Supporting Information

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SI Results

Human and mouse lesions of atherosclerosis contain both M1 and M2 macrophage phenotypes (1, 2). Previous work has suggested the presence of M2 macrophages, expressing suppressor of cytokine signaling 3 (SOCS-3) protein, in late-stage atherosclerotic lesions (3, 4). Additionally, it has been observed in the same mouse model that knockdown of SOCS-3 and its associated anti-inflammatory effects exacerbates atherosclerosis. Thus, we tested whether M2pep binds M2 macrophages isolated from late-stage atherosclerotic lesions of apolipoprotein E ($ApoE^{-/-}$) mice and elicited peritoneal macrophages from the same mice (Fig. S6). Macrophages were identified by staining for the macrophage marker CD68, and the M2 macrophage subset was identified by the CD301/Mgl1 marker. Both lesion and peritoneal $Mgl1^{+}$ macrophages showed a significant 1.5-fold to twofold differential in the ratio of M2pep vs. scrambled M2pep relative to $Mgl1^{-}$ cells, demonstrating preferential M2pep binding to M2 macrophages isolated from two different sites.

SI Materials and Methods

Generation of Macrophage Cell Types. Bone marrow cells were plated in 100-mm Petri dishes at 5 million cells per plate. Cells were differentiated into macrophages for 7 d using media containing RPMI, 20% (vol/vol) horse serum (CellGro), 1% (vol/vol) antimicrobial/antimycotic (CellGro), and 20 ng/mL recombinant mouse macrophage colony-stimulating factor (Shenandoah Biotech). Media were replenished on day 4. Seven-day differentiated macrophages were activated for 48 h in RPMI media containing 20% (vol/vol) horse serum and cytokines. The cytokines used were 25 ng/mL IFN-γ (R&D Systems) and 100 ng/mL LPS (InvivoGen) for M1 macrophages and 25 ng/mL IL-4 (R&D Systems) for M2 macrophages. All cells were cultured at 37 °C in a 5% CO_2 atmosphere.

Generation of K_d Curve. The K_d of M2pep with an oligoethyleneglycol-biotin tag was quantified in a titration flow cytometry assay. A total of 10^5 M2 macrophages per sample were incubated with varying concentrations of M2pep. The presence of M2pep was probed with FITC-conjugated streptavidin (eBioscience) and analyzed on a FACSCanto flow cytometer (Becton Dickinson). To calculate K_d, the median fluorescence intensities were fit by least-squares regression to the following equation: $MFI_{expected} = MFI_{min} + MFI_{range}\left(\frac{[peptide]}{[peptide] + K_d}\right)$.

Isolation of Atherosclerotic Lesion Cells. Peritoneal and lesion macrophages were isolated from $ApoE^{-/-}$ mice with advanced lesions of atherosclerosis (45–60 wk). Resident peritoneal macrophages were harvested by injection of 5 mL of PBS/EDTA and kept on wet ice before staining for flow cytometric analysis. The mouse aorta, brachiocephalic artery, and carotid arteries were dissected following collection of the peritoneal macrophages. After removing excess fat from the dissected aorta and its branches, the aorta was incubated for 5 min at 37 °C in Hepes-buffered DMEM with 1.3 mg/mL collagenase type III (Worthington), which allowed the adventitia to be easily removed from the aortic media and intima. The remaining vessel was chopped into small segments and then incubated for 1 h in Hepes-buffered Media 199 containing 125 U/mL collagenase type XI (Sigma), 60 U/mL hyaluronidase (Sigma), 60 U/mL DNase I (Sigma), and 450 U/mL collagenase type I (Worthington). The cells were passed through a 70-mm strainer to remove tissue debris and then stained for analysis by flow cytometry.

ELISA Phage-Binding Study. M1, M2, and unactivated macrophages and dendritic cells were incubated with 1× 10^5 pfu/μL M2pep Phage or Phage Library. The presence of phage was probed using a rabbit anti-M13 primary antibody and HRP-conjugated anti-rabbit secondary antibody (Sigma). Substrate was added and quenched, and absorbance at 450 nm and 540 nm was read on a plate reader.

