Human Natural Killer Cell Cytoskeletal Dynamics and Cytotoxicity are Regulated by LIM Kinase

Supplementary Materials

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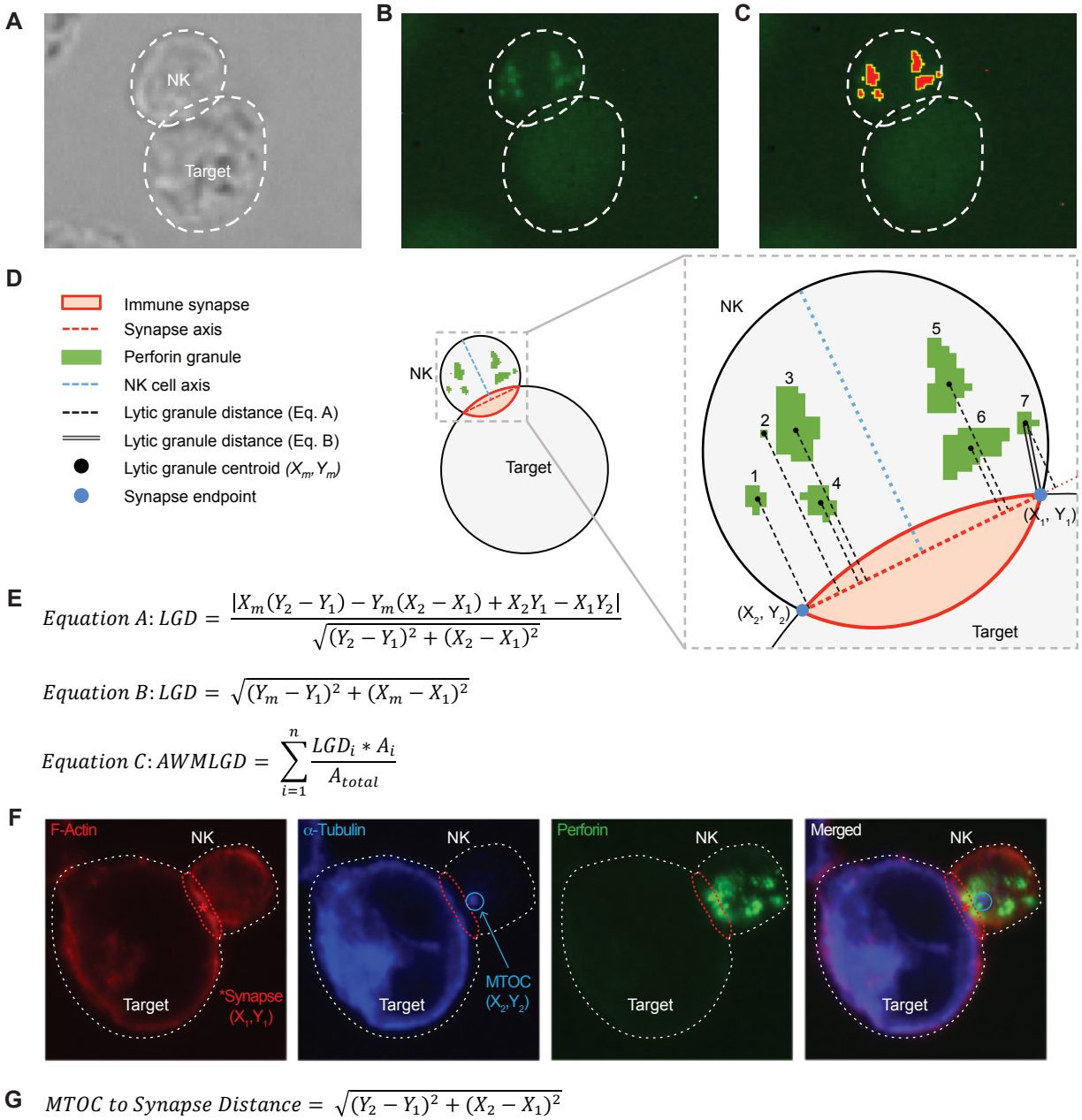
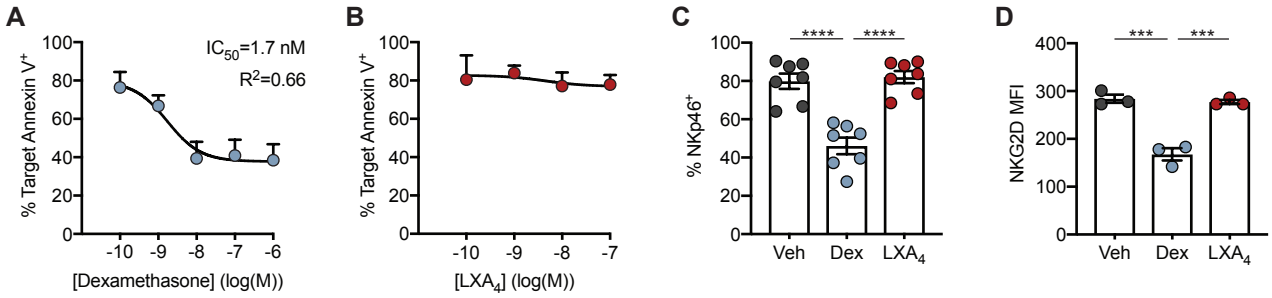
Supplemental Figure 1



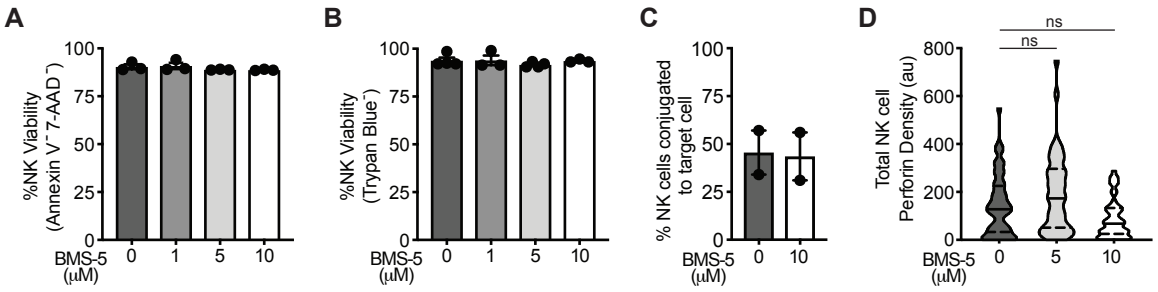
Supplemental Figure 1. **Analysis of NK cell immunofluorescence and confocal microscopy**. **(A)** Cell perimeters of NK:target conjugates were outlined as regions of interest (ROIs). **(B)** ROIs were applied to immunofluorescence images (perforin; green). **(C)** Centroid coordinates of each lytic granule were noted (X_m,Y_m). **(D)** The immune synapse was identified as the overlap of NK and target cell perimeters (red shaded area). Coordinates of synapse endpoints were noted (X_1,Y_1 and X_2,Y_2). The red dashed line connecting the points is the synapse axis. **(E)** Distance from each lytic granule to the synapse axis was calculated using Equation A (black dashed lines in panel D). For granules outside the synapse axis (*e.g.* Granule 7), Equation B was used to calculate the distance to the nearest synapse endpoint (black double line in panel D). Equation C to was used to calculate area-weighted mean lytic granule distance (AWMLGD). See Methods "Immunofluorescence microscopy". **(F)** Confocal microscopy was used to image NK:target conjugates for F-actin (phalloidin, red), perforin (green), and α-tubulin (blue). Phalloidin signal was measured within the immune synapse (red dashed line). Centroid coordinates of phalloidin signal within the synapse were noted (red star; X_1,Y_1). NK cell MTOC was identified (α-tubulin, blue circle; X_2,Y_2). Perforin density within the immune synapse was determined. **(G)** Distance between immune synapse center (X_1,Y_1) and the MTOC (X_2,Y_2) was calculated for each NK:target conjugate. See Methods section "Confocal Microscopy".

Supplemental Figure 2



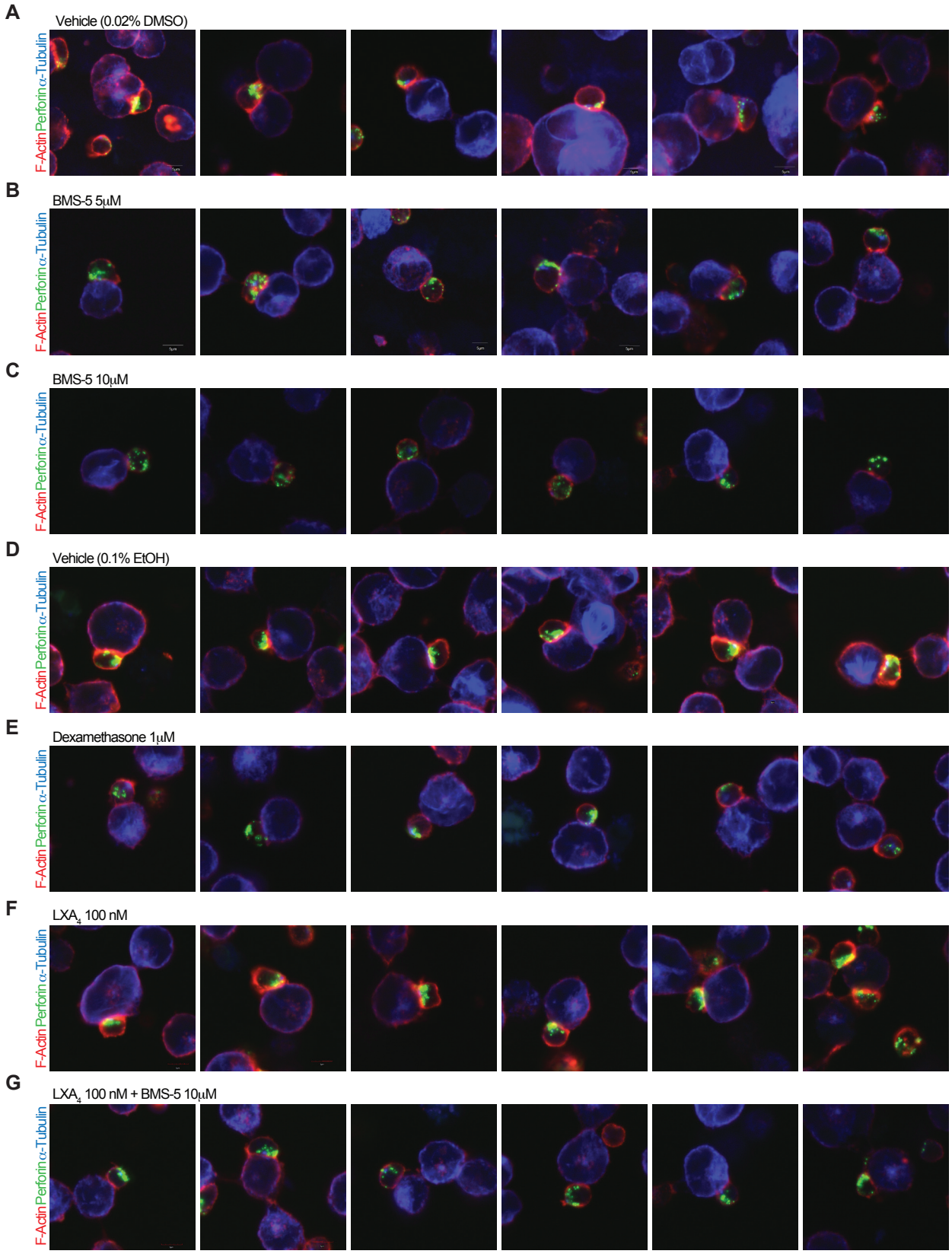
Supplemental Figure 2. Dexamethasone and LXA_4 concentration response curves and NK cell activating receptor expression. A concentration response curve was established for dexamethasone and LXA_4 effect on NK cells using K562 target cell apoptosis as the readout. NK cells isolated from healthy donors were exposed to serial-dilutions of **(A)** dexamethasone (100 pM to 1 μM, blue), or **(B)** lipoxin A_4 (100 pM to 100 nM, red) for 48 hours. **(A-B)** Treated NK cells were co-incubated with K562 target cells at 5:1 effector:target ratio for 4 hours and NK cell-mediated target cell apoptosis was assessed by flow cytometry staining for annexin-V and 7-AAD (% Target Annexin V^+). log(Dex) and log(LXA_4) concentration response curves were generated and nonlinear regression was performed using least squares regression fitting method to calculate the IC_{50} for dexamethasone. R^2 goodness of fit measure is shown. n=3 experiments. Symbols denote mean ± SEM. **(C-D)** NK cells were exposed to vehicle control (Veh, grey), 1 μM dexamethasone (Dex, blue), or 100 nM lipoxin A_4 (LXA_4, red) for 48 hours. **(C)** Percentage of NK cells expressing the activating receptor NKp46 was analyzed for each condition by flow cytometry. n=7 experiments. **(D)** Median fluorescence intensity (MFI) of NKG2D expression on NK cells was analyzed by flow cytometry. n=3 experiments. Bars show mean ± SEM with individual data points. ***p<0.005 and ****p<0.0001 by repeated measures one-way ANOVA with Tukey's multiple comparisons test.

