

Supplementary Materials for
**DNA-PKcs Controls an Endosomal Signaling Pathway for a
Proinflammatory Response by Natural Killer Cells**

Sumati Rajagopalan,* Mark W. Moyle, Irma Joosten, Eric O. Long*

*To whom correspondence should be addressed. E-mail: sumi@nih.gov (S.R.) and eLong@nih.gov (E.O.L.)

Published 23 February 2010, *Sci. Signal.* **3**, ra14 (2010)
DOI: 10.1126/scisignal.2000467

The PDF file includes:

- Fig. S1. The CD158d-gp49 chimera traffics to the same endosomal compartments as does wild-type CD158d.
- Fig. S2. Stimulation of CD158d in resting NK cells results in the phosphorylation of Akt at Ser⁴⁷³, but not Thr³⁰⁸.
- Fig. S3. Inhibition of the CD158d-dependent secretion of IFN- γ and IL-8.
- Fig. S4. CD158d colocalizes with Rab5 and Rab5Q79L in endosomes.
- Fig. S5. Akt is present in endosomes that contain CD158d and Rab5Q79L.
- Fig. S6. Colocalization profiles of CD158d, Rab5Q79L, and Akt.
- Table S1. Compounds tested for their ability to inhibit CD158d-dependent secretion of IFN- γ by resting NK cells.