1. Khallou-Laschet J, et al. (2010) Macrophage plasticity in experimental atherosclerosis. *PLoS ONE* 5(1):e8852.
2. Bouhlel MA, et al. (2007) PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab* 6(2):137–143.
3. Tang J, Raines EW (2005) Are suppressors of cytokine signaling proteins recently identified in atherosclerosis possible therapeutic targets? *Trends Cardiovasc Med* 15(7):243–249.
4. Tang J, et al. (2005) The absence of platelet-derived growth factor-B in circulating cells promotes immune and inflammatory responses in atherosclerosis-prone ApoE-/- mice. *Am J Pathol* 167(3):901–912.

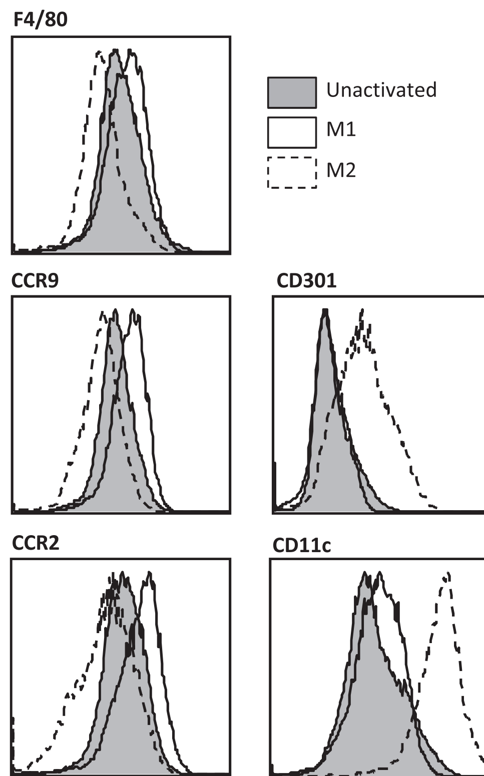

Fig. S1. Immunophenotyping of M1 and M2 cells by flow cytometry. All cells stain positively for F4/80. M1 cells are CCR9^{hi}CCR2^{hi}. M2 cells are CD301^{hi}CD11c^{hi}.

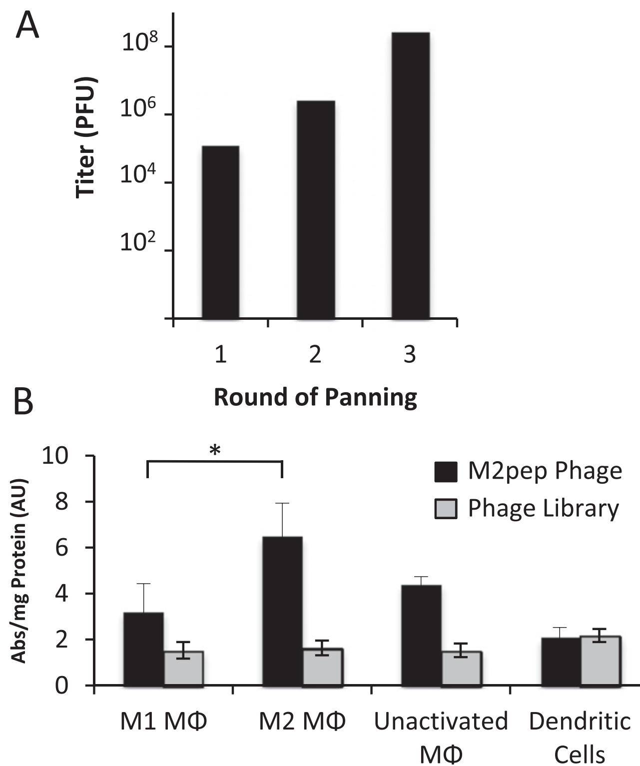

Fig. S2. Phage titer following each round of phage panning and selection of phage selective for M2 macrophages. (*A*) Increasing phage titer with each round of panning indicates selection of phage specific for M2 macrophages. (*B*) M2pep Phage binding to M1, M2, and unactivated macrophages and dendritic cells was quantified by whole-cell ELISA and normalized to protein content using a bicinchoninic acid colorimetric assay. AU, arbitrary units. *P = 0.041.

