Integrins $\alpha_v\beta_3$ and $\alpha_4\beta_1$ Act as Coreceptors for Fractalkine, and the Integrin-Binding Defective Mutant of Fractalkine Is an Antagonist of CX3CR1

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Integrins $\alpha_v\beta_3$ and $\alpha_4\beta_1$ Act as Coreceptors for Fractalkine, and the Integrin-Binding Defective Mutant of Fractalkine Is an Antagonist of CX3CR1

Masaaki Fujita, Yoko K. Takada, and Yoshikazu Takada


The membrane-bound chemokine fractalkine (FKN, CX3CL1) on endothelial cells plays a role in leukocyte trafficking. The chemokine domain (FKN-CD) is sufficient for inducing FKN signaling (e.g., integrin activation), and FKN-CD binds to its receptor CX3CR1 on leukocytes. Whereas previous studies suggest that FKN-CD does not directly bind to integrins, our docking simulation studies predicted that FKN-CD directly interacts with integrin $\alpha_v\beta_3$. Consistent with this prediction, we demonstrated that FKN-CD directly bound to $\alpha_v\beta_3$ and $\alpha_4\beta_1$ at a very high affinity ($K_D$ of $3.0 \times 10^{-10}$ M to $\alpha_v\beta_3$ in 1 mM Mn$^{2+}$). Also, membrane-bound FKN bound to integrins $\alpha_v\beta_3$ and $\alpha_4\beta_1$, suggesting that the FKN-CD/integrin interaction is biologically relevant. The binding site for FKN-CD in $\alpha_v\beta_3$ was similar to those for other known $\alpha_v\beta_3$ ligands. Wild-type FKN-CD induced coprecipitation of integrins and CX3CR1 in U937 cells, suggesting that FKN-CD induces ternary complex formation (CX3CR1, FKN-CD, and integrin). Based on the docking model, we generated an integrin-binding defective FKN-CD mutant (the K36E/R37E mutant). K36E/R37E was defective in ternary complex formation and integrin activation, whereas K36E/R37E still bound to CX3CR1. These results suggest that FKN-CD binding to CX3CR1 is not sufficient for FKN signaling, and that FKN-CD binding to integrins as coreceptors and the resulting ternary complex formation are required for FKN signaling. Notably, excess K36E/R37E suppressed integrin activation induced by wild-type FKN-CD and effectively suppressed leukocyte infiltration in thioglycollate-induced peritonitis. These findings suggest that K36E/R37E acts as a dominant-negative CX3CR1 antagonist and that FKN-CD/integrin interaction is a novel therapeutic target in inflammatory diseases.
we propose a model in which FKN on endothelial cells binds to leukocytes through CX3CR1 and/or integrins (αβ3 and αβ5), and in which integrins as well as CX3CR1 are directly involved in FKN signaling and leukocyte trafficking through binding to FKN-CD.

materials and methods

materials

Recombinant soluble αβ3 was synthesized in Chinese hamster ovary (CHO) K1 cells using the soluble αα and ββ expression constructs and purified by Ni-NTA affinity chromatography as described (33). 7E3 (anti-human integrin β3) (34) and anti-human β3 mAb AIIB2 (35, 36) hybridomas were obtained from the American Type Culture Collection. Anti-αα mAb SG73 has been described (37). K562 and U937 cells were obtained from the American Type Culture Collection. K562 erythroleukemia cells that express human αβ3 and CHO cells that express human β3 or β1,3,1,1 mutant have been described (39). Cyclic RGDfV was purchased from Enzo Life Sciences (Farmingdale, NY). Rabbit anti-human CX3CR1 was obtained from Torrey Pines Biolab (East Orange, NJ) or from AbD Serotec (Oxford, U.K.). Mouse anti-human FKN mAb (MAB365) was obtained from R&D Systems (Minneapolis, MN). Rabbit anti-human αα was obtained from Cell Signaling Technology (Danvers, MA). Anti-human integrin β3 (B1720) was purchased from BD Biosciences. Anti-human β3 mAb A10 was provided by B. Felding-Habermann (The Scripps Research Institute, La Jolla, CA).

synthesis of the chemokine domain of FKN (FKN-CD) and stromal cell-derived factor-1

The cDNA fragment of the chemokine domain of FKN was amplified using primers 5'-CGGGATCCACACACGACGGTTCGACG-3' and 5'-CGGAAAGCTTCAAGCTTGAGGGCAGC-3' with human FKN cDNA (Open Biosystems, Lafayette, CO) as a template, and subcloned into the BamHI/EcoRI site of PET28a expression vector. The protein was synthesized in BL21 induced by isopropyl β-D-thiogalactoside as an insoluble protein. The protein was solubilized in 8 M urea, purified by Ni-NTA affinity chromatography, and refolded as previously described (40). The amino acid sequence is [MGSSHHHHHHSSGLVPRGSHMASMT- KRAILETQRHQLCPDEKQVVWVDKAMQDRLQAAALTIRNG. The refolded protein was >90% homogeneous upon SDS-PAGE.

The cDNA fragment of stromal cell-derived factor-1 (SDF1) was amplified using primers 5'-CGGATCCACACACGACGGTTCGACG-3' and 5'-CGGAAAGCTTCAAGCTTGAGGGCAGC-3' with human SDF1 cDNA (Open Biosystems) as a template, and subcloned into the BamHI/EcoRI site of PET28a expression vector. The protein was synthesized in BL21 induced by isopropyl β-D-thiogalactoside as an insoluble protein. The protein was solubilized in 8 M urea, purified by Ni-NTA affinity chromatography, and refolded as described (39). The refolded protein was >90% homogeneous upon SDS-PAGE.

expression of full-length FKN

The cDNA fragment encoding full-length FKN was amplified using primers 5'-CTCTCAGAGGCTGTCGGCGCCCTT-3' and 5'-GCTCTAGAGGGTCTGTAATCA-3' as primers with human FKN cDNA as a template and subcloned into the XhoI/XbaI site of pBluescript. The full-length FKN expression construct was transfected into CHO cells together with neomycin-resistant plasmid using FuGENE (Promega). After selection with G418, cells stably expressing full-length FKN were further enriched by limited dilution to obtain >50% positive populations.

