	N-t	T-c	A	1 (0.7)	2 (1.3)
5A	T	V	L	F-t	I-c	P-c	R	K	T-c	A	11 (7.4)	10 (6.7)
5B	T	V	L	F-t	I-c	P-a	R	K	T-c	A	1 (0.7)	0
6A	T	V	L	F-t	I-c	P-c	R	N-t	T-c	A	6 (4.0)	7 (4.7)
6B	T	V	L	F-t	I-c	P-a	R	N-t	T-c	A	12 (8.1)	7 (4.7)
8A	I	V	V	F-t	I-c	P-c	R	N-t	T-c	A	4 (2.7)	5 (3.4)
8B	I	V	V	F-t	I-c	P-a	R	N-t	T-c	A	0	1 (0.7)
9A	I	V	V	F-t	I-c	P-c	R	K	T-c	A	2 (1.3)	0
11A	T	V	V	F-t	I-c	P-a	R	N-t	T-c	A	8 (5.4)	7 (4.7)
11B	T	V	V	F-c	I-c	P-a	R	N-t	T-c	A	1 (0.7)	3 (2.0)
11C	T	V	V	F-t	I-c	P-c	R	N-t	T-c	A	2 (1.4)	1 (0.7)
11D	T	V	V	F-t	I-c	P-c	R	N-t	T-t	A	0	1 (0.7)
11E	T	V	V	F-c	I-c	P-a	R	N-t	T-c	A	0	1 (0.7)
12B	T	V	V	F-t	I-c	P-c	R	N-t	T-c	E	4 (2.7)	1 (0.7)
12C	T	V	V	F-t	I-c	P-a	R	N-t	T-c	E	2 (1.3)	4 (2.7)
12D	T	V	V	F-c	I-c	P-c	R	N-t	T-c	E	0	1 (0.7)
12E	T	V	V	F-t	I-c	P-c	R	N-t	T-t	E	1 (0.7)	0
12F	T	V	V	F-c	I-c	P-a	R	N-t	T-c	E	1 (0.7)	0
16A	T	V	V	F-t	I-c	P-c	R	K	T-c	E	4 (2.7)	0
19A	T	V	L	F-t	I-c	P-a	R	N-t	T-c	E	6 (4.0)	9 (6.1)
19B	T	V	L	F-t	I-c	P-c	R	N-t	T-c	E	2 (1.4)	3 (2.0)
24A	T	A	L	F-t	I-c	P-a	R	N-t	T-c	E	0	6 (4.0)
25A	T	V	L	F-t	I-c	P-c	R	K	T-c	E	0	1 (0.7)

The nomenclature follows that of Sahagun-Ruiz et al. (2001) except our newly discovered haplotypes in which the SNP does not change the amino acid; labeled B, C, D, E and F. Haplotypes that were not detected in this study (such as 7, 10 and 13) were excluded from this table. Haplotypes 24A and 25A have not been previously reported. $P=0.206$, $\chi^2=35.0$, $df=29$

to be associated with the inability to recognize bacterial motifs and to respond to them through signal transduction events that result in proinflammatory defense (such as activation of the NF- κ B pathway). In a genome-wide association study testing over 300,000 autosomal SNPs in inflammatory bowel disease (Duerr et al. 2006), the Illumina HumanHap300Genotyping BeadChip included only one FPR SNP (*c.1037C>A/p.A346E*). Therefore, it remains to be determined whether FPR is associated with inflammatory bowel disease.

Because mycobacterial infection has been associated with certain cases of inflammatory bowel disease (Subramanian et al. 2006), we were interested in examining whether the mycobacterial peptide fMFEDAVAWF, derived from hypothetical protein MAP4176, would activate cells through FPR and whether any of the three most common

haplotypes (in Caucasians) would become differentially activated. These studies were carried out using stably transfected CHO cells. The CHO transfectants display many of the same responses toward bacterial peptides as neutrophils, including chemotaxis, and therefore provide an excellent model system for these studies. We first examined the concentration-dependent activation of ERK1/2 with the human mitochondrial cytochrome c oxidase peptide fMAFDRW, *E. coli* peptide fMLF, and *M. avium* ssp. *paratuberculosis* peptide fMFEDAVAWF. The responses between the haplotypes were similar toward the *E. coli* peptide, whereas FPR H2-mediated activation of ERK1/2 with the mycobacterial peptide required a significantly higher concentration than in the case of FPR H1 and FPR H3. The subsequent experiments measuring receptor downregulation further supported the notion that

FPR H2 was functionally different from FPR H1 and FPR H3 in response to the mycobacterial peptide; a significantly higher concentration of the peptide was required for half maximal downregulation of the receptor and the peptide exhibited a reduced affinity for FPR H2. The most conclusive evidence that these differences could have physiological implications in our defense against bacteria, was obtained with the chemotaxis assays. In these experiments, CHO cells expressing FPR H2 showed significantly reduced migration toward lower concentrations of fMFEDAVAWF compared with CHO cells expressing FPR H3, while the responses toward the *E. coli* peptide fMLF were comparable between the haplotypes.