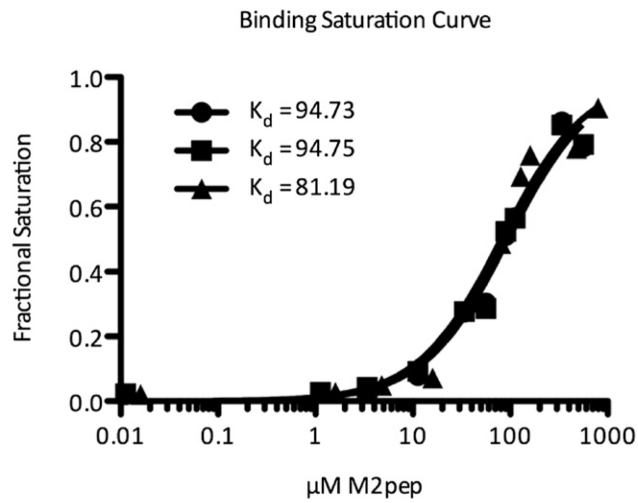

Fig. S3. K_d titration binding curve. The K_d of M2pep with an oligoethyleneglycol-biotin tag binding M2 macrophages was measured by means of a peptide concentration titration flow cytometry experiment. Three replicate experiments were performed.

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Fig. S4. Internalization of M2pep mediates intracellular delivery of a test protein. (*A*) M1 and M2 macrophages and CT-26 cancer cells were incubated with 200 μM of M2pep or scrambled M2pep (scM2pep) and analyzed by flow cytometry (n = 3 for each cell type). (*B*) Internalization of 20 μM Alexa Fluor 660-labeled M2pep or scM2pep was evaluated by confocal microscopy (blue, DAPI; red, F4/80; green, peptide). (*C*) Quantification of mean fluorescence intensity per cell demonstrates preferential uptake by M2 macrophages compared with M1 cells (*P = 0.0002) and CT-26 cells (*P = 0.000003): M2pep/M2MΦ (n = 8),

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M2pep/M1MΦ ($n = 3$), M2pep/CT-26 ($n = 5$), scM2pep/M2MΦ ($n = 5$), scM2pep/M1MΦ ($n = 3$), and scM2pep/CT-26 ($n = 2$). (*D*) Internalization of complexes of M2pep or scM2pep with phycoerythrin-cy7–conjugated streptavidin was quantified by confocal microscopy (blue, DAPI; red, F4/80; green, peptide).

Fig. S5. M2pep binds tumor-associated macrophages in vivo and does not accumulate in either liver or splenic macrophages. (*A*) Mice injected with M2pep show increased uptake in F4/80^{+} cells compared with mice administered scM2pep ($*P = 0.002$) or PBS ($*P = 0.003$): M2pep ($n = 5$), scM2pep ($n = 4$), and injection buffer ($n = 2$). (*B*) Additional confocal microscopy images of tumor sections from mice tail vein-injected with Alexa Fluor 660-labeled M2pep. (*C*) Both M2pep and scM2pep display diffuse uptake in the liver. (*D*) Both M2pep and scM2pep lack uptake in splenic cells.