Cell–cell binding assays

CHO cells that express full-length FKN were plated on wells of 96-well plates (5000 cells/well). K562 or U937 cells were labeled with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein triacetoxymethyl ester and added to the wells (100,000 cells/well). After incubating for 1 h at 37°C, bound K562 or U937 cells were quantified using a fluorescent microplate reader (excitation at 503 nm, emission 520 nm) after removing unbound cells by gentle rinsing.

binding of soluble αβ3 to immobilized FKN-CD

ELISA-type binding assays using soluble αβ3 were performed as described previously (40). Briefly, 96-well Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 100 μl 0.1 M NaHCO3 containing FKN or their mutants and were incubated for 2 h at 37°C. Remaining protein binding sites were blocked by incubating with PBS/0.1% BSA for 30 min at room temperature. After washing with PBS, soluble recombinant αβ3 (5 μg/ml) in HEPES-Tyrode buffer (10 mM HEPES, 150 mM NaCl, 12 mM NaHCO3, 0.4 mM Na2HPO4, 2.5 mM KCl, 0.1% glucose, 0.1% BSA) with 1 mM MnCl2 was added to the wells and incubated for 2 h at room temperature. After unbound αβ3 was removed by rinsing the wells with binding buffer, bound αβ3 was measured using anti-integrin β3 (mAb AV-10) followed by HRP-conjugated goat anti-mouse IgG and peroxidase substrates.

adhesion assays

Adhesion assays were performed as described previously (40). Briefly, to assess cell adhesion to immobilized FKN-CD, 96-well Immulon 2 microtiter plates were coated with 100 μl 0.1 M NaHCO3 containing FKN-CD or its mutant and were incubated for 2 h at 37°C. Remaining protein binding sites were blocked by incubating with PBS/0.1% BSA for 30 min at room temperature. After washing with PBS, K562 cells, CHO cells, or U937 cells in 100 μl RPMI 1640/0.1% BSA or DMEM/0.1% BSA were added to the wells and incubated at 37°C for 1 h. After unbound cells were removed by rinsing the wells with the medium used for adhesion assays, bound cells were quantified by measuring endogenous phosphatase activity (41). To determine cation dependence, HEPES-Tyrode buffer with 4 mM EDTA, 4 mM CaCl2, 4 mM MgCl2, or 4 mM MnCl2 was used instead of RPMI 1640. To assess the effect of blocking Abs and cyclic RGDfV, cells were pretreated with mAbs or rRGDfV at room temperature for 30 min before the assay.

Surface plasmon resonance study

Recombinant soluble integrin αβ3 was immobilized to Biacore sensor chip CM5 (Biacore Life Sciences, Piscataway, NJ) by the amine coupling method. Two-fold serially diluted FKN-CD or its mutant K36E/R37E in running buffer (HBS-P buffer containing 1 mM MnCl2, CaCl2, or MgCl2) was injected for 3 min at the flow rate of 30 μl/min. Then the sensor chip was washed with the running buffer alone at the same flow rate for another 15 min (the dissociation phase). Thirty-second injections of regeneration buffer (0.1 M NaOH, 1 M NaCl) at the same flow rate were used to regenerate the chip for another cycle of injection. The resonance unit elicited from reference flow cells was subtracted from the resonance unit elicited from the integrin flow cell to eliminate the nonspecific protein–flow cell interaction and the bulk refractive index effect. The recorded binding curves were analyzed using the BIAevaluation version 4.

Chemotaxis

Chemotaxis was measured in modified Boyden chambers (Transwells). Wide-type (wt) FKN-CD, K36E/R37E, or R47A (1 and 10 ng/ml, total 600 μl RPMI 1640 medium) was placed in the lower chamber, and U937 cells (1.5 × 105 cells/well) were placed in the upper chamber. After 4 h incubation at 37°C, the cells in the lower chamber were counted.

Pull-down assays

We incubated the wt and mutant FKN-CD (1 or 10 μg/ml, with 6His tag) with lysates of U937 cells and recovered FKN-CD with Ni-NTA-Sepharose and analyzed the bound CX3CR1 by Western blotting.

Coprecipitation of integrins, CX3CR1, and FKN-CD

U937 cells were cultured to nearly confluent in RPMI 1640/10% FCS. The cells were resuspended with RPMI 1640 and incubated with 1 μg/ml wt or K36E/R37E for 15 min. The cells were lysed with lysis buffer (20 mM HEPES [pH 7.4], 100 mM NaCl, 10% glycerol, 1% Nonidet P-40) and centrifuged to remove cell debris. The supernatant was recovered from the supernatant by protein binding, centrifugation to eliminate the nonspecific protein–flow cell interaction and the bulk refractive index effect. The recorded binding curves were analyzed using the BIAevaluation version 4.

Flow cytometry

U937 cells were cultured to nearly confluent in RPMI 1640/10% FCS. The cells were resuspended with RPMI 1640/10% BSA and incubated for 30 min at room temperature to block remaining protein binding sites. The cells were then incubated with wt FKN-CD (or SDF1) and/or K36E/R37E for 5 min at room temperature and then incubated with FITC-labeled ligands (fibrinogen γ-chain C-terminal domain that lacks residues 400–411, fi-
bronectin type III domains 8–11, or fibronectin H120 fragment) for 15 min at room temperature. For blocking experiments, the cells were pre-incubated with Abs (KH72, SG73, AIIB2, or 7E3) for 30 min at room temperature before incubation with FKN. The cells were washed with PBS/0.02% BSA and analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA).

