Supporting Information

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SI Materials and Methods

Synthesis of Pepducins. Peptides were synthesized on a 0.1-mmol scale using microwave-assisted solid phase peptide synthesis using Rink Amide MBHA resin (EMD Chemicals), fluorenylmethyloxycarbonyl chloride protected amino acids (CEM), and HBTU coupling chemistry. The protected amino acids were dissolved in DMF (0.2 M), the activator solution was prepared in dimethylformamide (0.45 M) and the activator base, *N*,*N*-diisopropylethylamine, was prepared in *N*-methylpyrrolidone (2 M). Palmitic acid was used to cap the N terminus with a lipid using the same coupling strategy as individual amino acids. The deprotection mix was prepared as a 20% vol/vol solution of piperidine in DMF with 0.1 M hydroxybenzotriazole hydrate. The synthesis was accomplished using the CEM Liberty Microwave Peptide synthesis system. The resin was agitated for approximately 2 h using a mixture consisting of (TFA:85%MSA/water: TIS:DDT 82:6:6:6; 10 mL). Filtration through a medium porosity Büchner funnel and trituration with 40 mL of diethylether provides crude pepducin. The crude pepducin (30–50 mg) was dissolved in 1.0 mL of dimethylsulfoxide and purified by injecting 900 μL of the solution onto the one-half inch C-5 Luna column using an ammonium acetate/acetonitrile gradient on an Agilent 1100 HPLC/MS. Desired fractions are combined using mass detection fraction collection, and lyophilized without further concentration, leaving 1–10 mg of white powder. Typically the peptides elute between 60% and 80% acetonitrile. Pepducin (0.5–1 mg) is dissolved in 0–30% acetonitrile (0.5–1.0 mL) and injected onto a Phenomenex C-5 Luna column, purity determined by area (220 nm) using an Agilent 1100.

Calcium Flux Assay. CCRF-CEM cells were maintained in RPMI-1640 medium supplemented with 10% FBS at density between 2×10^5 and 2×10^6 per mL. Cells were collected by centrifugation and resuspended in assay buffer [phenol red free RPMI-1640, 20 mM Hepes, pH 7.4, and 0.2% (wt/vol) BSA] at 2×10^6 per mL. For studies in U87-CD4 cells, the cells were transiently transfected using Fugene reagent (Roche). Twenty hours after transfection, cells were trypsinized and washed once with fresh medium before seeding into 384-well plates at 6×10^4 cells per well. Calcium 4 dye from Molecular Devices was added to the cells before seeding 100-μL aliquots onto 96-well black plates with clear bottom. Plates were centrifuged briefly to spin down cells before incubation for 60 min at 37 °C. Calcium response to CXCL12 or test articles was measured on a FlexStation III (Molecular Devices). Data were analyzed using GraphPad Prism. Dose–response curves were fitted using nonlinear curve fit (Y = Bottom + (Top-Bottom)/(1 + 10 ^ ((LogEC50-X) × HillSlope))). For experiments using PTX, cells were incubated in growth medium overnight at 37 °C with 5% CO_2 in the presence or absence of 100 ng/mL of PTX (Sigma).

cAMP Assay. Twenty four hours after seeding, cell medium was removed by gentle dumping and replaced with 30 μL of CXCL12 (Peprotech) or test articles in assay buffer [Hanks' balanced salt solution, 20 mM Hepes, pH 7.4, 0.1% (wt/vol) BSA]. After 15 min of incubation at 37 °C, 20 μL of water soluble analog of forskolin, NKH477, was added to final concentration of 10 μM followed by 30 min of incubation at room temperature. cAMP Dynamic 2 kit from Cisbio was used following the manufacturer's protocol. Briefly, 25 μL of cAMP-d2 was added to each well followed by 25 μL of anti-cAMP cryptate. After 1 h of incubation, plates were read on PheraStar Plus (BMG Labtech). Data were analyzed using GraphPad Prism. Dose–response curves were fitted using nonlinear curve fit (Y = Bottom + (Top-Bottom)/(1 + 10 ^ ((LogEC50-X) * HillSlope))). For experiments using PTX, cells were incubated in growth medium overnight at 37 °C with 5% CO_2 in the presence or absence of 100 ng/mL of PTX (Sigma).

