2. SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Related to Figure 1 and 2. (A) Similar expression of surface activation markers on neutrophils in the two groups evaluated by flow cytometry. Data is presented as fold induction relative to neutrophils from the non-risk donors. Fluorescence intensity histograms of **(B)** Human neutrophils or **(C)** K562 cells expressing α_M^{WT} with the CBR LFA1/7, CBRM1/29, CBRN1/6, CBRN3/4, CBRM1/20 and CBRM1/32 antibodies. Goat-anti mouse APC was used as a secondary antibody.

Figure S2. Related to Figure 2. (A) Surface expression (flow cytometry) of α_M in K562 cells bearing the common (α_M^{WT}) or risk-associated (α_M^{R77H}) variants. **(B)** Mac-1 activation (Mg^{2+}/EGTA and Mn^{2+}) on K562 cells evaluated by flow cytometry using the reporter antibodies: m24, Kim127 and MEM148. Antibody binding was performed at 4^oC or after cell fixation with 1% paraformaldehyde (PFA). Mean fluorescence intensities (MFI) were normalized by surface expression of Mac-1. MFI, after isotype control subtraction is graphed. Cumulative data from 3-4 independent experiments is shown. Representative histograms of m24 binding are shown. **(C)** Accumulation of cells at indicated shear flow rates (dynes/cm^2) was quantified after two minutes of flow. K562 cells lacking β_2 integrins (--), or stably expressing α_M^{WT} or α_M^{R77H} were compared on an ICAM-1 coated surface, following activation with Mg^{2+}/EGTA. N=3 independent experiments.

Figure S3. Related to Figure 2. (A) Surface expression (flow cytometry) of stable K562 cell lines expressing α_M^{WT} or risk-associated α_M^{R77H} variants coupled with the β_2^{WT} or β_2^{V124A} chain mutants. **(B)** Mac-1 activation (Mg^{2+}/EGTA and Mn^{2+}) of K562 expressing $\alpha_M^{WT}/\beta_2^{V124A}$ or

$\alpha_M^{R77H}/\beta_2^{V124A}$ evaluated by flow cytometry using the reporter antibodies: m24, Kim127 and MEM148. Mean fluorescence intensities (MFI) were normalized by surface expression of Mac-1. MFI, after isotype control subtraction is shown. Data represents cumulative data from 4 independent experiments. **(C)** Surface expression stable K562 cell lines expressing $\alpha_M^{WT_{\Delta}GFFKR}$ or $\alpha_M^{R77H_{\Delta}GFFKR}$ with β_2^{WT}. **(D)** Mac-1 activation evaluated by the reporter antibodies: m24, Kim127 and MEM148 of the $\alpha_M^{WT_{\Delta}GFFKR}$ or $\alpha_M^{R77H_{\Delta}GFFKR}$ expressing cell lines. The dotted line corresponds to MFI of staining for $\alpha_M^{WT}/\beta_2^{WT}$ plus Ca^{2+}/Mg^{2+}.

Figure S4. Related to Figure 3. (A-D) Adhesion frequency assay for zero-force kinetics. **(A)** Plot of mean ± SEM bond lifetime *vs* force of $\alpha_M^{WT}/\beta_2^{WT}$ bearing K562 cells dissociating from ICAM-1 beads blocked with 0.5% BSA or 1% PVP. **(B-D)** Mean ± SEM of adhesion frequency evaluated from 3 pairs of cell and bead each contacted 50 time are ploted *vs* contact duration. Site densities of Mac-1 (m_r) and ICAM-1 (m_l) were marked along each curve. Panel B shows results with CBR LFA-1/2 antibody activation. Panel C shows results with ΔGFFKR mutation. Panel D shows results with V124A mutation. **(E-P)** Semi-log survival frequency *vs* lifetime plots of α_M^{WT} (**E, F, I, J, M, N**) and α_M^{R77H} (**G, H, K, L, O, P**) Mac-1 binding to ICAM-1 under 0-12pN (**E, G, I, K, M, O**) and >12pN (**F, H, J, L, N, P**) forces. Survival frequency is calculated as the fraction of the binding events with a lifetime no shorter than a give t_b value. Panels E-H show results with CBR LFA-1/2 antibody activation. Panels I-L show results with ΔGFFKR mutation. Panels M-P show results with V124A mutation.

