Supporting Information

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SI Text

Methods ***Construction of Random Mutagenesis library of Mac-1 I Domain.*** To generate random mutagenesis library, 2 μM each of nucleotide analogues of 8-oxo-2′-deoxyguanosine-5′-triphosphate and (8-oxo-dGTP, TriLink) and 2′-deoxy-*p*-nucleoside-5′-triphosphate (dPTP, TriLink) mixed in 200 μM of dNTP were used to PCR amplify cDNA encoding Asp132—Thr322 of the Mac-1 I domain. 15 μg of PCR product and 4 μg of linearized pCTCON digested at NheI and BamHI sites were mixed in 5 μl water for high-efficiency yeast transformation. The cDNA library-vector mixture was added to a cuvette containing freshly prepared 3×10^8 yeast competent cells in 50 μl of 10 mM Tris (pH 7.5), 270 mM sucrose, and 1 mM $MgCl_2$. Electroporation was performed according to the published protocol (1). Transformation efficiency was approximately 4%, estimated by the number of colonies on selective dextrose (SD) medium (20 g/L dextrose, 6.7 g/L Difco yeast nitrogen base, 5 g/L Bacto casamino acids, 5.4 g/L Na_2HPO_4, 8.56 g/L $NaH_2PO_4 \cdot H_2O$) with 2% (wt/vol) agar. The Mac-1 I domain library cells were grown in SD media for 24 h at 30 °C with shaking, induced in SG media (20 g/L galactose, 6.7 g/L Difco yeast nitrogen base, 5 g/L Bacto casamino acids, 5.4 g/L Na_2HPO_4, 8.56 g/L $NaH_2PO_4 \cdot H_2O$) for 24 h at 30 °C with shaking, and screened for binding to HeLa cell monolayers. HeLa cells were grown to full confluency on a 24-well plate and were washed once with washing buffer (PBS, 0.5% BSA, 10 mM $MgCl_2$). A total of 5×10^7 yeast cells from the library of Mac-1 I domain were washed once with washing buffer, resuspended in 300 μl of washing buffer, and applied to the confluent HeLa cells in 24-well plate. Yeast cells were first allowed to settle down for at least 2 h at RT. The yeast cells were then washed off from HeLa cells by gentle shaking of the 24-well plate on an orbital shaker for 15 min. The washing cycle was repeated until uninduced yeast cells were completely washed off from HeLa cells. Yeast cells that remained adhered to HeLa cells were then collected with elution buffer (PBS, 0.5% BSA, 10 mM EDTA). The collected yeast cells were centrifuged and resuspended in SD medium, allowed to grow for 24 h, and induced in SG medium for 24 h. The yeast library underwent two rounds of sorting, after which enriched yeast cells were grown in SD plate. Individual clones from the plate were tested for binding to HeLa cells, similarly as described for library sorting. Microscopic images of yeast clones that remained bound on the surface of HeLa cells were acquired with a CCD camera (Spot Insight, Diagnostic Instruments) at 4 random spots. The acquired images were processed by Image-Pro Plus (Media Cybernetics) for automated cell counts. From the yeast clones the plasmids were extracted using Zymoprep II Yeast Plasmid Miniprep Kit (Zymo Research) for DNA sequencing of the Mac-1 I domain.

Quantitative Screening of Antibody Library in YS2H. To screen antibodies based on monomeric antigen–antibody interactions in yeast, the cDNA encoding scFv was PCR-amplified from the phagemid belonging to the library after the second and the fourth round sorting. Then, scFv and the F302L cDNAs were inserted into the YS2H vector as the prey and the bait, respectively. Two different tags (Myc and Flag) were used for immunofluorescence staining. In YS2H, Flag tag is fused to the I domain at the C-terminal and used to measure the level of antigen expression, and Myc tag is placed at the C-terminal of scFv and used to estimate the affinity between the I domain and scFv. The level of tag expression was measured by the binding of antiFlag monoclonal antibody and antiMyc antibody used at 5 μg/mL in PBS with 0.5% BSA and 10 mM $MgCl_2$. From mean fluorescence intensity (MFI) values of flag and myc tag expression, the affinity between antibody and the I domain was predicted using the first-order Langmuir equation, given by $[\mathrm{bait:prey}]/[\mathrm{bait}] = [\mathrm{prey}]/([\mathrm{prey}] + K_D)$ or $1/\mathrm{MFI}_{\mathrm{Myc}} = \alpha^{-1}\ (1 + K_D/[\mathrm{prey}])/\mathrm{MFI}_{\mathrm{Flag}}$, where α is the MFI ratio of antimyc to antiflag antibody binding to equal copies of Myc and Flag tags and $[\mathrm{prey}] = 10$ nM (2).