Thioglycollate-induced peritonitis

We injected wt FKN-CD and/or K36E/R37E in PBS i.p. to mice and 3 h later injected 1 ml 3% thioglycollate. Mice were killed 48 h after thioglycollate injection, and peritoneal exudate cells were harvested by peritoneal lavage using ice-cold RPMI 1640/10% FBS. Cells were counted on a hemocytometer and then differential cell counts were conducted after staining with Hem-3 (Fischer Scientific). Data are shown as means ± SE, and statistical analysis was performed using one-way ANOVA and Tukey post hoc analysis.

In vitro stability of FKN-CD

6His-tagged wt FKN-CD (1 µg) was added to whole mouse serum (20 µl) and incubated for 15–60 min and analyzed by Western blotting using anti-6His Ab. In another experiment, 6His-tagged wt FKNCD (1 µg) was incubated with U937 cells (1 × 10^5 cells/sample) in RPMI 1640 medium in the presence or absence of FCS (10%) for 5–60 min. Bound wt FKN-CD was detected using Alexa 488-anti-6His Ab and flow cytometry.

Coprecipitation of membrane-bound FKN and integrin β3 or α4

Cell lysates of FKN-CHO cells and U937 cells were mixed and incubated for 1 h at 4°C and then the mixture was incubated with anti-β3 or anti-α4 mAbs for an additional 1 h at 4°C. The immune complex was recovered by incubating with protein A-Sepharose for 30 min at 4°C and analyzed by Western blotting with anti-CX3CR1 Ab.

FIGURE 1. αvβ3 binds to FKN-CD. (A) A model of FKN-CD/integrin αvβ3 interaction predicted by docking simulation using AutoDock3. The headpiece of integrin αvβ3 (PDB code 1LG5) was used as a target. The model predicts that the FKN-CD (PDB code 1F2L, red) binds to the RGD-binding site of the integrin αvβ3 headpiece (blue and green). The Lys36 and Arg47 of FKN-CD are located at the interface between FKN-CD and αvβ3 and were selected for mutagenesis studies. Arg47 that is involved in CX3CR1 binding is not located in the predicted integrin-binding site. (B) Binding of FKN-CD to soluble αvβ3. FKN-CD binding to recombinant soluble αvβ3 was measured in ELISA-type assays. Wells of 96-well microtiter plates were coated with FKN-CD, and the remaining protein-binding sites were blocked with BSA. Soluble recombinant integrin αvβ3 (5 µg/ml) was added to the wells in the presence of 1 mM Mn^2+ and incubated for 2 h at room temperature. Bound αvβ3 was determined using anti-β3 Ab and HRP-conjugated anti-mouse IgG. Data are shown as means ± SE of three independent experiments. (C) Adhesion of K562 cells that express integrin αvβ3 to FKN-CD. Wells of 96-well microtiter plates were coated with FKN-CD, and the remaining protein-binding sites were blocked with BSA. Cells were added to the wells and incubated for 1 h at 37°C in RPMI 1640, and bound cells were quantified by using phosphatase assays. Data are shown as means ± SE of three independent experiments. (D) Cation dependency of FKN-CD/αvβ3 interaction. Adhesion assays of αvβ3-K562 cells to FKN-CD were performed as described in (C) in Tyrode-HEPES containing 4 mM cations. FKN-CD was used at the 2.5 µg/ml coating concentration. Data are shown as means ± SE of three independent experiments. (E) SPR study of integrin αvβ3/FKN-CD interaction. Binding of FKN-CD to αvβ3 in SPR is shown. Soluble αvβ3 was immobilized on a CM5 sensor chip. Wild-type FKN-CD was individually 2-fold serially diluted from 1.1 µM to 138 nM in HBS-P buffer with 1 mM Ca^2+.
Docking simulation of interaction between FKN-CD and integrin αβ3 was performed using AutoDock3 as described (41). Treatment differences were tested using ANOVA and a Tukey multiple comparison test to control the global type I error using Prism 5.0a (Graphpad Software).

Results

FKN-CD binds to integrin αβ3

The chemokine domain of FKN (FKN-CD) is sufficient for FKN binding to cells (42, 43). We performed docking simulation of the interaction between integrin αβ3 (Brookhaven Protein Data Bank [PDB] code 1L5G) and FKN-CD (PDB code 1F2L) using AutoDock3. The simulation predicted that FKN-CD binds to αβ3 at a high affinity (docking energy, −24.7 Kcal/mol) (Fig. 1A). The prediction is not consistent with previous reports. It has been thought that FKN-mediated adhesion is independent of integrins (18). We thus studied whether FKN-CD directly binds to integrin αβ3. We found that recombinant soluble αβ3 bound to immobilized FKN-CD in a dose-dependent manner in ELISA-type binding assays (Fig. 1B). Heat denaturation markedly reduced αβ3 binding to FKN-CD, suggesting that proper folding of the protein is required for integrin binding. K562 erythroleukemic cells do not express CX3CR1 (21). We found that K562 cells (αβ3−) that express exogenous αβ3 (αβ3-K562, αβ3+/αα3+) strongly adhered to FKN-CD, but mock-transfected K562 cells showed only weak adhesion to FKN-CD (Fig. 1C). The adhesion of αβ3−/K562 cells to FKN-CD showed cation dependency, and there is significant adhesion of αβ3−/K562 to FKN-CD in the presence of Ca2+ and EDTA: Mn2+ > Mg2+ = Ca2+ = EDTA (Fig. 1D). To assess the affinity of FKN-CD to bind to αβ3, we performed surface plasmon resonance (SPR) studies using a sensor chip immobilized with soluble αβ3 (Fig. 1E). FKN-CD bound to αβ3 at a very high affinity: KD of 1.1 × 10−8 M in 1 mM Ca2+ (Fig. 1E), KD of 6.9 × 10−9 M in 1 mM Mg2+, and KD of 3.0 × 10−10 M in 1 mM Mn2+ (data not shown) in contrast to previous reports. However, mAb 7E3 (anti-β3 blocking Ab) and cyclic RGDV, an antagonist specific to αβ3 (44), did not effectively block the adhesion of αβ3−/K562 cells to FKN-CD (data not shown).