SUPPLEMENTAL TABLE

		Human Neutrophils	
		Shear Flow	**Static**
iC3b	**CBR LFA1/7**	0 ± 0	0 ± 0
	CBRM 1/29	41.9 ± 5.1 ***	48.8 ± 9.1 ***
	CBRM1/32	56.3 ± 7.5 ***	21.9 ± 6.0
	CBRM1/20	6.9 ± 4.6	-6.3 ± 14.1
	CBRN1/6	39.5 ± 3.6 **	4.4 ± 7.0
	CBRN3/4	44.7 ± 12.0 **	-2.6 ± 11.2
	CBRM1/29 and 1/32	60.7 ± 7.6	73.3 ± 9.8 ***
		K562 cells α_M^{WT}	
iC3b	**CBR LFA1/7**	0 ± 0	0 ± 0
	CBRM 1/29	74.5 ± 9.1 **	26.4 ± 17.3
	CBRM1/32	96.7 ± 2.2 ***	44.8 ± 23.7 *
	CBRM1/20	29.8 ± 9.3	28.9 ± 6.1
	CBRN1/6	82.7 ± 11.4 ***	20.5 ± 2.2
	CBRN3/4	52.7 ± 22.8 *	32.5 ± 8.7
ICAM-1	**CBR LFA1/7**	0 ± 0	n.d.
	CBRM 1/29	94.5 ± 3 **	n.d.
	CBRM1/32	96.1 ± 2.2 **	n.d.
	CBRM1/20	9.4 ± 29.1	n.d.
	CBRN1/6	76.5 ± 9.6 **	n.d.
	CBRN3/4	63.1 ± 14.9 *	n.d.

SUPPLEMENTAL TABLE LEGEND

Table S1. Related to Figure 1 and 2. K562 cells expressing α_M^{WT} or human neutrophils isolated from healthy volunteers were activated with Mn^{2+} or fMLP respectively. Binding to iC3b or ICAM-1 (K562 cells only) coated surface was assessed under shear flow (0.38dynes/cm^2) and static conditions. CBRM1/29 antibody blocks the αI-domain and CBRM1/32, CBRM1/20, CBRN1/6 and CBRN 3/4 block different regions of the β-propeller domain. The percentage of inhibition compared to the CBR LFA1/7 antibody control is given. For human neutrophils, a combination of CBRM1/29 and 1/32 is used to demonstrate the relative contribution of Mac-1 to neutrophil binding to iC3b. For experiments performed under shear flow, % of inhibition at 8 minutes is shown. n=3-5 independent experiments. n.d., not determined. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

EXTENDED EXPERIMENTAL PROCEDURES

Reagents and Antibodies: Human IgM (Jackson ImmunoResearch), Protein A (Pierce Thermo Scientific), Human ICAM-1Fc (R&D) and fMLP (Sigma-Aldrich) were purchased. Serum, a source of iC3b, was obtained from healthy volunteers under approved human subjects protocols approved by the Brigham and Women's Hospital Institutional Review Board. CBRM1/29, CBRM1/32, CBRM1/20, CBRN1/6, CBRN3/4, CBRLFA1/2 and CBRLFA1/7 were a generous gift from Dr. T. Springer (Children's Hospital, Harvard University, Boston MA) and were used as described (Lu et al., 2001; Lu and Springer, 1997; Oxvig et al., 1999). Antibodies to human α_M (CBRM1/5 and ICRF44), CD3, CD14, CD32, CD16, CD11a, and CD62L, and goat anti-mouse IgG conjugated antibodies were from BioLegend. Antibodies to human CD18 (β_2), MAb 24 (m24) and MEM148 were from Abcam. Leukadherin-1 (LA-1) was from Calbiochem (EMD Millipore).