Expression of the Mac-1 I Domains and scFv. Wild-type Mac-1 I domain (Asp132-Ala318) and the mutants of F302L and I316G with six histidine (His tag) at the C-terminal were cloned into pET20b (EMD Chemicals). The scFvs in the phagemid vector, pIT2 were cloned into pET20b with His and Myc tags fused at the C-terminal. These proteins were expressed as soluble forms and purified by Nickel-nitrilotriacetic acid (NTA) column. Both proteins were expressed in BL21(DE3) (Invitrogen) as soluble proteins. BL21-transformed cells were grown at OD_{600} to 0.4, to which 1 mM isopropyl β-D-1-thiogalactopyranoside was added. Cells were induced to express protein at 30 °C by culturing for 6 hours with shaking at 250 rpm. After induction, cells were spun down, resuspended in binding buffer (50 mM sodium phosphate at pH 8.0, 300 mM sodium chloride, and 10 nM imidazole), sonicated to break the cell wall, and spun at 10,000 g for 15 min to remove cell debris. Proteins in the supernatant were purified with NTA column followed by gel filtration chromatography using Superdex 75 column connected to AKTA Purifier (GE Healthcare). By comparing to the molecular weight markers for gel filtration chromatography (Sigma–Aldrich), the molecular weight of single-chain antibodies (AM01 and AM17) was estimated to be 25–30 kDa, indicating these antibodies exist as monomer in solution. The purity and the size of proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SPR

Surface Plasmon Resonance (SPR) Analysis. CM5 sensor chip was prepared using an amine coupling kit (BIAcore) to immobilize Mac-1 ligands [inactivated complement component 3b (iC3b, EMD Biosciences), fibronectin (Invitrogen), and ICAM-1) or antibodies against Mac-1 (AM01, CBRM1/5 (3), and mAb44 (4)]. SPR was measured using Biacore (BIA2000) as described previously (1). To measure the affinity of I domain variants to Mac-1 ligands or antibodies, the I domains were injected over the chip in injection buffer (20 mM Tris HCl, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$) at a flow rate of 10 μl/ min at RT. To examine the potency of antibodies in blocking I domain binding to ligands, the I domains at 200 nM were preincubated with antibodies (AM01, CBRM1/5, and mAb44) at 62.5—1000 nM (per valency using the values of 30 kDa for scFv and 75 kDa for immunoglobulins) for 15–30 minutes at RT and the mixture was injected over the chip coated with Mac-1 ligands. The chip surface was regenerated by flowing 20 μl of 10 mM Tris-glycine, pH 2.0 buffer. The IC_{20} values of the antagonists in blocking Mac-1 binding to ICAM-1, fibronectin, and iC3b were determined by fitting the equation $A = A_{MAX} - (A_{MAX}/[1 + IC_{50}/L])$ to the data (GraphPad Prism), where "A" is the sensogram data and "L" is the molar concentration of inhibitors.

Antibody Binding to Neutrophils. To separate neutrophils, 20 mL of whole blood were drawn from volunteers (under the permission

of Cornell Institutional Review Board), cooled down to RT, and loaded on top of 20 mL of 1-Step® Polymorphs (Accurate Chemical and Scientific Corporation). The gradient was centrifuged at 500 g for 30 min at 22 °C. After centrifuge, neutrophils in the polymorphonuclear fraction were taken out and washed with Hank's balanced salt solution without calcium and magnesium ions (Invitrogen). Purified neutrophils were suspended in PBS, 0.5% BSA with 1 mM MgCl2, 1 mM MgCl2 plus1 mM EGTA, or 1 mM EDTA, incubated with single-chain antibodies at RT for 10 min, washed, and then fixed with 3.7% formaldehyde for 10 min. Then neutrophils were incubated in respective labeling buffer with 5 μg/mL antiHis antibody on ice for 45 min, washed, and subsequently incubated with 5 μg/mL goat antimouse antibody conjugated with PE. The cells were washed and resuspended in PBS for flow cytometry analysis.

Immunofluorescence Flow Cytometry. Antibodies used in this study were the anti- Myc antibody 9E10 (ATCC), antiFlag (Genscript), and phycoerythrin-labeled goat polyclonal antimurine antibodies (Santa Cruz Biotechnology). After induction, yeast cells were harvested, washed in 100 μl of the labeling buffer (PBS containing 0.5% BSA with 10 mM $MgCl_2$ or 10 mM EDTA), and then incubated with ligands in 50 μl of the labeling buffer for 20 min with shaking at 30 °C. Cell were then washed and incubated with secondary antibodies at 5 μg/mL in 50 μl of the labeling buffer for 20 min at 4 °C. Finally, cells were washed once in 100 μl and suspended in 100 μl of the labeling buffer for flow cytometry (Epics XL flow cytometer, Beckman Coulter).

Adhesion Inhibition Assay. The 96-well V-bottom plate (Greiner) was coated with fibrinogen (100 μg/mL) or 2% BSA in PBS, pH 7.4 overnight at 4 °C, and washed three times before incubation with neutrophils. Neutrophils were suspended in PBS at 10^7 cells/mL with 1 mM $MgCl_2$ or 1 mM $MgCl_2$ and 1 mM EGTA to induce active conformation of Mac-1 (5). To this mixture, antibodies at 20 μg/mL and 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) at 2 μg/mL were added. After 15 min incubation at RT, neutrophils were washed in PBS with 1 mM $MgCl_2$ plus with or without 1 mM EGTA and added to the V-bottom wells, and the plate was then subjected to centrifugation at 55 g for 1 min to cause nonadherent or weakly bound cells but not strongly adherent cells to accumulate at the tip of the V-bottom well (1). The number of nonadhered cells was measured by a fluorescent plate reader (Synergy HT, Biotek). To detach all the adherent cells to measure the fluorescence from the total number of cells in each well, the plate was centrifuged at 873 g for 20 min.

Migration Assay. Cell migration assay was carried out as previously described (6). In brief, delta T dish (Fisher Scientific) was coated with 100 μg/mL fibrinogen (Sigma) in PBS, pH7.4 at 4 °C overnight and washed three times with PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. 5×10^5 neutrophils were diluted in 1 ml L-15 media (Gibco), to which antibodies were added at 20 μg/mL. After incubation at room temperature for 15 min, neutrophils were transferred to the delta T dish and prewarmed to 37 °C for 5 min. The images of neutrophil migration were recorded for 20 minutes on every 10 seconds using Nikon TE-2000U inverted microscope. During 20 min recording, 10 nM formyl-Met-Leu-Pro (fMLP) was added at 1 min after neutrophils were added to the dish. From the recorded differential interference contrast (DIC) microscopic images, the frames of the last 12 minute interval were chosen for analysis. The movie was contrast enhanced for automated object tracking using Image-Pro Plus to trace the migration path of individual cells. Only the neutrophils present in the image field for the entire 12-minute of the movie were included in analysis.