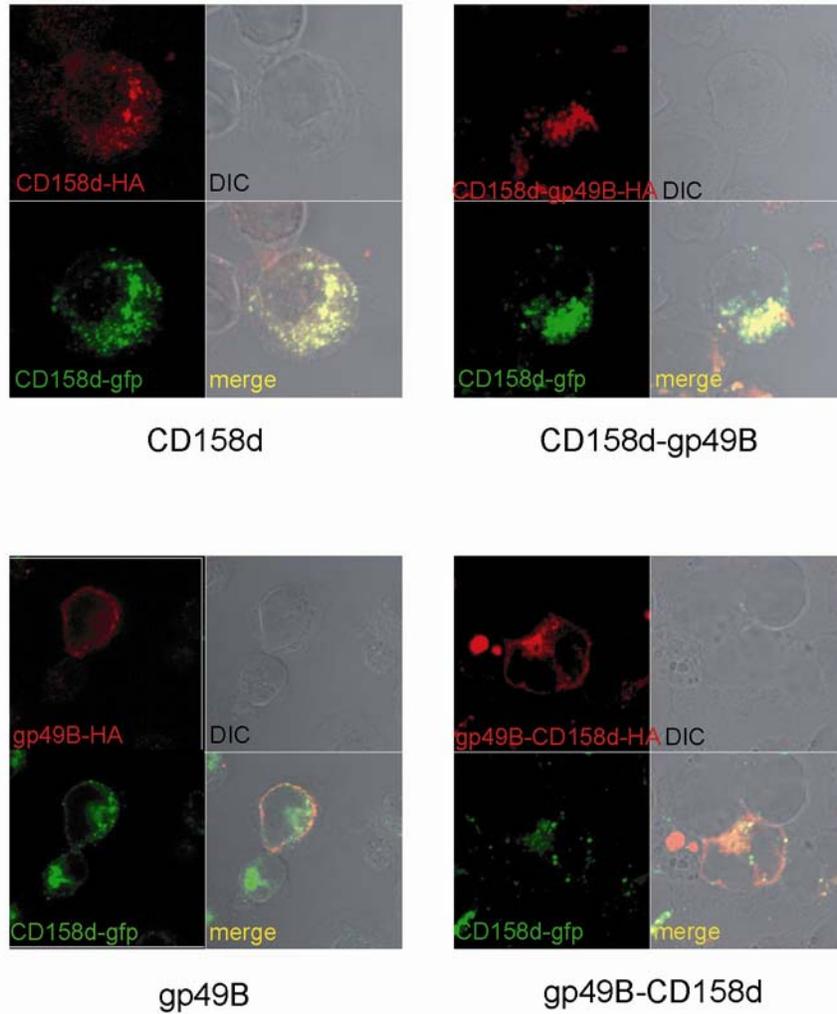


Fig. S1. The CD158d-gp49 chimera traffics to the same endosomal compartments as does wild-type CD158d. HEK 293T cells stably expressing CD158d-GFP were transiently transfected with the indicated plasmids and then cultured for 48 hours. Cells were incubated with an antibody against the HA tag, and receptor localization was detected by confocal microscopy. Single confocal sections are shown. The data are representative of three independent experiments.

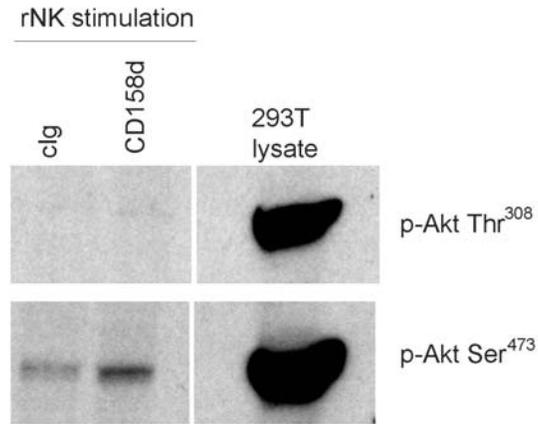


Fig. S2. Stimulation of CD158d in resting NK cells results in the phosphorylation of Akt at Ser⁴⁷³, but not Thr³⁰⁸. The phosphorylation of Akt in resting NK (rNK) cells stimulated for 16 hours with control antibody (cIg) (10 µg/ml) or antibody against CD158d (10 µg/ml) was analyzed by Western blotting with antibodies against phospho-Akt Thr³⁰⁸ (pAktT308) and phospho-Akt Ser⁴⁷³ (pAktS473). Western blotting analysis of whole-cell lysates of HEK 293T cells is also shown, as positive controls. The data are representative of experiments from three different donors.

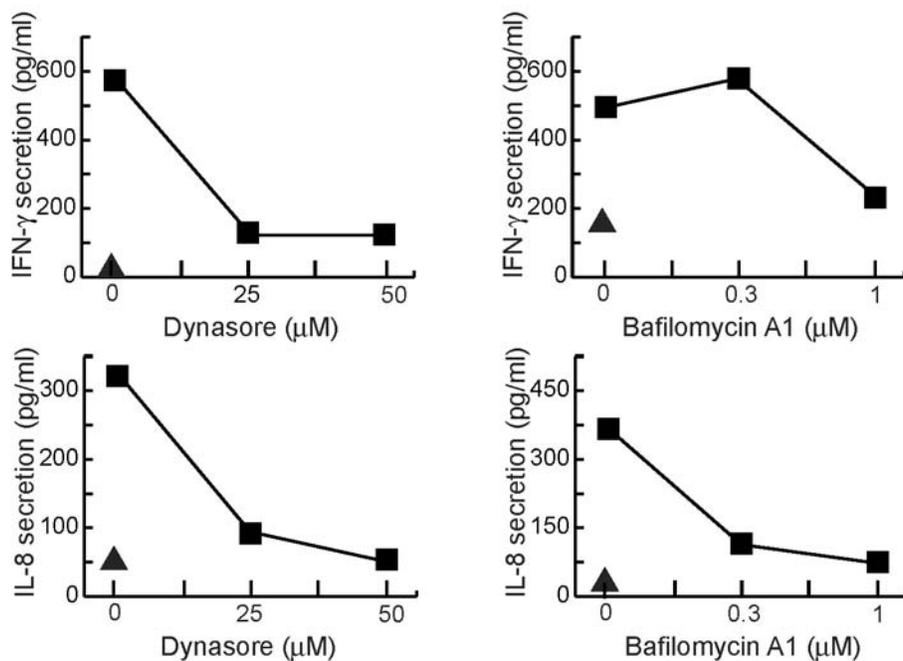


Fig. S3. Inhibition of the CD158d-dependent secretion of IFN- γ and IL-8. Inhibition of the secretion of IFN- γ and IL-8 by resting NK cells treated with the indicated concentrations of Dynasore and Bafilomycin A1 was determined by ELISA. The inhibitors were added for 1 hour at 37°C prior to stimulation of the cells with a mAb against CD158d for 16 hours, in the continued presence of inhibitor. The triangles denote the amounts of IFN- γ and IL-8 secreted by cells incubated with the isotype-matched control mAb and not treated with any inhibitor. The data are representative of experiments performed with resting NK cells isolated from three different donors.

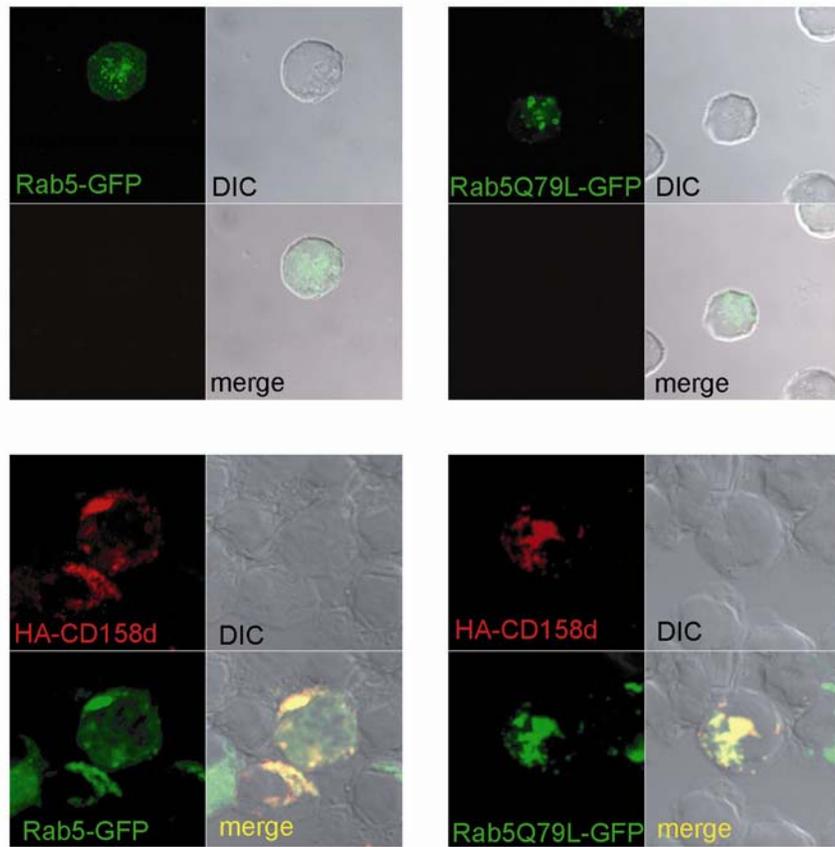


Fig. S4. CD158d colocalizes with Rab5 and Rab5Q79L in endosomes. Confocal sections of HEK 293T cells transiently transfected with plasmids encoding Rab5-GFP or Rab5Q79L-GFP either alone or cotransfected with a plasmid encoding HA-tagged CD158d. Forty-eight hours after transfection, CD158d was detected with an antibody against the HA tag (red). The data are representative of three experiments.