Lentiviral constructs: cDNAs of α_M^{WT} and α_M^{R77H}, were cloned in the lentiviral plasmid pWPI (modified from the Addgene, plasmid #12254, from Didier Trono laboratory, by removing the EGFP cassette), whereas the cDNA for $\beta_2^{WT/L132A}$ and ^{V124A}, were cloned in the pWPI containing an EMCV IRES-EGFP cassette (Addgene plasmid #12254). GFFKR mutants ($\alpha_M^{WT_{\Delta}GFFKR}$ and $\alpha_M^{R77H_{\Delta}GFFKR}$) were generating by including a stop codon before the GFFKR sequence, by standard PCR. 293T cells (clone 17, from ATCC) were transfected with the lentiviral construct (pWPI) in addition to the packaging plasmids psPAX2 and pMD2.G (Addgene, plasmids #12260 and #12259 from the laboratory of Didier Trono) using Lipofectamin (Invitrogen). 24hrs after transfection the cells were washed. Media was collected at 48 and 72hrs, and K562 cells were

infected with the supernatant of the transfected cells that had been passed through a 0.25μm filter.

Ligand coating of surfaces: iC3b coating was performed on 96 well opaque bottom plates (Costar) or glass coverslips. Human IgM (10μg/mL) diluted in carbonate-bicarbonate buffer (pH 9.4) was incubated overnight at 4^{o}C. Fresh serum was diluted 1:1 with PBS (Ca^{2+} and Mg^{2+}) and incubated at 37^{o}C for 2hrs (heat inactivated serum was used as control). Blocking of unspecific binding to the surface was achieved by incubating with 1% PVP (Sigma) for 1hr at 37^{o}C. ICAM-1 coating on glass coverslips was as follows. Coverslips were incubate with Protein A (1μg/mL, used to orient the ICAM-1Fc molecules), for 1hr at 37^{o}C, followed by ICAM-1 (10μg/mL) overnight at 4^{o}C. Blocking of unspecific binding to the surface was achieved by incubating with 2% BSA (Sigma-Aldrich) and 0.5%PVP for 1hr at 37^{o}C.

Shear flow adhesion assay: For all experiments performed under shear flow (using both K562 cells and human neutrophils), 0.38 dynes/cm^2 was selected from a range of shear stresses (0.19-0.67 dynes/cm^2) and binding every two minutes for the indicated times was evaluated because this shear stress allowed both Mac-1 specific binding and resulted in the most significant binding defect in Mac-1^{R77H} cells (Figure S2C). Live-cell imaging of cell adhesion was recorded by a video camera coupled to a Nikon TE2000 inverted microscope equipped with a 20×0.75 NA phase contrast objective and VideoLab software (Mitov).

Spreading assay: Neutrophil adhesion was performed over an iC3b coated surface for 30 min. Cells were washed and fixed with 4% paraformaldehyde for 10 minutes. After saponin permeabilization (0.1%, for 2 minutes), the cells were stained with Alexa Fluor-568 phalloidin (Invitrogen) and the coverslips were mounted with DAPI.

Crawling assay: This was performed as previously described (Phillipson et al., 2006). Briefly, glass coverslips were coated with 10% human plasma for 4 hours. Neutrophils were placed on the coated surface in HBSS (without Ca^{2+}/Mg^{2+}) for 15 minutes at 37^{o}C, and activated with 10μM fMLP for 5 minutes. Glass coverslips containing the activated neutrophils were placed in a flow chamber and high shear flow (10 dynes/cm^2) was applied for 5 minutes. Shear flow was stopped, and neutrophil movement over the surface was recorded for 10 minutes. Neutrophil velocities were analyzed using ImageJ.

RBC and bead preparation for BFP experiments: Human red blood cells (RBCs) were isolated from whole blood of healthy volunteers by finger prick according to protocols approved by the Institutional Review Board of Georgia Institute of Technology. Freshly isolated human RBCs were biotinylated by covalently linking with biotin-PEG3500-SGA (JenKem USA) for 30 minutes incubation at room temperature (Chen et al., 2010). Biotinylated RBCs were then incubated with nystatin (Sigma-Aldrich) in N2 buffer (265.2 mM KCl, 38.8 mM NaCl, 0.94 mM KH_2PO_4, 4.74 mM Na_2HPO_4, 27 mM sucrose; pH 7.2, 588 mOsm) for 30 minutes in 0^{o}C. Nystatin-loaded biotinylated RBCs were washed twice with the N2 buffer and re-suspended in the N2 buffer for the BFP experiments.