A previous study by Rabiet et al. (2005) identified several novel mitochondrial agonists that activated HL-60 cells transfected with FPR (haplotype 3) and a FPR homolog, formyl peptide receptor-like 1 (FPRL1). FPRL1 which requires fMLF in the micromolar range for activation was activated by three mitochondrial peptides with almost equal potency as FPR. However, when the activation efficiency was compared using peptides derived from *Listeria monocytogenes*, only FPR was activated in the low nanomolar range (Rabiet et al. 2005). These results suggest that FPRL1 may play a crucial role, together with FPR, in the regulation of inflammatory processes involving primarily endogenous ligands released during tissue damage, whereas FPR alone responds to exogenous pathogens.

The coding sequence for *FPR1* contains six nonsynonymous and four synonymous SNPs. The reason for this large diversity in receptor sequence is unknown, but we hypothesize that evolution favors these polymorphisms to allow FPR binding of peptides from a wide variety of microorganisms and other infection–inflammation sources. Some of the nonsynonymous SNPs cause significant changes in the amino acids, such as p.R190W and p.N192K, which may partly determine ligand binding specificity and affinity. Our previous studies suggest that the ligand binding site of FPR maps in the transmembrane region, potentially in close proximity to amino acids 190 and 192 (Miettinen et al. 1997; Mills et al. 2000). Because H1 and H3 have an arginine (R) at position 190, this amino acid may interact with the aspartic acid (D) or glutamic acid (E) side chain, or the phenylalanine (F) C-terminus of fMFEDAVAWF and increase the affinity of the peptide, compared to tryptophan (W). Because we have previously determined that R201 is the site of formyl group binding (Mills et al. 2000), R190 is appropriately positioned to interact with the C-terminus of fMFEDAVAWF provided that the peptide binds parallel to helix V. In addition, because fMFEDAVAWF has a charge of -3 , R190 may facilitate the entry of this negatively charged peptide into the binding pocket. The mitochondrial peptide fMAFDRW has a charge of -1 and is two residues shorter than

fMFEDAVAWF and exhibited similar activation of all three haplotypes. Therefore, FPR from different individuals may show variation in binding properties to microbial and mitochondrial peptides, which may affect the neutrophils' response to microbial invaders or tissue injury. Reduced or enhanced ligand binding may thus affect the outcome of a bacterial infection and in combination with other susceptibility factors contributes to inflammation.

The role of FPR variants has been previously examined in aggressive periodontitis (AP) but not in other inflammatory disorders, to our knowledge. A study by Zhang et al. (2003) found a significant association of two SNPs, c. A568 (p.R190) and c.G576 (p.K192), with the AP phenotype in 38 African–American patients (compared to 23 controls) but not in Brazilian or Turkish patients when expressed together. A role of FPR in AP can easily be rationalized because AP is associated with bacterial infections (Henderson et al. 2003). However, the role of FPR in RA has not been established due to lack of a clear correlation between RA and bacterial contamination. Certain bacteria, such as *Mycoplasma pneumoniae*, have been associated with RA, and it has been theorized that infectious agents may initiate the disorder. A case-control study showed that the presence of antibodies against *M. pneumoniae* was associated with RA ($P < 0.001$, OR 2.34; Ramirez et al. 2005). A correlation between RA and other *Mycoplasma* strains has also been reported (Haier et al. 1999; Horowitz et al. 2000). Thus, a relationship between bacteria and RA has been shown, but it remains to be established whether bacterial infection is the cause or the result of RA. A connection between RA and mitochondrial peptides and cathepsin G in the synovial fluid appears plausible, although these peptides are clearly not the cause of RA, but may instead exacerbate the ongoing inflammation. Because chronic inflammation is characterized by the continuous infiltration of neutrophils, any information that can lead to inhibition of this activity would be medically important.

In summary, the large genetic heterogeneity of FPR may affect our capability to respond to bacteria of different origin and may contribute to certain inflammatory disorders due to diminished innate immune defense at early stages of bacterial infection or tissue injury. However, our preliminary studies suggest that FPR haplotypes are not involved in the pathogenesis of RA, although the possible contributions of certain rare haplotypes (such as 16A and 24A) will require a much larger study. In addition, the role of FPR haplotypes in inflammatory bowel disease remains to be determined.

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