Receptor Internalization. Receptors were transiently transfected into HEK-293 cells using Lipofectamine Plus reagent (Invitrogen) and were plated 24 h after transfection on glass coverslips coated with poly-D-lysine (Sigma). The next day, cells were treated with either vehicle alone or with varying concentrations of ATI-2341 for 30 min at 37 °C followed by fixation with methanol for 5 min at −20 °C. GFP fluorescence was visualized directly using a Zeiss Axiovert inverted microscope. Images were processed using Adobe Photoshop and Adobe Illustrator.

Human PMN Chemotaxis Assay. Human PMN cells were purified using a Ficoll density centrifugation method and resuspended in assay buffer [phenol red free RPMI-1640, 20 mM Hepes, pH 7.4, and 0.5% (wt/vol) BSA]. Transwell 96-well plates with 3.0-μm pore size polycarbonate membranes (Corning) were used for the assays. CXCL12 or ATI-2341 were diluted in assay buffer and added to the bottom well, whereas 5×10^4 cells were added to the top well. Cells were allowed to move to the bottom well for 30 min in a 37 °C incubator with 5% CO_2 before the receiver plates were separated from the inserts. Cells translocated to the bottom wells were quantified using Cyquant dye (Invitrogen). Fluorescence intensity was measured on a FlexStation III.

PMN Peritoneal Recruitment Model in Mice. Peritoneal lavages were collected following i.p. injection of 10 mL of DPBS. Number of white blood cells (WBCs) per milliliter of lavage fluid was counted using Cellometer Auto T-4 (Nexcelom Bioscience). Percentage of PMNs (PMNs %) in peritoneal lavages was determined by flow cytometry after staining cells with FITC-conjugated antibodies specific to Gr.1 neutrophil marker. Total number of PMNs accumulated in the peritoneal cavity was calculated according to equation PMNs = WBCs × PMNs(%) × 10 mL.

Colony-Forming Units Assay in Mice. PBMCs were prepared by lysing 200 μL of whole blood with Pharmlyse (BD Biosciences), then washing twice with Isocove's medium containing 2% FBS. Total cell pellets were resuspended in 3 mL of Methocult medium supplemented with rmCSF, rmIL-3, and rhIL-6 (StemCell Technologies), plated in duplicate, and incubated at 37 °C, 5% CO_2 for 12 d. Colonies were counted once by an investigator blinded to the identity of the groups. Total colonies per milliliter were calculated by multiplying the number of colonies identified by the volume of blood prepared.

PMN Mobilization Assay in Mice. Male 6- to 8-wk-old BALB/c and DBA/2 mice were purchased from Charles River Laboratories. All mice were fed ad libitum, housed on a 12-h light/dark cycle, and allowed to acclimate to the housing facility for at least 1 wk before experiments. All studies were done in accordance with Institutional Animal Care and Use Committee guidelines. Pepducins were injected i.v. in 10 mM of ammonium acetate pH 7, 0.2% Tween 80, 8.5% sucrose (0.1 mL per mouse). AMD-3100 was formulated in distilled water and injected i.v. (2 μmol/kg). Mice were killed by CO_2 asphyxiation and blood was collected via the vena cava 1.5 h following compound injection. Neutrophil and lymphocyte counts were obtained by Hemavet (model 950FS, Drew Scientific).

Characterization of Lymphocyte Subpopulations in Mice. BALB/c mice received i.v. injections of either vehicle, AMD-3100 (2 μmol/kg) or ATI-2341 (2 μmol/kg). Ninety minutes later mice were killed using CO_2 asphyxiation and heparanized blood was collected from the inferior vena cava. A total WBC count (WBCC) was determined using a Hemavet HV950FS cell counter (Drew Scientific). To determine the percentage of major lymphocyte subsets (LSP), blood samples were stained with the fluorescently labeled antibodies specific to CD45 (leukocyte common antigen, all WBCs), B220 (B-lymphocytes subset), CD4 (Th lymphocyte subset), and CD8 (Tc lymphocytes subset) and analyzed by flow cytometry (BD FACSCanto II). Absolute lymphocyte subset count (LSC) for each lymphocyte subset was calculated as following: LSC = WBCC × LSP.

PMN Mobilization Assay in Cynomolgus Monkey. In nonhuman primate studies, nonnaïve male cynomolgus monkeys (*Macaca fascicularis*) received i.v. infusion of vehicle or ATI-2341. At the indicated time points, blood was collected for hematological profiling using an Advia 120 automated analyzer.

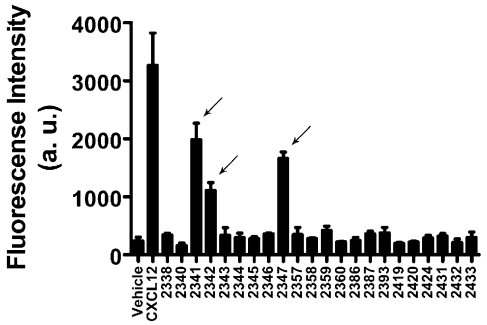

Fig. S1. Screening of a CXCR4 pepducin library. A library of pepducins derived from the intracellular loops of human CXCR4 was screened in CCRF-CEM cells using 1 μM of compound in a 96-well Transwell chemotaxis assay. Migrating cells were quantified using Cyquant dye. The averages of relative fluorescent intensity of triplicate wells were plotted. Active compounds are indicated by an arrow.

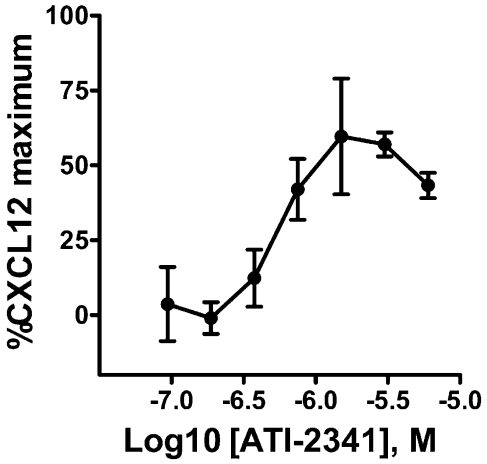

Fig. S2. ATI-2341 dose-dependently induces chemotaxis of human PMNs. Migration of human PMNs to the receiving wells of a 96-well Transwell system was quantified by the use of Cyquant dye and subsequent measurement of fluorescent intensity on a FlexStation III. Data were normalized to maximal CXCL12-stimulated migration and are representative of $n = 3$ independent experiments.

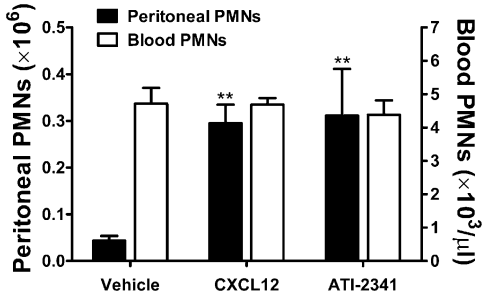

Fig. S3. Intraperitoneal administration of ATI-2341 does not increase blood PMN count. BALB/c mice received i.p. injections of vehicle, CXCL12 (30 μmol/kg), or ATI-2341 (300 nmol/kg). Three hours later the number of peritoneal PMNs (closed bars) and blood PMNs (open bars) were determined as described in *Materials and Methods*. The data are presented as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's posttest, $^{**}P < 0.01$, 8–10 mice per group.

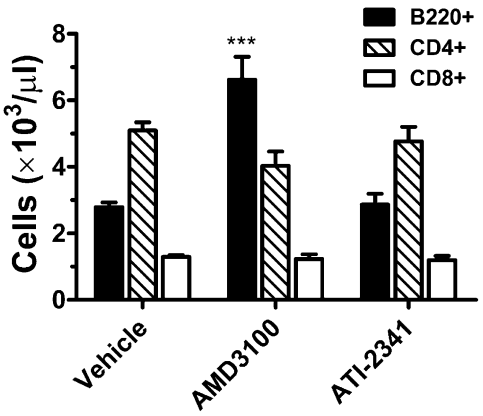

Fig. S4. Treatment with AMD-3100 but not ATI-2341 induces an increase in blood B-cell count. BALB/c mice received i.v. injections of either vehicle, AMD-3100 (2 μmol/kg) or ATI-2341 (2 μmol/kg). Ninety minutes later heparanized mouse blood was collected and a differential lymphocyte count was determined as described in *Materials and Methods*. The data are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's posttest, ***$P < 0.001$, 8–10 mice per group.