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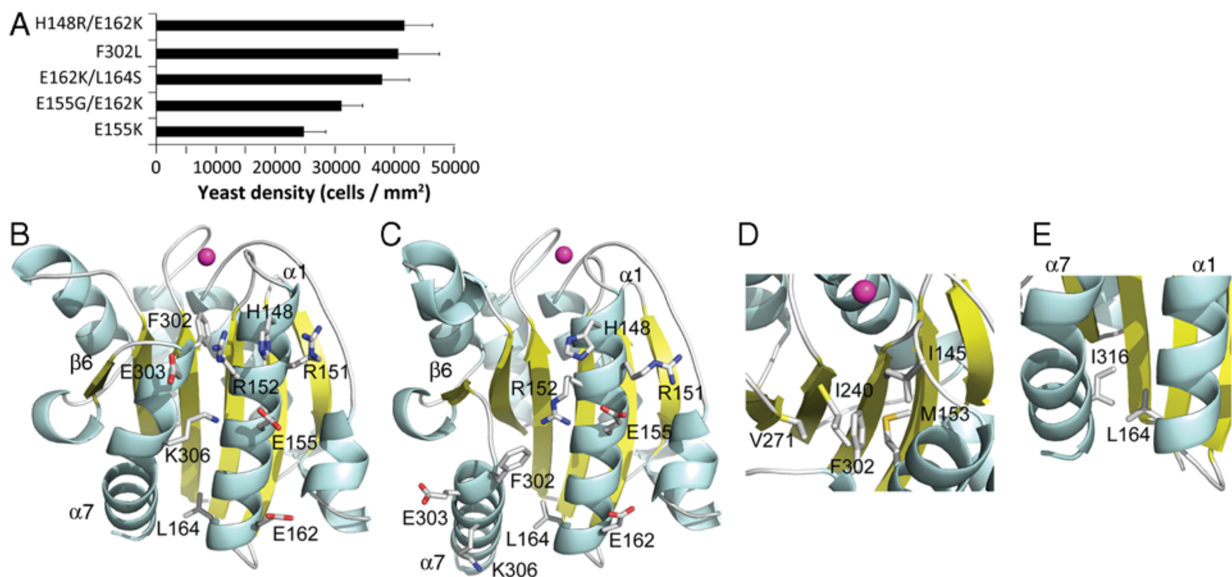

Fig. S1. Identification of activation mutations in Mac-1 I domain. From sequence analysis of twelve clones, five unique clones containing one or two mutations were isolated (*A*), which contained mutations along α1-helix (H148R, E155G/K, E162K, L164S) and at the loop between the β6 strand and the α7-helix (F302L) (B–E). Mac-1 I domain was drawn in cartoon using PyMOL (DeLano Scientific) with α-helices and β-strands colored in cyan and yellow, respectively. The closed (*B*) and open (*C*) conformations were modeled based on the crystal structures as described previously (7). The residues where activation mutations were found and the residues in their vicinity were drawn in sticks. The activation of the I domains by the mutations along the α1-helix has not been isolated before: The activation by E155K/G and presumably by H148R (*B*) may be due to the disruption of favorable electrostatic attraction, as Glu-155 forms interaction with Lys-306 only in the closed position of the α7-helix (*B* and *C*); the insertion of Arg into His-148 and as a result Arg triad (at 148, 151, and 152) would likely to create unfavorable electrostatic interaction in a closed conformation. In the open conformation, the residues at 148, 151, and 152 move away from each other (*C*). L164S would induce high affinity conformation by presumably disrupting the interaction of Leu164 with Ile-316 (*E*), which creates van der Waals interaction with neighboring hydrophobic residues in the closed position of the α7-helix.

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                                     CDR1                  CDR2
#1    EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTIEQRGIKTRY 60
#17   EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSTIEQRGIKTRY 60
#2    EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSSIADAGAYTYY 60
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                                         CDR3
#1    ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSNHAFDYWGQGTLVTVSSGGGG 120
#17   ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSNHAFDYWGQGTLVTVSSGGGG 120
#2    ADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKADTDFDYWGQGTLVTVSSGGGG 120
      **************************************::  ******************

         linker                            CDR1
#1    SGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLI 180
#17   SGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLI 180
#2    SGGGGSGGGGSTDIQMTQSPSSLSASVGDRVTITCRASQSISSYLNWYQQKPGKAPKLLI 180
      ************************************************************

        CDR2                                     CDR3
#1    YKASALQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQRARFPLTFGQGTKVEIKR 240
#17   YAASTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQAYDYPATFGQGTKVEIKR 240
#2    YAASTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQAYDYPATFGQGTKVEIKR 240
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Fig. S2. Sequence alignment of single-chain antibodies isolated against the Mac-1 high affinity I domain (F302L). Complementarity determining regions (CDRs) and a linker are indicated with a line above the sequence.