The specificity loop of β3 plays a role in recognition of FKN-CD

To determine whether FKN-CD is a ligand for integrin αβ3, we identified amino acid residues in β3 that are critical for FKN-CD binding. We previously showed that when a disulfide-linked loop of β3 I-like domain (residues 187−193) is swapped with a corresponding sequence in β3 integrin (designated the β3−/−/− mutant) (Fig. 2A), ligand-binding specificity of the mutated integrin αβ3−/−/− is altered to that of αβ3 (45). Hence, the loop was designated “the specificity loop.” Consistent with the idea that the specificity loop determines ligand specificity, the loop is in the ligand-binding sites in the αβ3 crystal structure and exposed to the surface (46). The specificity loop is diverse in sequence and is present in all β subunits except for β4, in which the loop is deleted and replaced with two remnant residues. The β3−/−/− mutant binds to several αβ3 ligands (40, 41, 45, 47−49), which do not bind to wt β3 integrins in CHO cells. We tested whether the specificity loop of β3 is involved in FKN-CD binding to αβ3. We found that CHO cells that express β3−/−/− (β3−/−/−-CHO cells) adhered to FKN-CD at the level comparable to that of CHO cells that express β3 (Fig. 2B). It has been reported that CHO cells do not express CX3CR1 (50). We demonstrated that anti-human β3 mAb A11B2 significantly reduced adhesion of β3−/−/−-CHO cells to FKN-CD (Fig. 2C).

Amino acid residues in FKN-CD that are involved in integrin binding

To identify the integrin-binding site in FKN-CD, we introduced mutations within the integrin-binding site of FKN-CD, which has been predicted by docking simulation (Table I). We selected Lys36, Arg37, Lys54, and Lys59 of FKN-CD for mutagenesis and mutated them individually or in combination to Glu or Ala. We found that Lys36/Arg37, Lys3/54, and Lys59 of FKN-CD had little or no effect on integrin binding (Fig. 3A–D). In adhesion assays, the K36E/R37E mutation, in which the β1 sequence in the specificity loop is changed to the corresponding β3 sequence, changes the specificity of β3 integrins to that of β1 integrins (see text for details) (Fig. 3A). As a control, we mutated Arg47, which is involved in CX3CR1 binding (42, 43), to Ala (R47A). The R47A mutation had little or no effect on integrin binding (Fig. 3A–D). In adhesion assays, the K36E/R37E mutant was defective in supporting adhesion of wt K562, strongly reduced adhesion of αβ3−/K562 cells as well (Fig. 3C). K562 cells that express endogenous integrin αβ3 weakly adhered to wt FKN (Figs. 1C, 3D), suggesting that the binding is specific. These findings suggest that the FKN-CD binding site in β3 overlaps with those of other known αβ3 ligands.

Other methods

Docking simulation of interaction between FKN-CD and integrin αβ3 was performed using AutoDock3 as described (41). Treatment differences were tested using ANOVA and a Tukey multiple comparison test to control the global type I error using Prism 5.0a (Graphpad Software).
findings suggest that the K36E/R37E mutation of FKN-CD effectively suppresses integrin binding and that the integrin- and the CX3CR1-binding sites of FKN-CD are distinct.

We tested whether the K36E/R37E mutation affects the binding of FKN-CD to CX3CR1 by pull-down assays. We incubated the wt and K36E/R37E FKN-CD (with 6His tag) with lysates of U937 cells and recovered FKN-CD with Ni-NTA-Sepharose and analyzed the bound CX3CR1 by Western blotting. Wild-type FKN-CD and K36E/R37E bound to CX3CR1 whereas R47A (a negative control) was defective in this function, suggesting that the K36E/R37E mutation does not affect the binding to CX3CR1 (Fig. 3E).

FKN-CD binds to $\alpha_4\beta_1$, and the K36E/R37E mutation reduces the interaction

We have shown so far that FKN-CD binds to $\alpha_4\beta_3$, but it is not a major integrin in leukocytes. We tested the possibility that $\alpha_4\beta_1$, a major integrin in immune-competent cells, interacts with FKN-CD. K562 cells ($\alpha_5\beta_1^+$) expressing exogenous $\alpha_4\beta_1$ (ex-K562, $\alpha_4\beta_1^+\alpha_5\beta_1^+$) adhered to FKN-CD (Fig. 4A) stronger than did mock-transfected K562 cells (Fig. 3D). The K36E/R37E mutant was defective in binding to $\alpha_4\beta_1$, suggesting that the $\alpha_4\beta_1$-binding site in FKN-CD is similar to that of $\alpha_5\beta_3$. Anti-$\alpha_4$ mAb S733 and anti-$\beta_1$ mAb AIIB2 suppressed FKN-CD binding to ex-K562 cells in adhesion assays (Fig. 4B). When ex-K562 cells were incubated with FITC-labeled soluble FKN-CD, mAb AIIB2 and S733 suppressed the binding of FKN-CD (data not shown). These findings suggest that $\alpha_4$-specifically binds to FKN-CD. The $\alpha_4\beta_1$/FKN-CD interaction was cation-dependent: ex-K562 adhesion to FKN-CD is the strongest in Mn$^{2+}$ and was not completely suppressed by Ca$^{2+}$ or EDTA (Fig. 4C).