Fig. S6. M2pepKLA delays tumor growth compared with treatment with M2pep. (*A*) Treatment with M2pepKLA as described in Fig. 5 mediates delayed tumor growth compared with M2pep, with a statistically different tumor size at day 9 (*$P = 0.009$) and day 10 (*$P = 0.038$). (*B*) In a separate study, mice were dosed with M2pepKLA three times over 7 d, resulting in a statistically significant tumor growth delay at day 14 (*$P = 0.025$). (*C*) In the separate study with dosing three times over 7 d, there was a trend for delayed mortality in the group treated with M2pepKLA. M2pepKLA ($n = 5$), scM2pepKLA ($n = 6$), M2pep ($n = 6$), PBS/5%DMSO ($n = 5$).

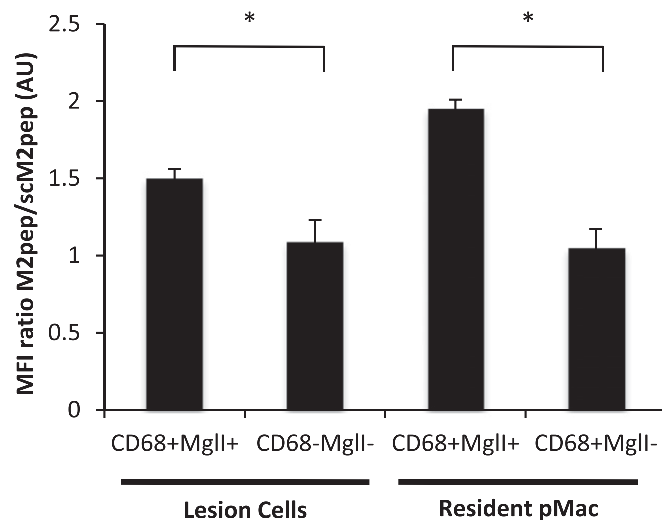

Fig. S7. M2 macrophages are detected in peritoneal and lesion macrophages of ApoE$^{-/-}$ mice with advanced lesions of atherosclerosis. Lesion and resident peritoneal cells were isolated from 45- to 60-wk-old ApoE$^{-/-}$ mice on a chow diet ($n = 3$ separate pools each from 2 mice) and analyzed by flow cytometry. Cells were stained for CD68 (macrophages), for Mgl1 (M2 macrophages), and with M2pep or scM2pep. The mean fluorescence intensity (MFI) ratio of M2pep/scM2pep is shown for different gated populations with $^*P = 0.034$ and $^*P = 0.0013$ for lesion and peritoneal macrophages (pMac), respectively.

Table S1. Antibody clone numbers, vendors, and conjugations

Marker	Clone	Vendor	Conjugation	Detection
B220	RA3-6B2	BD Pharmingen	FITC, PE	B cells
CCR2	475301	R&D Systems	PE	M1 macrophages, M2c macrophages
CCR9 (CD199)	CW-1.2	eBioscience	FITC	M1 macrophages
CD11b	M1/70	eBioscience	eFluor450	Leukocytes
CD11c	N418	eBioscience	PE	M2 macrophages
CD3	17A2	BD Pharmingen	PE	T cells
CD301 (MglI)	ER-MP23	AbDserotec	FITC	M2 macrophages
CD68	FA-11	AbDserotec	PE	Macrophages
CD86	GL1	eBioscience	PE-Cy5	M2b macrophages
F4/80	CI:A3-1	AbDserotec	FITC	Macrophages
F4/80	CI:A3-1	Abcam	None	Macrophages
F4/80	BM8	Life Technologies	Alexa Fluor 647	Macrophages
Goat anti-rabbit		Sigma	FITC	
Goat anti-rat		Life Technologies	Alexa Fluor 555	
Ly6C	AL-21	BD Pharmingen	APC-cy7	Macrophage subset
Ly6G	1A8	BD Pharmingen	FITC, PE, PerCP Cy5.5	Neutrophils
MHCII	M5.114.15.2	eBioscience	FITC	Macrophage subset
NeutrAvidin		Life Technologies	PE	Peptide
Rabbit anti-M13		Sigma	None	Phage
Streptavidin		eBioscience	FITC, PE-cy7	Peptide

APC, allophycocyanin; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex.