Supplemental Data

METHODS

Subcellular fractionation. LAK cells were homogenized by 10 strokes of a Dounce homogenizer in 5 ml of a solution consisting of 0.25 M sucrose, 0.5 mM EDTA, 10 mM HEPES, pH 7.4 and 1 mM PMSF (homogenate buffer). The homogenate was then centrifuged at 1000 x *g* for 10 min at 4 ℃ and the supernatant was loaded on the top of a continuous 0.4-1.6 M sucrose gradient containing 1 mM EDTA, 10 mM HEPES, pH 7.4. After centrifugation at 100,000 x *g* for 20 h in a SW40Ti rotor (Beckman), 11 fractions were collected from the bottom of the tube. For Western blot analysis, an equal volume of 40 μl of each fraction were separated on either 10% or 13% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked in blocking buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 2 h at room temperature and then incubated with anti-NaK-ATPase (1:500; NOVUS Biologicals), anti-Hsp60 (1:2000; Stressgen), anti-Calreticulin (1:2000; Santa Cruz Biotechnology), anti-LAMP-1 (1:1000; BD Biosciences), anti-Rap1 (1:2000; Santa Cruz Biotechnology), or anti-CD38 (1:2000; Santa Cruz Biotechnology) antibodies in the blocking buffer overnight at 4 ^{o}C. Blots were then washed with blocking buffer and incubated with HRP-conjugated anti-rabbit, anti-rat, anti-mouse, or anti-goat antibodies (1:2000; Santa Cruz Biotechnology) in the blocking buffer at room temperature for 1 h. The immunoreactive proteins with respective secondary Abs were determined using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and exposed to an LAS 1000 Image Reader Lite (Fujifilm, Japan).

Expression and Construction of Green Fluorescent Protein (GFP)-fused CD38. The expression construct encoding CD38-enhanced green fluorescent fusion protein was constructed by the following. The coding sequence of CD38 was PCR-amplified from CD38 (CD38 cDNA in pFLAG-CMV2 vector) using the sense primer, 5′-CCCAAGCCT**ATG**GCTAACTATGAATTTAGCCA-3′ (the *Hin*dIII site is underlined, the start codon of CD38 is bolded), and the antisense primer, 5′-ACGCGTCGACCACGTATTAAGTCTACACGATGG -3′ (the *Sal*I site is underlined). The PCR products were eluted from agarose using Gel & PCR Purification System (SolGent, Korea) and subcloned using the T&A cloning kit (RBC). Then, after restriction digestion with *Hin*dIII and *Sal*I, the PCR product was ligated with identically predigested pEGFP-N1 vector (CLONTECH) to create pEGFP-CD38. LAK cells were transfected with GFP-CD38 using transfection reagent (Invitrogen).

Supplementary Figures and Legends

Figure S1. Time course of cADPR and NAADP transport into LAK cells. Transport experiments were executed by a rapid oil-stop procedure as described under "Experimental Procedures." and cADPR and NAADP transported were measured using a cyclic enzymatic assay described in the manuscript. LAK cells were treated 200 μM cADPR (*A*) and 100 μM NAADP (*B*) at 37 ℃. The means ± S.E. of three independent experiments are shown.

Figure S2. Dipyridamole blocks on cADPR- and NAADP-induced Ca^{2+} and cell migration. *A, B*, NAADP-induced Ca^{2+} increase prevented by dipyridamole, an inhibitor of equilibrative nucleoside transporters, in $CD38^{+/+}$ (*A*) and $CD38^{-/-}$ (*B*) LAK cells. LAK cells were preincubated with 100 μM dipyridamole for 15 min before the treatment with 50 nM NAADP. *Arrows* indicate the time points of the addition of NAADP. *n* indicates the number of cells examined for Ca^{2+} measurement. *C, D*, cADPR- or NAADP-induced cell migration was inhibited by dipyridamole in $CD38^{+/+}$ (*C*) and $CD38^{-/-}$ (*D*) LAK cells. The means ± S.E. of three independent experiments are shown. * $p < 0.01$, control *versus* cADPR or NAADP; ** $p < 0.05$, cADPR *versus* cADPR plus dipyridamole ; # $p < 0.01$, NAADP *versus* NAADP plus dipyridamole.