Table 1. Amino acid residues predicted to be involved in FKN-CD/$\alpha_\beta_3$ interaction

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<th>FKN-CD</th>
<th>Integrin $\alpha_\gamma$</th>
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<tr>
<td>Thr$^6$, Lys$^{10}$, Val$^{11}$, Tyr$^{17}$, Gln$^{29}$, Asn$^{30}$, Gln$^{31}$, Ala$^{32}$, Ser$^{33}$, Gly$^{35}$, Lys$^{36}$, Arg$^{37}$, Ala$^{38}$, Ile$^{39}$, Asp$^{52}$, Pro$^{53}$, Lys$^{54}$, Glu$^{55}$, Gln$^{56}$, Trp$^{57}$, Val$^{58}$, Lys$^{59}$, Asp$^{60}$, Met$^{62}$, Gln$^{63}$, His$^{64}$, Asp$^{66}$, Arg$^{67}$</td>
<td>Asp$^{146}$, Ile$^{147}$, Asp$^{148}$, Ala$^{149}$, Asp$^{150}$, Gly$^{151}$, Phe$^{177}$, Tyr$^{178}$, Try$^{179}$, Gln$^{180}$, Arg$^{211}$, Ala$^{212}$, Gly$^{214}$, Ala$^{215}$, Ile$^{216}$, Asp$^{218}$, Ser$^{219}$, Tyr$^{220}$, Ser$^{221}$, Met$^{223}$, Thr$^{224}$, Arg$^{225}$, Asn$^{226}$, Asp$^{227}$, Ala$^{228}$, Thr$^{229}$, Glu$^{230}$, Asn$^{231}$, Val$^{232}$, Leu$^{233}$, Ser$^{234}$, Met$^{235}$, Asp$^{236}$, Ser$^{237}$</td>
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Amino acid residues in integrin $\alpha_\beta_3$ and FKN-CD within 6 Å of each other in the docking model were identified using Swiss-PDB viewer.
This is in contrast to
human CX3CR1 and integrins (in the binding of FKN-CD to U937 cells that express both These results suggest that both CX3CR1 and integrins play a role equally well and much less efficiently to K36E/R37E (Fig. 3).

CX3CR1): both cell lines bound to both wt FKN-CD and R47A to U937 cells showed cation dependency that is similar to those of independent experiments. (performed as described in Fig. 1. Data are shown as means ± SE of three independent experiments. (Fig. 1. FKN-CD was used at the 2.5 µg/ml coating concentration. mlgG (20 µg/ml), SG73 (anti-human αv, 10 µg/ml), and AIIB2 (anti-human β1, 20 µg/ml) were used at the indicated final concentrations. Data are shown as means ± SE of three independent experiments. (C) Cation requirement of αvβ3/FKN-CD interaction. Adhesion assays were performed as described in Fig. 1 in Tyrode-HEPES containing 4 mM cations. Data are shown as means ± SE of three independent experiments. (K562 and CHO cells). It has been reported that FKN-CD binding to its specific receptor CX3CR1 plays a role in leukocyte adhesion and migration (18, 21). We thus determined the relative contribution of CX3CR1 and integrins to the binding of U937 cells (which express CX3CR1) to FKN-CD. U937 cells efficiently adhered to wt FKN-CD in a dose-dependent manner (Fig. 5A), whereas U937 cells adhered to K36E/R37E or R47A less efficiently (∼40–45%). This is in contrast to αvβ3 or αvβ5-K562 cells (which do not express CX3CR1): both cell lines bound to both wt FKN-CD and R47A equally well and much less efficiently to K36E/R37E (Fig. 3). These results suggest that both CX3CR1 and integrins play a role in the binding of FKN-CD to U937 cells that express both CX3CR1 and integrins (αvβ1 and αβ3). The binding of FKN-CD to U937 cells showed cation dependency that is similar to those of αvβ3 and αvβ1 binding (Fig. 5B). We found that mAb SG73 (anti-αv) and AIIB2 (anti-β1) effectively suppressed the binding of FKN-CD to U937 cells (Fig. 5C, 5D). These results suggest that αvβ1 is involved in FKN-CD binding to U937 cells. We could not determine the contribution of other integrins (e.g., αvβ3 and αvβ3) to FKN-CD binding to U937 cells because antagonists to αvβ1 (mAb 7E3 or cRGDfV) or that of αvβ3 (mAb KH72) did not affect the binding of U937 cells to FKN-CD (data not shown).

We tested whether the K36E/R37E mutation affects the ability of FKN-CD to induce chemotaxis. We found that both wt FKN-CD and K36E/R37E induced chemotaxis of U937 cells, whereas the R47A mutant was very defective in this function (Fig. 5E). These results suggest that chemotaxis is CX3CR1-dependent but not integrin-dependent.

FKN-CD induces coprecipitation of integrins αv and β3 and CX3CR1 in U937 cells and the coprecipitation requires direct binding to FKN
We tested the hypothesis that integrin and CX3CR1 can bind simultaneously to FKN-CD by coimmunoprecipitation. We stimulated U937 cells with wt or mutant FKN-CD and immunopurified integrin αv or β3 from cell lysates and analyzed the purified materials by Western blotting. We detected CX3CR1 in the purified materials from cells stimulated by wt FKN-CD, but not those from cells that were not stimulated or those from cells stimulated by K36E/R37E or R47A (Fig. 5F, 5G). These results suggest that FKN-CD, CX3CR1, and integrins (αvβ3 or αvβ3) make a ternary complex upon FKN-CD stimulation, and that the ternary complex formation requires simultaneous binding of CX3CR1 and integrins to FKN-CD.