To obtain ICAM-1/streptavidin (SA) coated glass beads, borosilicate glass beads (Duke Scientific) were covalently coupled with mercapto-propyl-trimethoxy silane (United Chemical Technologies). ICAM-1 was covalently linked with NHS-PEG-MAL (JenKem USA) by incubation in carbonate/bicarbonate buffer (pH 8.5) for 30 minutes. The complex together with streptavidin-maleimide (Sigma-Aldrich) was covalently linked to the glass beads in phosphate buffer (pH 6.8) for overnight incubation at room temperature. The coated beads were re-suspended in phosphate buffer plus 0.5% BSA or 1% PVP.

Molecular density measurement: To measure the Mac-1 density, K562 cells were incubated with a PE-conjugated anti-hCD11b (α_M) antibody at 10μg/ml in the D-PBS (0.493mM $MgCl_2$, 1.47mM KH_2PO_4, 137.9mM NaCl, 8.06mM $Na_2HPO_4 \cdot 7H_2O$, 0.884mM $CaCl_2$, 2.68mM KCl, pH 7.4) at room temperature for 30 minutes and washed for 3 times in the D-PBS. To measure the ICAM-1 density, beads were incubated with a PE-conjugated anti-ICAM-1 monoclonal antibody (HA58, eBioscience) at 10μg/ml in the phosphate buffer at room temperature for 30 minutes and washed for 3 times in phosphate buffer. The fluorescent intensities of K562 cells or beads were measured by a BD LSR flow cytometer (BD Biosciences). The intensities were compared to standard calibration beads (BD Quantibrite PE Beads, BD) to determine the respective site densities of Mac-1 or ICAM-1 on K562 cells and beads, respectively by dividing total number of molecules per cell or bead to the cell or bead surface area. The cell or bead surface area was calculated from the radii measured with a customized LabView (National Instrument) program (7.28μm for a K562 cell and 1μm for a bead).

Biomembrane force probe: In brief, a biotinylated RBC attached to a streptavidinylated (SA) glass bead is aspirated by a micropipette to form an ultra-sensitive force probe (Figure 4A). The probe bead is coated with ICAM-1. A Mac-1-expressing K562 cell is aspirated by an opposing micropipette driven by a piezoelectric translator (Physical Instrument) with sub-nanometer precision via a capacitive sensor feedback control (Figure 3A). The probe bead and the target cell are aligned in a cell chamber containing 2mM Mg^{2+}/EGTA, and the probe bead position with 3nm precision in real-time was tracked by an inverted microscope (Nikon TiE, Nikon) through two cameras. Two cameras (Prosilica) capture real-time images (Figure 3A) at rates of 30 and 1600 frames per second, (fps) respectively, with images limited to pixelation of 100 x 30 region of contact between the bead and the RBC. Data was analyzed with LabView (National

Instrument).

The RBC spring constant k is determined as described (Chen et al., 2008; Evans et al., 1995). k was set to 0.3pN/nm and 0.25pN/nm for clamping forces higher or lower than 10pN, respectively.

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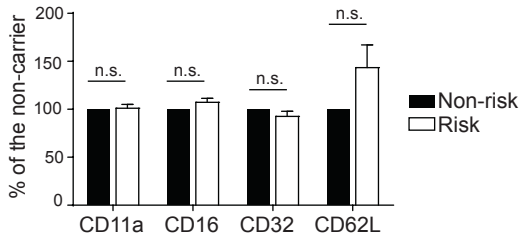
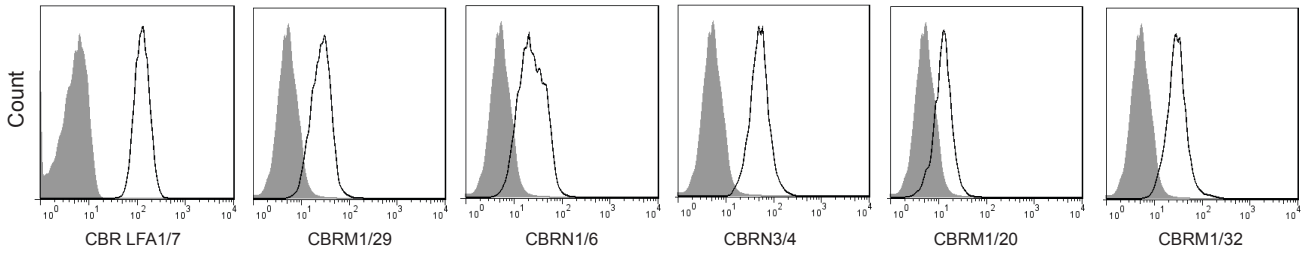
Figure S1

A



B Human neutrophils



C K562 cells expressing α_MWT

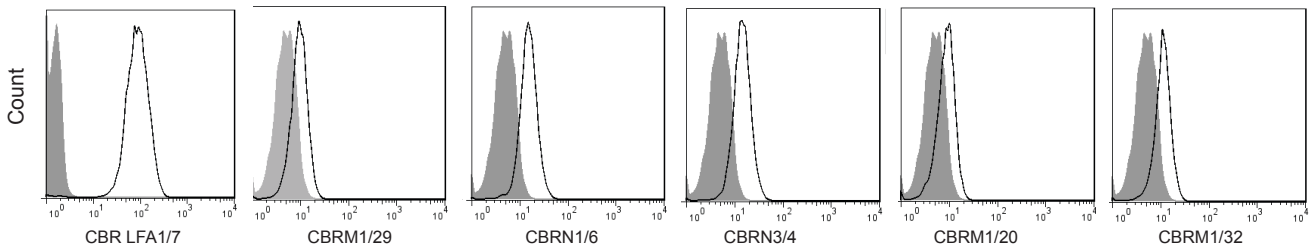
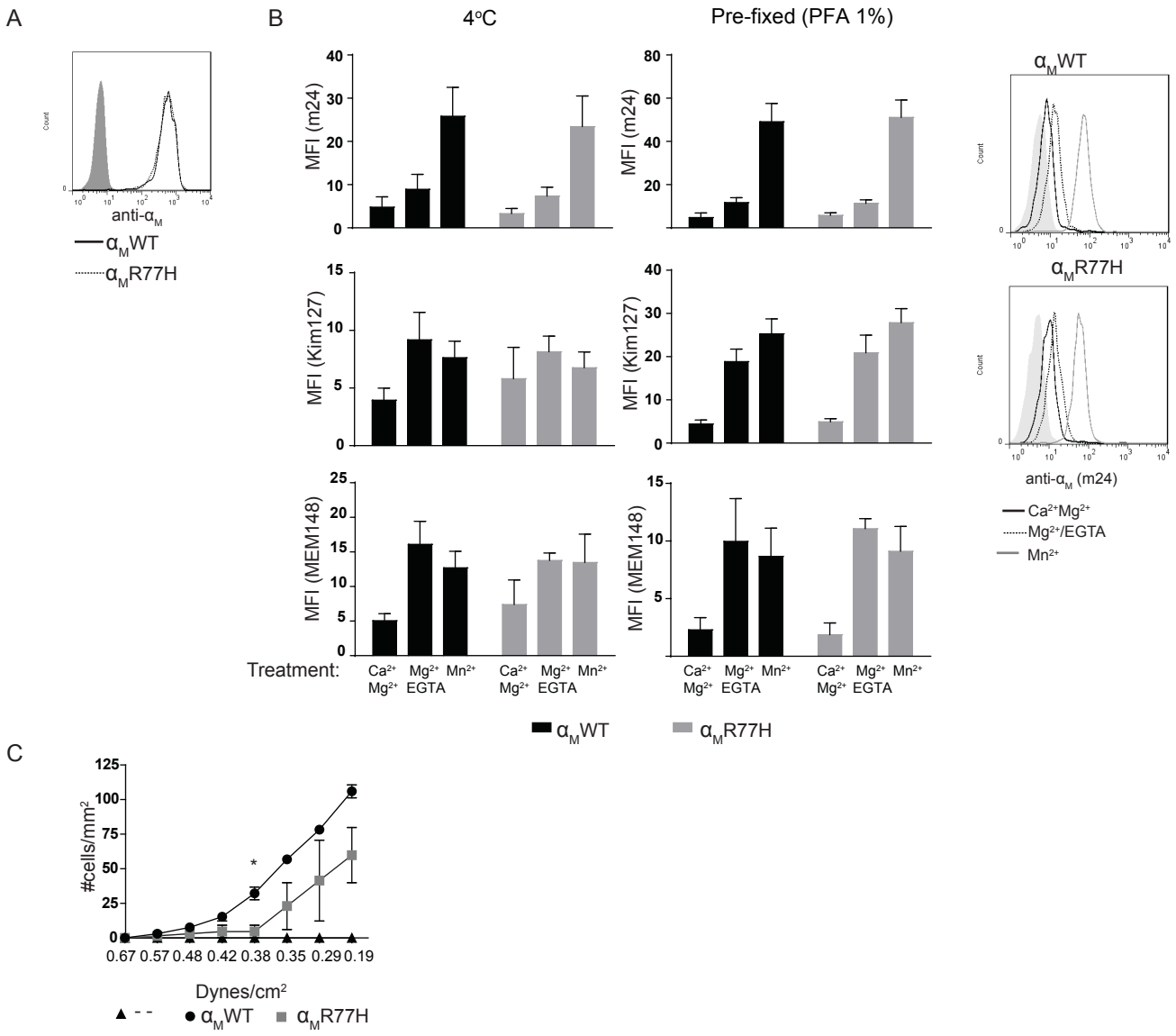


Figure S2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B
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Pre-fixed (PFA 1%)
Count
anti-αM
αMWT
αMR77H
MFI (m24)
MFI (Kim127)
MFI (MEM148)
Treatment:
Ca2+ Mg2+
Mg2+ EGTA
Mn2+
αMWT
αMR77H
anti-αM (m24)
Ca2+Mg2+
Mg2+/EGTA
Mn2+
C
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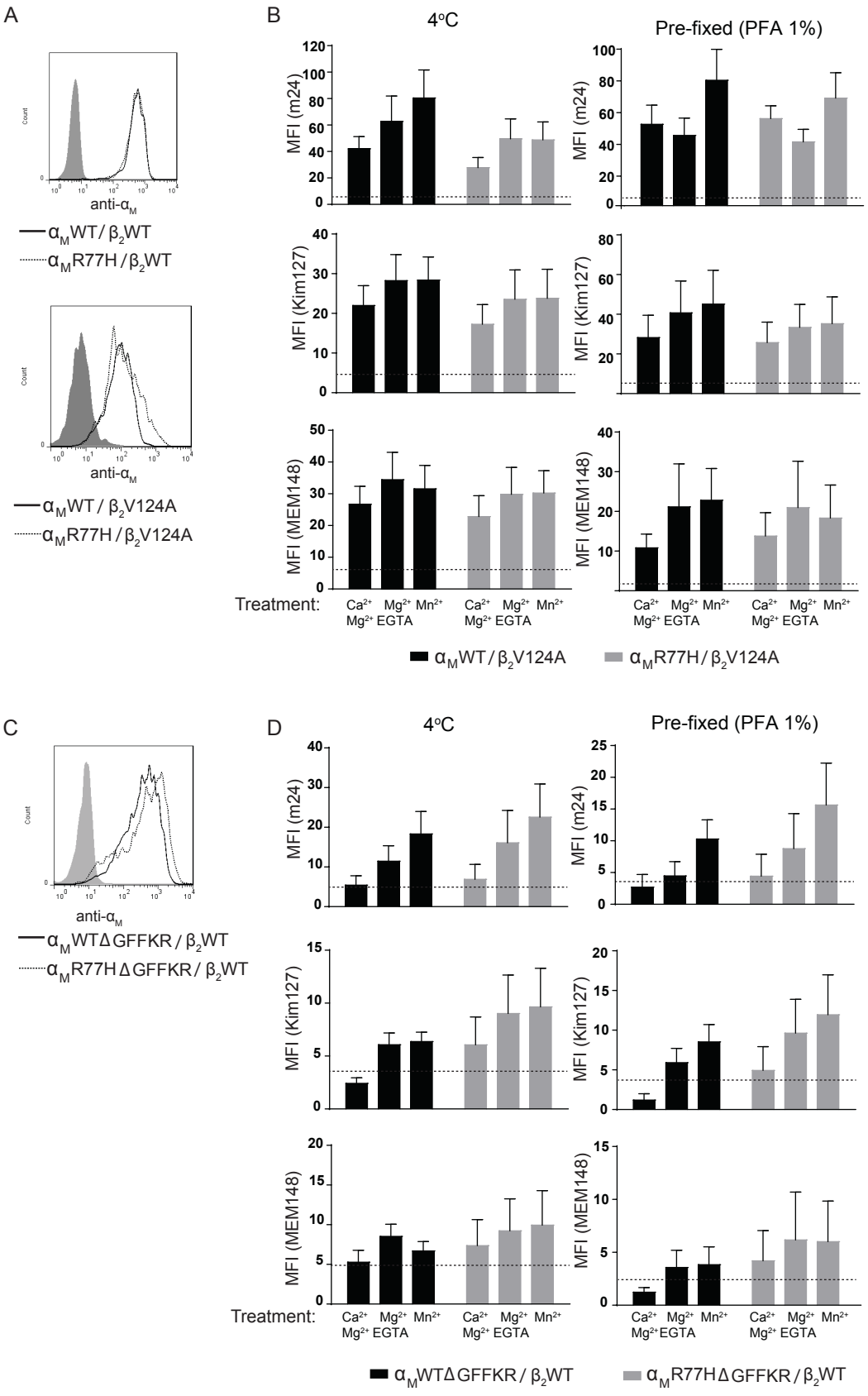
Dynes/cm2
αMWT
αMR77H