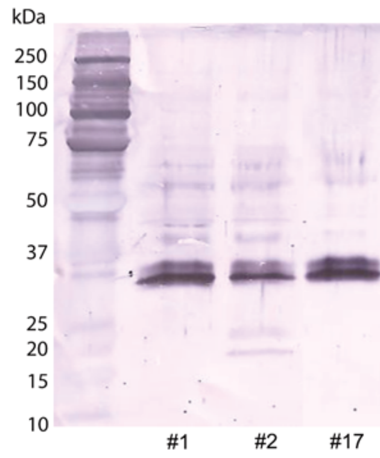

Fig. S3. Western blot detection of antibodies from yeast culture supernatant. Anti-Mac-1 antibodies were detected with antiHis tag antibody as a primary and HRP-conjugated goat antimouse antibody as a secondary antibody. Each lane was loaded with 30 μl of culture. Assuming 10 ng of scFv (30 kDa) for each band, the amount of antibodies secreted into the media was estimated to be approximately 10 nM (330 μg/mL).

Table S1. SPR measurement of the binding of physiological ligands and antibodies to the wild-type and active I domain mutants.

		MgCl$_2$			MgCl$_2$		EDTA	
		ICAM-1	iC3b	fibronectin	AM01	mAb44	AM01	mAb44
WT	k_{on} ($M^{-1}\,s^{-1} \times 10^{-3}$)	ND	ND	ND	198.0 ± 8.7	1247.5 ± 17.5	ND	2290.0 ± 120.3
	k_{off} $s^{-1} \times 10^{3}$	ND	ND	ND	12.90 ± 0.42	0.44 ± 0.14	ND	0.87 ± 0.13
	K_D ($M \times 10^{9}$)	ND	1820 ± 25.4	ND	65.8 ± 4.68	0.358 ± 0.012	ND	0.375 ± 0.048
I316G	k_{on} ($M^{-1}\,s^{-1} \times 10^{-3}$)	92.3 ± 32.7	231.3 ± 11.6	163.0 ± 20.2	477.3 ± 37. 1	1242.9 ± 47.2	ND	1778.6 ± 54.0
	k_{off} $s^{-1} \times 10^{3}$	110.9 ± 55.6	9.69 ± 0.69	53.50 ± 10.62	0.93 ± 0.03	5.65 ± 0.53	ND	9.13 ± 0.57
	K_D ($M \times 10^{9}$)	1050 ± 199	41.8 ± 1.43	363 ± 86.8	1.96 ± 0.09	4.63 ± 0.515	51.5 ± 7.09	5.21 ± 0.457
F302L	k_{on} ($M^{-1}\,s^{-1} \times 10^{-3}$)	61.1 ± 11.8	47.9 ± 4.6	50.9 ± 11.1	463.8 ± 37.9	464.4 ± 18.2	ND	1217.5 ± 42.5
	k_{off} $s^{-1} \times 10^{3}$	17.1 ± 2.6	1.63 ± 0.25	9.44 ± 0.20	0.93 ± 0.08	2.06 ± 0.14	ND	2.88 ± 0.15
	K_D ($M \times 10^{9}$)	339 ± 110	33.5 ± 1.98	222 ± 42.0	2.01 ± 0.052	4.47 ± 0.349	51.5 ± 7.07	2.36 ± 0.068

The values are mean ± S.E., estimated from BIAevaluation software from Biacore.

Table S2. SPR measurement of the effectiveness of antibodies (IC_{50}) in inhibition of ligand binding to the I domain.

		mAb44	AM01	AM17
I316G	ICAM-1	284 ± 4	294 ± 5	1097 ± 17
	iC3b	432 ± 7	433 ± 6	1351 ± 19
	fibronectin	271 ± 4	275 ± 4	937 ± 19
F302L	ICAM-1	226 ± 3	242 ± 3	898 ± 18
	iC3b	223 ± 3	237 ± 4	948 ± 21
	fibronectin	203 ± 3	222 ± 3	698 ± 20

The values are in nM concentration. Mean ± 95% confidence interval are shown.

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