K36E/R37E suppresses integrin activation induced by FKN-CD (a dominant-negative effect)
If direct integrin binding to FKN-CD and subsequent ternary complex formation is involved in FKN signaling, it is predicted that the integrin-binding defective FKN-CD mutant (K36E/R37E) suppresses signals induced by wt FKN-CD. It has been reported that FKN binding to CX3CR1 enhances cell adhesion by activating integrin through inside-out signaling (30–32). We tested whether K36E/R37E suppresses integrin activation that is induced by wt FKN-CD. We used integrin ligands that are specific to αvβ3 (fibronectin type III domains 8–11) (51, 52), αβ3 (fibronectin H120 fragment) (53), and αvβ3 (fibronerin γ-chain C-terminal domain that lacks residues 400–411) (48). We measured the binding of soluble labeled ligands to U937 cells in flow cytometry. Wild-type FKN-CD enhanced ligand binding to these integrins in a dose-dependent manner, whereas K36E/R37E was defective in this function (Fig. 6A–C, 6E, 6F). Notably excess K36E/R37E suppressed the ligand binding to the integrins induced by wt FKN-CD in a dose-dependent manner (Fig. 6A–C, 6E, 6F). These findings suggest that K36E/R37E is a dominant-negative antagonist to CX3CR1.

We next tested whether the inhibitory effect of K36E/R37E on integrin activation is receptor-specific. SFDF1 (CXCL12), a member of the CXC chemokine family, activates integrin αvβ3 in a CXCR4-dependent manner (54–56). We found that K36E/R37E did not affect SFDF1-induced binding of fibronectin H120 fragment to integrin αvβ3 in U937 cells (Fig. 6G). This suggests that the inhibition of αvβ3 activation by K36E/R37E is specific to CX3CR1 and does not affect CXCR4-mediated integrin activation.

K36E/R37E suppresses leukocyte infiltration in thioglycollate-induced peritonitis
The in vivo inhibitory action of K36E/R37E was evaluated further in a noninfectious peritonitis model (57). We injected K36E/R37E...
to mice and 3 h later injected thioglycollate. Forty-eight hours after thioglycollate injection, peritoneal exudate cells were harvested by peritoneal lavage. Thioglycollate treatment markedly enhanced total leukocyte cell number (Fig. 7A). About 70% of total leukocytes 48 h after thioglycollate injection were monocytes/macrophage (Fig. 7B) and 10–20% were neutrophils, consistent with a previous report (57). K36E/R37E strongly inhibited the thioglycollate-induced accumulation of leukocytes and wt FKN-CD

FIGURE 5. U937 monocytic cells bind to FKN-CD. (A) Adhesion of U937 cells (CX3CR1+) to FKN-CD. Adhesion assays were performed in RPMI 1640 as described in Fig. 1. Data are shown as means ± SE of three independent experiments. The data suggest that integrins and CX3CR1 contribute to U937 adhesion to FKN-CD. (B) Cation requirement of adhesion of U937 cells to FKN-CD. Adhesion assays were performed as described in Fig. 1 in Tyrode-HEPES containing 4 mM cations. FKN-CD was used at the 2.5 µg/ml coating concentration. Data are shown as means ± SE of three independent experiments. (C) Effect of anti-α4 Ab on adhesion of U937 cells to FKN-CD. Adhesion assays in RPMI 1640 were performed as described in Fig. 1. FKN-CD (20 µg/ml) was used for coating. Cells were preincubated with SG73 (10 µg/ml) or AIIB2 (20 µg/ml) before adhesion assays. Data are shown as means ± SE of three independent experiments. The data suggest that α4β1 antagonists partially suppress U937 adhesion to FKN-CD. (D) Inhibition of the binding of labeled FKN-CD to U937 cells. U937 cells were incubated with FITC-labeled FKN-CD (5 µg/ml) in the presence of mAb AIIB2 (anti-β1, 20 µg/ml) or SG73 (anti-α4, 10 µg/ml), and the binding of FKN-CD was measured in flow cytometry. The number is percentage positive cells. The histograms shown are representatives of three experiments. (E) Wild-type FKN-CD and K36E/R37E induce chemotaxis of U937 cells at comparable levels, but R47A does not. Chemotaxis was measured in modified Boyden chambers (Transwells). Wild-type FKN, R36E/R37E, or R47A (1 and 10 ng/ml, total 600 µl RPMI 1640 medium) was placed in the lower chamber, and U937 cells (5 × 10⁵ cells/well) were placed in the upper chamber. After 4 h incubation at 37˚C, cells in the lower chamber were counted. (F and G) FKN-CD induces coprecipitation of integrins and CX3CR1, whereas K36E/R37E does not. Integrins were immunopurified from cell lysates using anti-α4 (F) or anti-β3 (G), and immunopurified materials were analyzed by Western blotting.
enhanced it. In mice treated with WT FKN and excess K36E/R37E (20-fold) the total and monocyte/macrophage levels were below those of thioglycollate only (Fig. 7), suggesting that excess K36E/R37E suppresses leukocyte recruitment induced by exogenous and endogenous FKN. The effect of wt FKN-CD and/or K36E/R37E to neutrophils was similar to that of monocytes/macrophages but was not conclusive, probably because neutrophil levels were already off the peak and the number of neutrophils are low 48 h after thioglycollate injection (data not shown). These results suggest that K36E/R37E can induce the dominant-negative effect in an in vivo model of inflammation and neutralize endogenous and exogenous FKN. Interestingly, WT FKN-CD (without thioglycollate) significantly enhanced the total and monocytes/macrophages, confirming that FKN-CD is able to recruit leukocytes by itself. These data demonstrate K36E/R37E suppresses the recruitment of inflammatory cells in a mouse model of inflammation.

Is FKN-CD present in circulation after 48 h? To address this question, we tested whether the serum samples of mice that have been injected with wt FKN-CD or K36E/R37E (6His-tagged) still contain 6His-tagged proteins by Western blotting. We did not detect any signals (data not shown). We studied the in vitro stability of FKN-CD by incubating FKN-CD with medium with or without serum (FCS or mouse serum). We found that FKN-CD was quickly degraded in these conditions (half-life of $\sim 30$ min) (Supplemental Fig. 1). Thus, it is highly likely that wt FKN-CD and K36E/R37E trigger their signals and rapidly disappear from circulation in vivo.

Do integrins interact with membrane-bound FKN?

Our results so far suggest that the isolated FKN-CD interacts with integrins. We studied whether integrins interact with the membrane-bound FKN because FKN is usually expressed in a membrane-bound form on the cell surface. We stably expressed wt full-length FKN in CHO cells (designated FKN-CHO cells). We found that $\alpha_v\beta_3$-K562, $\alpha_v\beta_5$, and U937 cells bound to FKN-CHO cells better than to mock-transfected CHO cells in cell–cell binding assays (Supplemental Fig. 2). Control K562 cells only
weakly bound to FKN-CHO cells. This suggests that integrins bind to membrane-bound FKN on the cell surface. We found that membrane-bound FKN induced ternary complex formation with CX3CR1 and integrins α4β1/α5β1. This indicates that the chemokine domain of natural membrane-bound FKN can simultaneously interact with integrins and CX3CR1.

Taken together, the present study suggests that FKN-CD is a ligand for αβ1, and αβ1 in contrast to previous studies, and the direct integrin binding of FKN-CD or full-length FKN is involved in FKN signaling. An integrin-binding defective mutant is defective in FKN signaling, whereas it still binds to CX3CR1 and is a dominant-negative mutant.

**Discussion**

In this study, we first predicted that FKN-CD may bind to integrin α4β1 at a high affinity using docking simulation. We then demonstrated that FKN-CD binds to integrins and supports cell adhesion in a CX3CR1-independent manner, as FKN-CD bound to recombinant soluble αβ1 and supported adhesion of αβ1-K562 cells that do not express CX3CR1. We found that mutating two amino acid residues (Lys36 and Arg37) in FKN-CD in the predicted integrin-binding site (the K36E/R37E mutation) suppressed binding to αβ1. The K36E/R37E mutant still bound to CX3CR1, suggesting that the integrin-binding site and the CX3CR1-binding site in FKN-CD are distinct. The FKN-CD binding site in αβ1 overlaps with those of other known αβ1 ligands, as the specificity loop of β1 is involved in recognition of FKN-CD. Furthermore, SPR studies suggest that FKN-CD binds to αβ1 at an extremely high affinity, and the K36E/R37E mutation markedly reduces the binding function of FKN-CD to αβ1. These findings establish that FKN-CD is a high-affinity ligand for αβ1.

However, mAb 7E3 specific to integrin β1 or a small molecule inhibitor cyclic RGDIV did not suppress the binding of FKN-CD to αβ1. mAb 7E3 has been mapped within the ligand binding site of β3 (58, 59), and the K36E/R37E mutation marked reduced binding to αβ1 because FKN-CD has high avidity for αβ1. Another possibility is that 7E3 did not block the access of FKN-CD to αβ3. Cyclic RGDIV has a Ka of 2.4 × 10^-9 M to αβ3 (60). It is again possible that the affinity of cyclic RGDIV may not be high enough to effectively suppress FKN-CD. Another possibility is that the RGD-binding site was not critically involved in FKN-CD binding, and therefore cyclic RGDIV did not induce steric hindrance to FKN-CD in αβ3. Consistent with this idea, anti-β1 mAb AIIB2, which affects conformation of β1 rather than blocking access of ligands to the binding site (36), did suppress the binding of β1,3.1-CHO cells to FKN-CD.

We also demonstrated that FKN-CD bound to another integrin, α4β1. In this case, function-blocking anti-α4 mAb SG73, which is mapped within the ligand-binding site of α4 (37), and anti-β1 mAb AIIB2 suppressed this interaction in α4-K562 and U937 cells. Because α4β1 is expressed in leucocytes, α4β1/FKN-CD interaction is expected to contribute to FKN binding to leucocytes during trafficking of leucocytes. Because the K36E/R37E mutant was defective in α4β1 binding, it is suggested that α4β1 binds to FKN-CD in a manner similar to that of αβ1. Because α4β1 is a major FKN-CD-binding integrin in U937 cells, it is likely that α4β1 is important in FKN signaling in macrophages/monocytes, T cells, and NK cells, in which both CX3CR1 and α4β1 are expressed.

We demonstrated that FKN-CD binds to αβ1 or α4-K562 cells through αβ1 or α4β1 and, to a much less extent, through α4β1 in a CX3CR1-independent manner. In the case of U937 cells that express both CX3CR1 and integrins (α4β1 and α4β3), we demonstrated that FKN-CD binds to cells in an integrin-dependent and/or CX3CR1-dependent manner.

Importantly, we obtained similar results in integrin binding using the membrane-bound FKN. This suggests that 1) integrins can access to the chemokine domain in the membrane-bound FKN, and 2) integrins, membrane-bound FKN, and CX3CR1 can make a ternary complex. We did not, however, show if integrins access to FKN in cis and/or in trans in the present study.

Why was direct integrin binding to the chemokine domain of FKN overlooked? It has been proposed that FKN binding to CX3CR1-transfected K562 cells is integrin-independent, as EDTA does not block the binding (18). In this study, we demonstrated that EDTA did not fully suppress FKN-CD binding to integrins αβ1 and α4β1. Also, previous studies used the chemokine domain (76 aa residues) fused with large secretory placenta alkaline phos-
phatase (484 aa residues) (CX3CL1-SEAP) (21, 32). It has been reported that the macrophage-like cell line THP-1 cells efficiently adhered to immobilized CX3CL1-SEAP and that anti-β2 Abs did not suppress the adhesion (21). It has also been reported that THP-1 cells effectively bind to TNF-α-stimulated HUVECs, and anti-β1, and -β2 Abs partially suppressed the binding (21). Because TNF-α-stimulated endothelial cells express VCAM-1, ICAM-1, and FKN, it is unclear whether the Abs suppressed integrin binding to VCAM-1, ICAM-1, or FKN. The present study identified that anti-β1 AIIB2 and anti-αi SG73 suppress integrin/FKN-CD interaction, but it did not determine which of other anti-integrin Abs effectively suppress integrin binding to FKN-CD at this point. It is possible that the mAbs used (anti-β1) in previous studies did not effectively block the access of FKN to integrins because FKN-CD is smaller than other ligands (e.g., fibronectin) or because the affinity of the Abs to β1 integrins is lower than that of FKN-CD. We also suspect that CX3CL1-SEAP is defective in integrin binding possibly owing to steric hindrance. One possible reason why we detected integrin/FKN-CD interaction in the present study may be that we used the chemokine domain with a relatively small tag (34 aa residues). FKN is expressed as the membrane-bound form. Importantly, we found that membrane-bound FKN induced ternary complex formation with CX3CR1 and integrins αβ/αβ (Supplemental Fig. 2). This indicates that the chemokine domain of natural membrane-bound FKN can simultaneously interact with integrins and CX3CR1. Thus, FKN/integrin interaction is biologically relevant.

In a current model of the FKN-mediated leukocyte trafficking, FKN captures leukocytes in a selectin- and integrin-independent manner (14). Interaction between FKN and CX3CR1 can also activate integrins, resulting in firmer adhesion. Leukocytes then extravasate through the vascular wall and into the tissue to a chemokine gradient. FKN may facilitate extravasation of circulating leukocytes by mediating cell adhesion through the initial tethering and final transmigration steps (14). In this model integrins are involved only in the firm adhesion step. The present study suggests that FKN/integrin interaction may be involved 1) in the initial capturing of leukocytes through direct binding to FKN, and 2) in the activation of integrins through CX3CR1 as well. Interestingly, K36E/R37E induced chemotaxis at the level comparable to that of wt FKN-CD, whereas R47A did not. This suggests that FKN-induced chemotaxis is CX3CR1-dependent and that FKN-CD/integrin interaction may not be involved in chemotaxis.

What is the role of integrins in FKN signaling? In U937 cells (CX3CR1+), we demonstrated that integrins (αβ/αβ), FKN-CD, and CX3CR1 generated a ternary complex, whereas the K36E/R37E mutant was defective in ternary complex formation, suggesting that the direct integrin/FKN interaction plays a critical role in the ternary complex formation. Based on these findings we propose a novel model of FKN signaling, in which FKN binding to CX3CR1 recruits integrins to the complex, resulting in the formation of a ternary complex (CX3CR1-FKN/integrin) on the cell surface. If the ternary complex formation is required for proper FKN signaling, it is predicted that the FKN-CD mutant that is defective in integrin binding will exert as an antagonist of this signaling pathway. Consistent with the prediction, we demonstrated that excess K36E/R37E suppressed integrin activation in vitro, as well as leukocyte infiltration in thioglycollate-induced peritonitis in vivo. Also, K36E/R37E suppressed the increase in leukocyte recruitment induced by wt FKN-CD. These findings suggest that the K36E/R37E mutant acts as a dominant-negative antagonist of FKN signaling and neutralizes endogenous and exogenous FKN. The K36E/R37E mutant may have potential as a therapeutic agent in inflammatory diseases. Collagen-induced rheumatoid arthritis and experimental autoimmune myositis are disease models with well-defined FKN contribution in which anti-FKN Abs suppress inflammation (61, 62). In future studies we will evaluate the potential of K36E/R37E to suppress chronic inflammation and compare the efficacy of K36E/R37E with that of anti-FKN Abs in these disease models. It is also imperative to study molecular mechanisms of integrin contribution in FKN signaling in future studies.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental data

Fig. S1

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FKN-CD binding

Fig. S1. In vitro stability of FKN-CD
a) wt FKN-CD (1 μg) was added to whole mouse serum (20 μl) for the indicated time and analyzed by western blotting using anti-6His antibodies. b) 6His-tagged wt FKN-CD (1 μg) was incubated with U937 cells (1 x 10⁵ cells per sample) in RPMI 1640 medium in the presence or absence of FCS (10%) for the indicated time. Bound wt FKN-CD was detected using Alexa488-anti-6His antibodies and flow cytometry. The histogram shown is a representative of three experiments. The numbers represent % positive cells.
**Fig. S2. Interaction of membrane-bound FKN to integrins.**
a) Expression of membrane-bound FKN in CHO cells. Full-length FKN was stably expressed in CHO cells and the expression of FKN was determined by flow cytometry using anti-FKN MAB365. b)-e) CHO cells that express membrane-bound wt FKN were plated to wells (5000 cells per well) and incubated with fluorescent labeled floating cells (U937, α4-K562, αvβ3-K562, and K562 cells, 1 x 10⁵ cells per well) in RPMI 1640 medium for 1 h at 37°C. Bound floating cells were counted after gentle rinsing using fluorescent plate reader. f) and g) Co-precipitation of membrane-bound FKN and integrin β3 (f) or α4 (g). Cell lysates of FKN-CHO cells and U937 cells were mixed and incubated for 1 h at 4°C, and then the mixture was incubated with anti-β3 or anti-α4 mAb for additional 1 h at 4°C. The immune complex was recovered by incubating with protein A-Sepharose for 30 min at 4°C, and analyzed by western blotting.