Figure S3. Effects of ACA on IL8-induced formation of cADPR and NAADP, Ca^{2+} increase, and cell migration. *A, B*, ACA, a specific inhibitor of TRPM2, did not inhibit IL8-stimulated increase of $[cADPR]_i$ and $[NAADP]_i$. Levels of cADPR and NAADP were determined after treatment of LAK cells with 10 pM IL8. LAK cells were preincubated with 20 μM ACA for 20 min before incubation with IL8. The means ± S.E. of three independent experiments are shown. * $p < 0.05$, control *versus* IL8 or IL8 plus ACA. *C,* IL8-stimulated increase in $[Ca^{2+}]_i$ with (open circle) and without (closed circle) ACA. *Arrows* indicate the time points of the addition of IL8. *n* indicates the number of cells examined for Ca^{2+} measurement. *D,* ACA inhibited IL8-induced cell migration. The means ± S.E. of three independent experiments are shown. * $p < 0.01$, control *versus* IL8; ** $p < 0.05$, IL8 *versus* IL8 plus ACA.

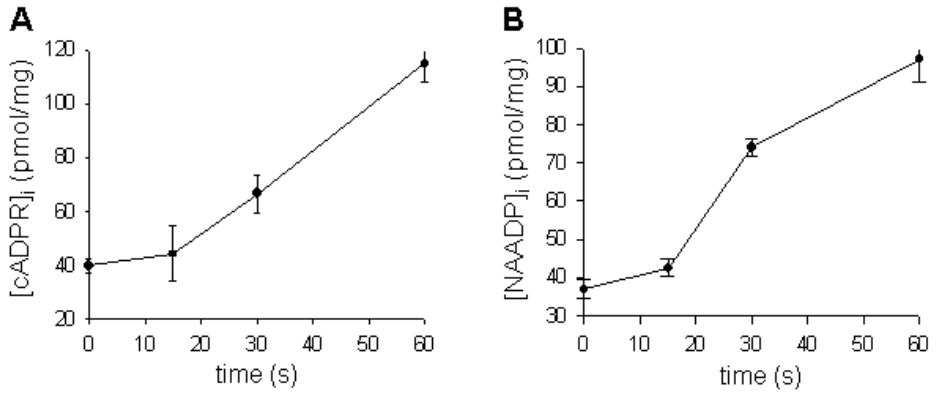
Figure S4. CD38 is localized in lysosomes as well as plasma membrane. LAK cells were transfected with GFP-CD38 using lipofectamine. After 72 h, LAK cells were serum-starved with serum free RPMI1640 for 18 h. LAK cells were loaded with Lysotracker Red DND-99 (75 nM) for 1 h at 37 ℃ to label lysosomes, treated with 10 pM IL8 for 90 s, and then fixed

with 3.7% paraformaldehyde. Images were taken using Zeiss confocal microscope equipped with a Plan-Apochromat X63 objective.

Figure S5. IL8 induced internalization of CD38 to early endosomes, but do not translocation of CD38 to lysosomes. LAK cells were treated with buffer *(A)* or 10 pM IL8 *(B)* for 90 s, homogenized, and a postnuclear supernatant was fractionated on a sucrose density gradient (0.4-1.6 M). Fractions were analyzed by Western blot to identify cellular organelles and identified by using antibodies against each markers, plasma membrane (NaK-ATPase), early endosomes (EEA1), and lysosomes (LAMP-1). The location of CD38 in fractions was identified with both Western blotting and measuring ADP-ribosyl cyclase activity. The means ± S.E. of three independent experiments are shown. * $p < 0.05$, control *versus* IL8.

Figure S6. Localization of Rap1 in lysosome-containing fractions from LAK cells. LAK cells were homogenized, and a postnuclear supernatant was fractionated on a sucrose density gradient (0.4-1.6 M). Fractions were analyzed by Western blot to identify cellular organelles and identified by using antibodies against each markers, plasma membrane (NaK-ATPase), mitochondria (Hsp60), endoplasmic reticulum (Calreticulin), and lysosomes (LAMP-1). Locations of Rap1 and CD38 were analyzed by western blotting.

Figure S1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100
80
60
40
20
0
10
20
30
40
50
60
time (s)
B
[NAADP]i (pmol/mg)
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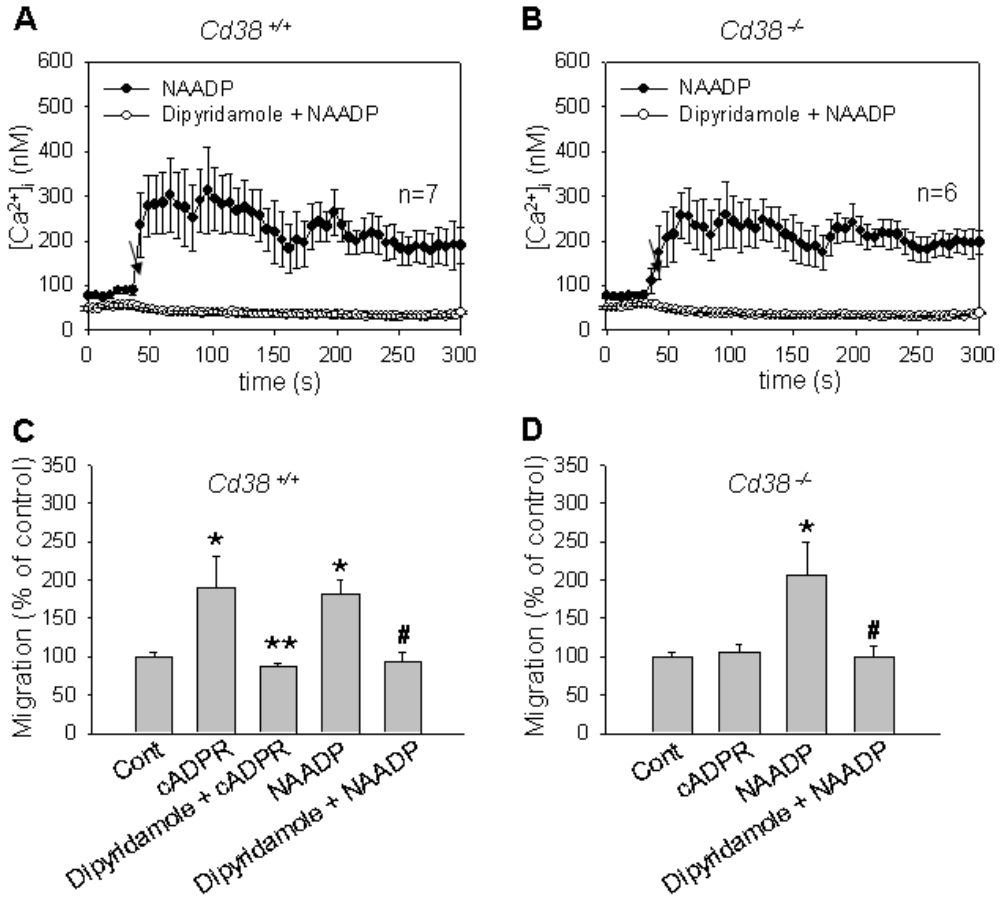
90
80
70
60
50
40
30
0
10
20
30
40
50
60
time (s)

Figure S2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NAADP
Dipyridamole + NAADP
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[Ca2+]i (nM)
time (s)
B
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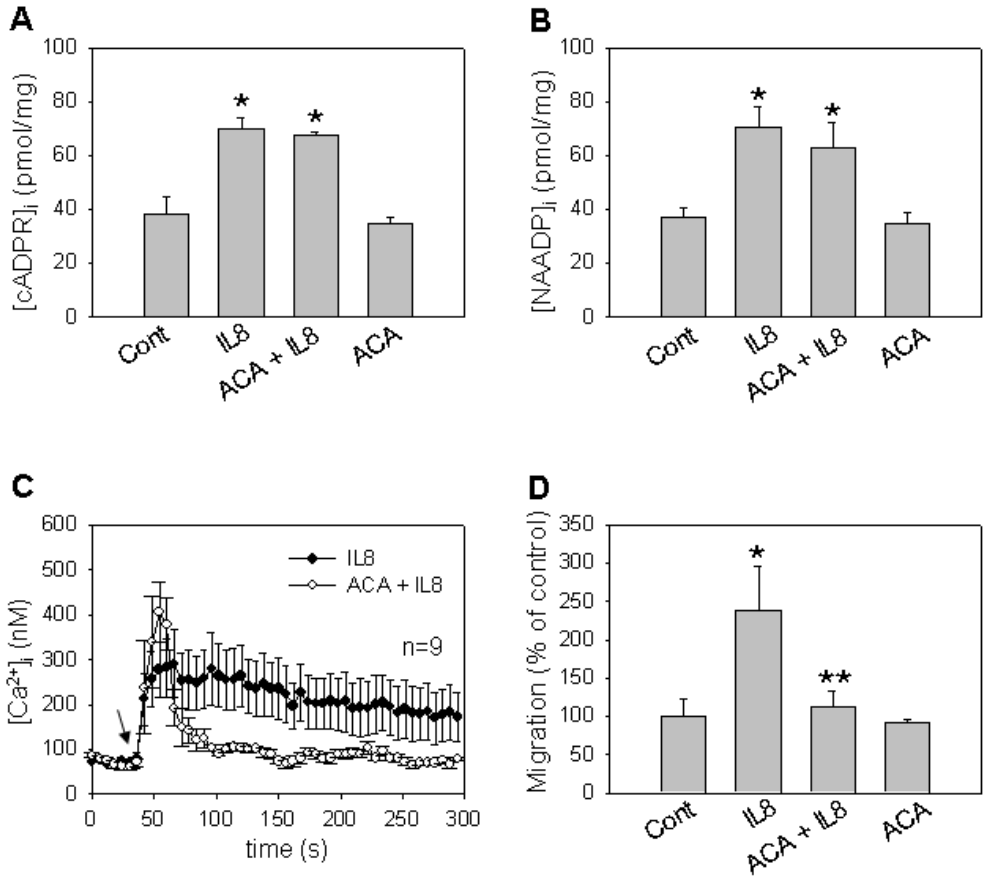
NAADP
Dipyridamole + NAADP
n=6
[Ca2+]i (nM)
time (s)
C
Cd38 +/+
Migration (% of control)
Cont
cADPR
Dipyridamole + cADPR
NAADP
Dipyridamole + NAADP
D
Cd38 -/-
Migration (% of control)
Cont
cADPR
NAADP
Dipyridamole + NAADP

Figure S3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[cADPR]i (pmol/mg)
100
80
60
40
20
0
*
*
Cont
IL8
ACA + IL8
ACA
B
[NAADP]i (pmol/mg)
100
80
60
40
20
0
*
*
Cont
IL8
ACA + IL8
ACA
C
[Ca2+]i (nM)
600
500
400
300
200
100
0
IL8
ACA + IL8
n=9
0
50
100
150
200
250
300
time (s)
D
Migration (% of control)
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300
250
200
150
100
50
0
*
**
Cont
IL8
ACA + IL8
ACA

Figure S4

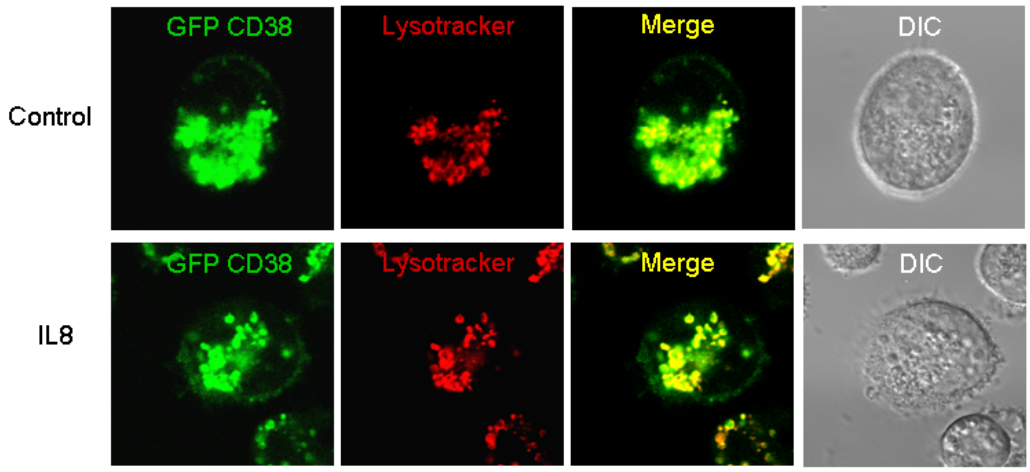

GFP CD38
Lysotracker
Merge
DIC
Control
GFP CD38
Lysotracker
Merge
DIC
IL8

Figure S5

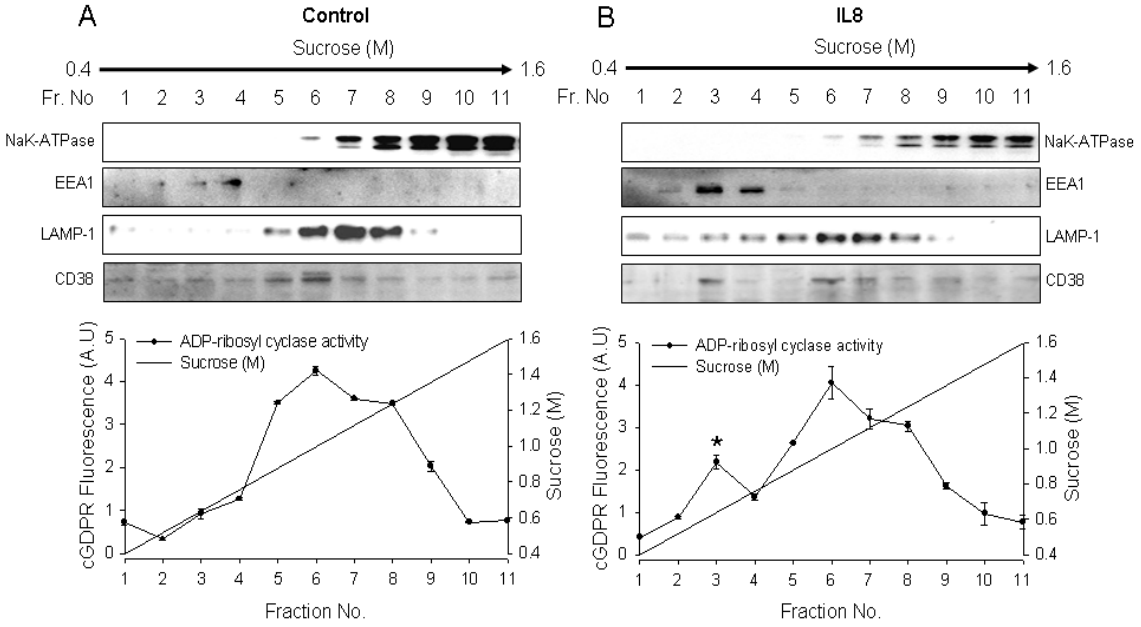
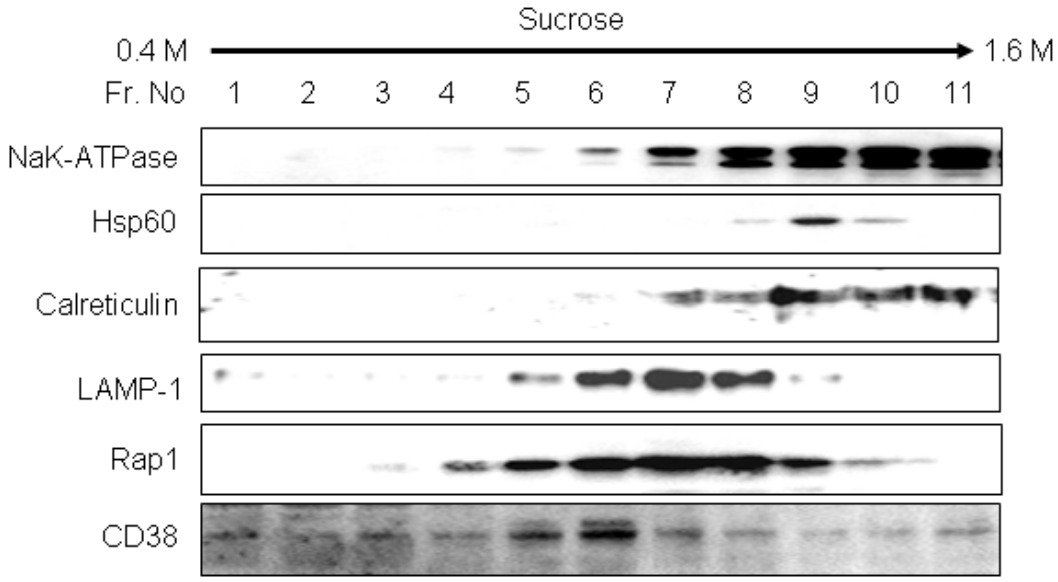

A
Control
Sucrose (M)
0.4
1.6
Fr. No 1 2 3 4 5 6 7 8 9 10 11
NaK-ATPase
EEA1
LAMP-1
CD38
ADP-ribosyl cyclase activity
Sucrose (M)
cGDPR Fluorescence (A.U)
Sucrose (M)
Fraction No.
B
IL8
Sucrose (M)
0.4
1.6
Fr. No 1 2 3 4 5 6 7 8 9 10 11
NaK-ATPase
EEA1
LAMP-1
CD38
ADP-ribosyl cyclase activity
Sucrose (M)
cGDPR Fluorescence (A.U)
Sucrose (M)
Fraction No.

Figure S6


Sucrose
0.4 M
1.6 M
Fr. No 1 2 3 4 5 6 7 8 9 10 11
NaK-ATPase
Hsp60
Calreticulin
LAMP-1
Rap1
CD38