Figure S3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Count
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αMWT/β2WT
αMR77H/β2WT
Count
anti-αM
αMWT/β2V124A
αMR77H/β2V124A
B
4°C
Pre-fixed (PFA 1%)
MFI (m24)
MFI (Kim127)
MFI (MEM148)
Treatment:
Ca2+ Mg2+ Mn2+
Mg2+ EGTA
αMWT/β2V124A
αMR77H/β2V124A
C
Count
anti-αM
αMWTΔGFFKR/β2WT
αMR77HΔGFFKR/β2WT
D
4°C
Pre-fixed (PFA 1%)
MFI (m24)
MFI (Kim127)
MFI (MEM148)
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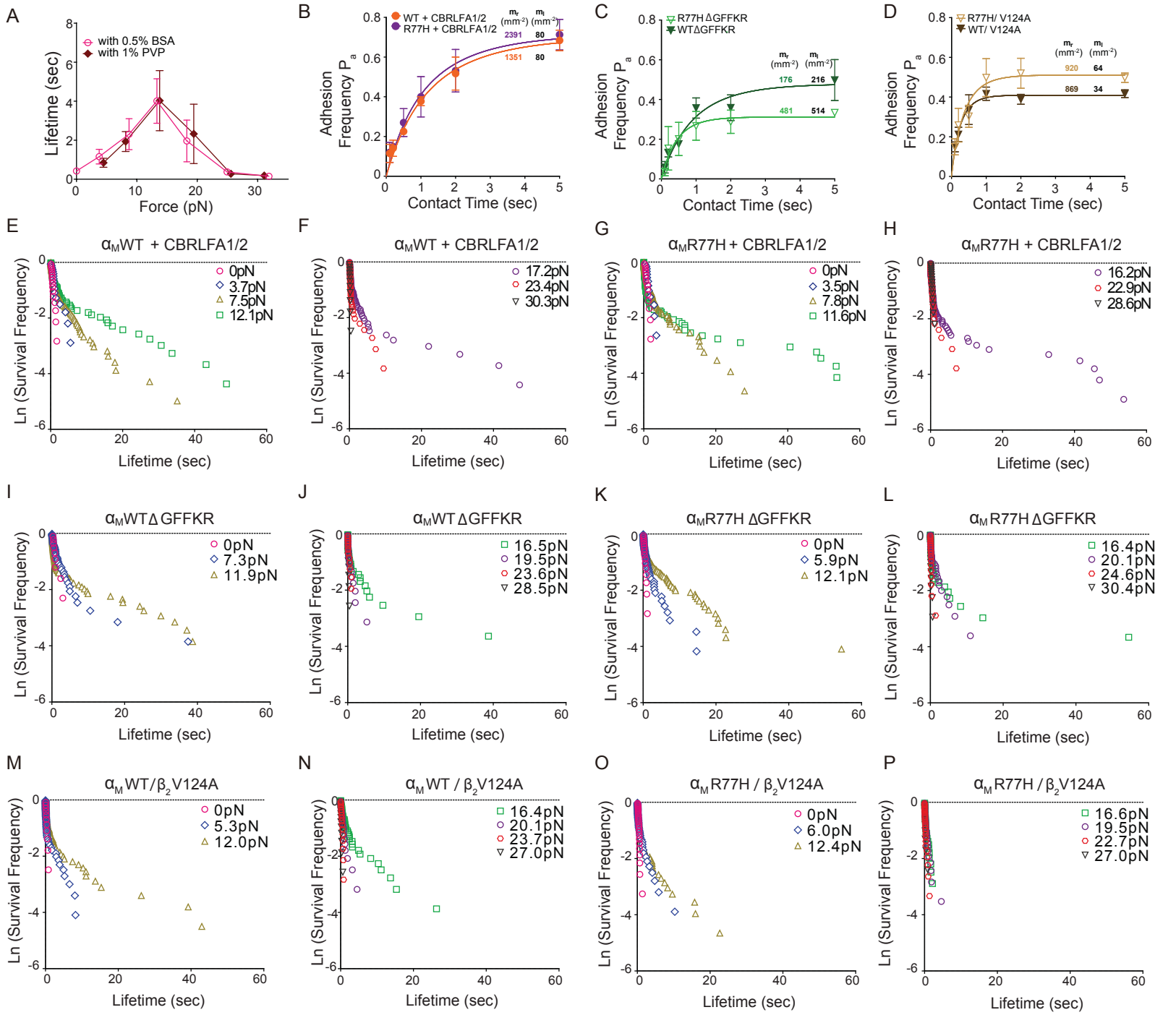
αMR77HΔGFFKR/β2WT