Supplemental Figure 3



Supplemental Figure 3. **LIMK inhibition does not impair NK cell viability, NK:target conjugate formation, or NK cell perforin content.** NK cells were exposed to the LIMK inhibitor BMS-5 for 48 hours and NK cell viability was assessed by **(A)** flow cytometry staining for annexin V and 7-AAD and **(B)** trypan blue evaluation. NK cell viability was ≥ 85% in all conditions; n=3 individual experiments. NK cells exposed to BMS-5 for 48 hours were subsequently co-incubated with K562 target cells for 2 hours. **(C)** The percentage of NK cells conjugated to target K562 cells was evaluated by confocal microscopy in n=2 experiments. Total NK cells observed was > 200 per condition. **(D)** Confocal microscopy was used to image NK:target conjugates for intracellular perforin (AF488, green) in BMS-5 5μM and 10μM conditions relative to control. Total NK cell perforin integrated density (area x MFI) was quantified for each NK:target conjugate. n=3 experiments, 49-51 total NK:target conjugates imaged and quantified per condition. Bars show mean ± SEM and violin plots depict density distribution with median (line) and interquartile ranges (dashed line). ns = not significant.

Supplemental Figure 4



Supplemental Figure 4. Supplemental confocal microscopy images. NK cells were exposed to noted conditions for 48 hours before co-incubation with K562 target cells for 2 hours. Confocal microscopy was utilized to image F-actin (phalloidin AF555, red), perforin (AF488, green), and α-tubulin (AF647, blue) in NK:target conjugates. Merged images are shown for 6 representative images for the following conditions: **(A)** Vehicle (0.02% DMSO), **(B)** BMS-5 5μM, **(C)** BMS-5 10μM, **(D)** Vehicle (0.1% EtOH), **(E)** Dexamethasone 1μM, **(F)** LXA_4 100nM, **(G)** LXA_4 100nM with BMS-5 10μM.

Supplemental Table 1. Cytoskeletal Protein Array

	NK cell exposure		
Protein	**Veh**	**Dex**	**LXA_4**
Actin alpha-1 skeletal muscle (N-term)	+	+	+
Actin alpha-2/3 (N-term)	-	-	-
Actin Pan (a/b/g) (Ab-55/53)	-	-	+
Actin Pan (a/b/g) (Phospho-Tyr55/53)	-	-	-
ACTN1 (F-Actin) (inter)	-	-	-
Beta actin	+	+	+
c-Raf (Ab-296)	-	-	+
c-Raf (Ab-43)	-	+	+
c-Raf (Phospho-Ser296)	-	+	-
c-Raf (Phospho-Ser43)	-	-	+
Calmodulin (Ab-79/81)	-	-	+
Calmodulin (Phospho-Thr79/Ser81)	-	+	-
CaMK1-alpha (Ab-177)	-	+	-
CaMK1-alpha (Phospho-Thr177)	-	-	+
CaMK1-beta (inter)	-	-	-
CaMK2 (Ab-286)	-	-	+
CaMK2 (Phospho-Thr286)	-	-	+
CaMK2 (Phospho-Thr305)	-	+	-
CaMK2-beta/gamma (inter)	-	-	-
CaMK2-beta/gamma/delta (Ab-287)	-	-	+
CaMK2-beta/gamma/delta (Phospho-Thr287)	-	-	+
CaMK4 (Ab-196/200)	-	-	-
CaMK4 (Phospho-Thr196/200)	-	-	+
CaMK5 (inter)	-	-	-
Cofilin (Ab-3)	-	-	+
Cofilin (Phospho-Ser3)	-	-	+
Cortactin (Ab-421)	-	-	+
Cortactin (Ab-466)	-	-	+
Cortactin (Phospho-Tyr421)	+	+	+
Cortactin (Phospho-Tyr466)	-	-	+
CrkII (Ab-221)	-	-	+
CrkII (Phospho-Tyr221)	-	-	+
CrkL (Phospho-Tyr207)	-	-	+
ERK1-p44/42 MAP Kinase (Ab-202)	-	-	+
ERK1-p44/42 MAP Kinase (Ab-204)	-	-	+
ERK1-p44/42 MAP Kinase (Phospho-Thr202)	-	+	+
ERK1-p44/42 MAP Kinase (Phospho-Tyr204)	-	-	+
ERK1/2 (N-term)	-	+	+
ERK3 (Ab-189)	-	-	-
ERK3 (Phospho-Ser189)	-	-	+
ERK8 (Phospho-Thr175/Tyr177)	-	-	+
Ezrin (Ab-353)	-	-	-
Ezrin (Ab-478)	-	-	-
Ezrin (Ab-566)	+	+	+
Ezrin (Phospho-Thr566)	+	+	+
Ezrin (Phospho-Tyr353)	-	-	+
Ezrin (Phospho-Tyr478)	-	-	+
FAK (Ab-397)	-	-	+
FAK (Ab-407)	-	+	-
FAK (Ab-576)	-	-	-
FAK (Ab-861)	-	-	+
FAK (Ab-910)	+	+	+
FAK (Ab-925)	-	-	+
FAK (Phospho-Ser910)	+	+	+
FAK (Phospho-Tyr397)	-	-	-
FAK (Phospho-Tyr407)	-	-	+
FAK (Phospho-Tyr576)	-	-	+
FAK (Phospho-Tyr861)	-	-	+
FAK (Phospho-Tyr925)	-	-	+
Filamin A (Ab-2152)	-	-	+
Filamin A (Phospho-Ser2152)	+	+	+
Gab2 (Ab-159)	-	-	-
Gab2 (Phospho-Ser159)	-	-	+
GAPDH	-	-	-
GTPase activating protein (Ab-387)	-	-	+
GTPase activating protein (Phospho-Ser387)	-	-	-
LIMK1 (Ab-508)	-	+	+
LIMK1 (Phospho-Thr508)	+	-	+
LIMK1/2 (Ab-508/505)	+	+	+
MEK1 (Ab-217)	-	-	+
MEK1 (Ab-221)	-	-	+

	NK cell exposure		
Protein (continued)	**Veh**	**Dex**	**LXA_4**
MEK1 (Ab-286)	-	+	+
MEK1 (Ab-291)	-	-	+
MEK1 (Ab-298)	-	+	+
MEK1 (Phospho-Ser217)	-	-	+
MEK1 (Phospho-Ser221)	-	-	+
MEK1 (Phospho-Ser298)	-	-	-
MEK1 (Phospho-Thr286)	-	+	+
MEK1 (Phospho-Thr291)	-	-	+
MEK2 (Ab-394)	-	-	+
MEK2 (Phospho-Thr394)	-	-	+
MEKKK 1 (inter)	-	-	-
MEKKK 4 (inter)	-	-	-
Merlin (Ab-10)	-	+	+
Merlin (Ab-518)	-	-	+
Merlin (Phospho-Ser10)	+	+	+
Merlin (Phospho-Ser518)	-	-	+
MKK3/MAP2K3 (Ab-189)	-	+	+
MKK3/MAP2K3 (Ab-222)	-	-	-
MKK3/MAP2K3 (Phospho-Ser189)	-	-	-
MKK3/MAP2K3 (Phospho-Thr222)	-	-	-
MKK6 (Ab-207)	-	+	+
MKK6 (Phospho-Ser207)	+	+	+
MKK7/MAP2K7 (Ab-271)	-	-	-
MKK7/MAP2K7 (Phospho-Ser271)	-	-	+
MKK7/MAP2K7 (Phospho-Thr275)	-	+	+
Myosin regulatory light chain 2 (Ab-18)	-	-	+
Myosin regulatory light chain 2 (Phospho-Ser18)	-	+	+
NCK2 (C-term)	-	-	-
p130Cas (Ab-165)	-	-	-
p130Cas (Ab-410)	-	-	-
p130Cas (Phospho-Tyr165)	-	-	-
p130Cas (Phospho-Tyr410)	-	+	-
Paxillin (Ab-118)	-	-	-
Paxillin (Ab-31)	-	-	+
Paxillin (Phospho-Tyr118)	-	-	+
Paxillin (Phospho-Tyr31)	-	-	+
PI3-kinase p85-alpha (Phospho-Tyr607)	-	-	+
PI3-kinase p85-subunit alpha/gamma (Ab-467/199)	-	-	-
PI3-kinase p85-subunit alpha/gamma (Phospho-Tyr467/Tyr199)	-	-	+
PIP5K (inter)	-	-	+
PIP5K (Phospho-Ser307)	-	-	-
PKA CAT (Ab-197)	-	+	+
PKA CAT (Phospho-Thr197)	-	-	+
PKC alpha (Ab-657)	-	-	-
PKC alpha (Phospho-Tyr657)	-	+	+
PKC alpha/beta II (Ab-638)	-	-	+
PKC alpha/beta II (Phospho-Thr638)	-	-	-
PKC pan activation site	-	-	+
PKC pan activation site (Phospho)	-	-	+
PLC beta-3 (Ab-1105)	-	-	-
PLC beta-3 (Ab-537)	-	-	-
PLC beta-3 (Phospho-Ser1105)	+	+	-
PLC beta-3 (Phospho-Ser537)	-	-	+
Rac1/cdc42 (Ab-71)	-	-	+
Rac1/cdc42 (Phospho-Ser71)	-	-	+
Rho/Rac guanine nucleotide exchange factor 2 (Ab-885)	-	-	-
Rho/Rac guanine nucleotide exchange factor 2 (Phospho-Ser885)	-	-	+
Src (Ab-418)	+	+	+
Src (Ab-529)	-	-	+
Src (Ab-75)	-	-	-
Src (Phospho-Ser75)	-	-	-
Src (Phospho-Tyr216)	-	-	+
Src (Phospho-Tyr418)	-	-	+
Src (Phospho-Tyr529)	-	-	+
VASP (Ab-157)	+	+	+
VASP (Ab-238)	-	-	+
VASP (Phospho-Ser157)	-	-	+
VASP (Phospho-Ser238)	-	-	+
WASP (Ab-290)	-	-	+
WASP (Phospho-Tyr290)	-	-	-
WAVE1 (Ab-125)	-	-	-
WAVE1 (Phospho-Tyr125)	-	+	-

Supplemental Table 1. Cytoskeletal Protein Array. NK cells from healthy donors were exposed to Vehicle, Dexamethasone, or LXA_4 for 48 hours and protein was extracted for screening in this cytoskeletal protein array (see Methods). + denotes positive signal and – denotes lack of signal above background threshold for each of the 141 site-specific and phosphor-specific cytoskeletal proteins included in the cytoskeletal protein array.