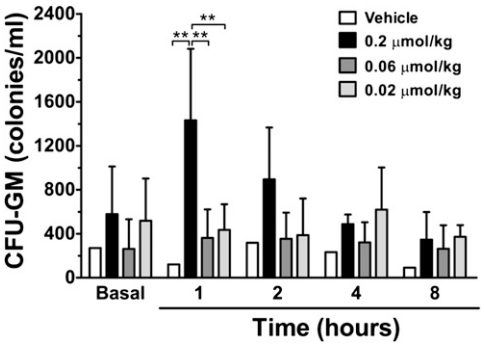

Fig. S5. ATI-2341 induces mobilization of CFU-GM in non-human primates. Nonnaïve cynomolgus monkeys received i.v. infusions of either vehicle (one monkey) or three different doses (0.2, 0.06, and 0.02 μmol/kg) of ATI-2341 (three monkeys per group). At the indicated time points blood was collected and processed for the CFU-GM assay. Two-way repeat measurements ANOVA with the Bonferroni posttest were used for the statistical analysis. The data are presented as mean ± SD (**$P < 0.01$).

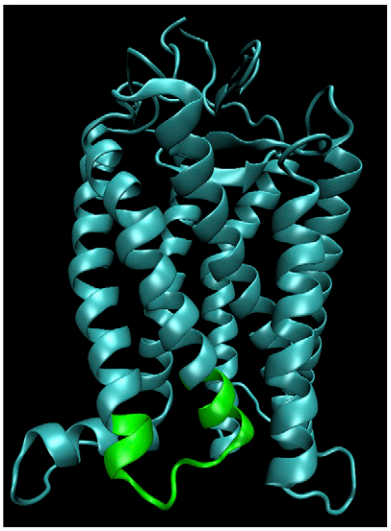

Fig. S6. Location of ATI-2341 peptide sequence on a homology model of CXCR4. Mapping of the peptide sequence of ATI-2341 within the i1 loop region using a homology model for CXCR4 that was developed on the basis of the structure of rhodopsin. Residues 64–82 of the CXCR4 intracellular loop 1 region corresponding to the peptide portion of ATI-2341 are highlighted.

Table S1. The effect of ATI-2341 on blood PMN and lymphocyte counts in non-human primates

	PMNs (×1,000 cells/μL)				Lymphocytes (×1,000 cells/μL)			
Time (h)	Vehicle	0.02	0.06	0.2	Vehicle	0.02	0.06	0.2
Basal	2.2	1.8 ± 0.2	2.0 ± 0.5	6.3 ± 4.5	6.9	10.5 ± 1.4	11.4 ± 4.9	9.3 ± 2.9
0.5	7.4	8.3 ± 2.1	11.8 ± 1.0	9.4 ± 7.4	5.3	9.7 ± 2.1	11.4 ± 4.6	11.9 ± 5.5
1	9.8	11.3 ± 0.5	17.2 ± 1.4	21.0 ± 5.5	5.0	8.1 ± 2.1	10.3 ± 2.9	11.6 ± 5.0
2	10.1	11.4 ± 0.6	20.8 ± 6.7	26.5 ± 2.5	3.8	7.2 ± 1.7	8.0 ± 2.5	7.4 ± 2.4
4	6.8	9.2 ± 1.3	12.1 ± 2.3	17.2 ± 3.1	4.5	8.3 ± 0.7	6.9 ± 1.7	5.5 ± 1.3
8	3.4	4.7 ± 1.0	5.3 ± 3.0	7.7 ± 1.6	6.6	12.9 ± 2.9	9.2 ± 2.4	8.0 ± 3.3

Cynomolgus monkeys received a 15-min i.v. infusion of either vehicle (n = 1) or ATI-2341 at 0.02 μmol/kg (n = 3), 0.06 μmol/kg (n = 3), and 0.2 μmol/kg (n = 3). Blood was collected before the infusion (basal) and at 0.5, 1, 2, 4, and 8 h after treatment for the determination of polymorphonuclear neutrophil (PMN) and lymphocyte counts. The values represent means ± SD.