Figure S4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with 1% PVP
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Force (pN)
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WT + CBRLFA1/2
R77H + CBRLFA1/2
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80
1351
80
Adhesion Frequency P_a
Contact Time (sec)
C
R77H ΔGFFKR
WTΔGFFKR
176
216
481
514
Adhesion Frequency P_a
Contact Time (sec)
D
R77H/ V124A
WT/ V124A
920
64
869
34
Adhesion Frequency P_a
Contact Time (sec)
E
αMWT + CBRLFA1/2
0pN
3.7pN
7.5pN
12.1pN
Ln (Survival Frequency)
Lifetime (sec)
F
αMWT + CBRLFA1/2
17.2pN
23.4pN
30.3pN
Ln (Survival Frequency)
Lifetime (sec)
G
αMR77H + CBRLFA1/2
0pN
3.5pN
7.8pN
11.6pN
Ln (Survival Frequency)
Lifetime (sec)
H
αMR77H + CBRLFA1/2
16.2pN
22.9pN
28.6pN
Ln (Survival Frequency)
Lifetime (sec)
I
αMWTΔGFFKR
0pN
7.3pN
11.9pN
Ln (Survival Frequency)
Lifetime (sec)
J
αMWTΔGFFKR
16.5pN
19.5pN
23.6pN
28.5pN
Ln (Survival Frequency)
Lifetime (sec)
K
αMR77H ΔGFFKR
0pN
5.9pN
12.1pN
Ln (Survival Frequency)
Lifetime (sec)
L
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16.4pN
20.1pN
24.6pN
30.4pN
Ln (Survival Frequency)
Lifetime (sec)
M
αMWT/β2V124A
0pN
5.3pN
12.0pN
Ln (Survival Frequency)
Lifetime (sec)
N
αMWT / β2V124A
16.4pN
20.1pN
23.7pN
27.0pN
Ln (Survival Frequency)
Lifetime (sec)
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6.0pN
12.4pN
Ln (Survival Frequency)
Lifetime (sec)
P
αMR77H / β2V124A
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19.5pN
22.7pN
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Ln (Survival Frequency)
Lifetime (sec)