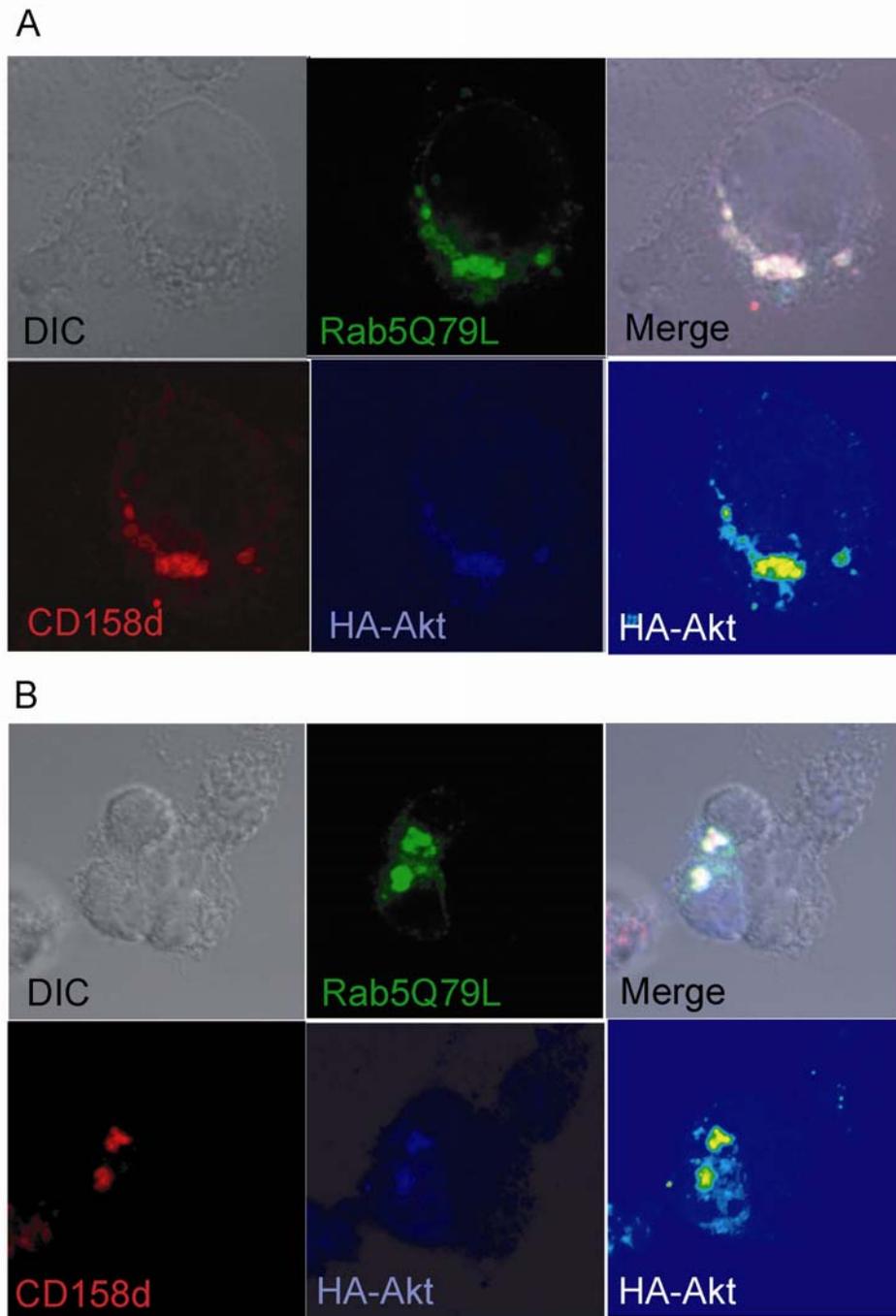


Fig. S5. Akt is present in endosomes that contain CD158d and Rab5Q79L. Two single confocal sections of HEK 293T cells cotransfected with plasmids encoding Rab5Q79L-GFP (green), CD158d (red), and HA-tagged Akt (blue). An intensity scale (red = high, blue = low) is also displayed in the bottom right panel to reflect the abundance of Akt. The colocalization coefficient for Akt and Rab5Q79L is 0.862 in (A) and 0.618 and 0.501 for the top and bottom cells, respectively, in (B). The data are representative of three experiments.

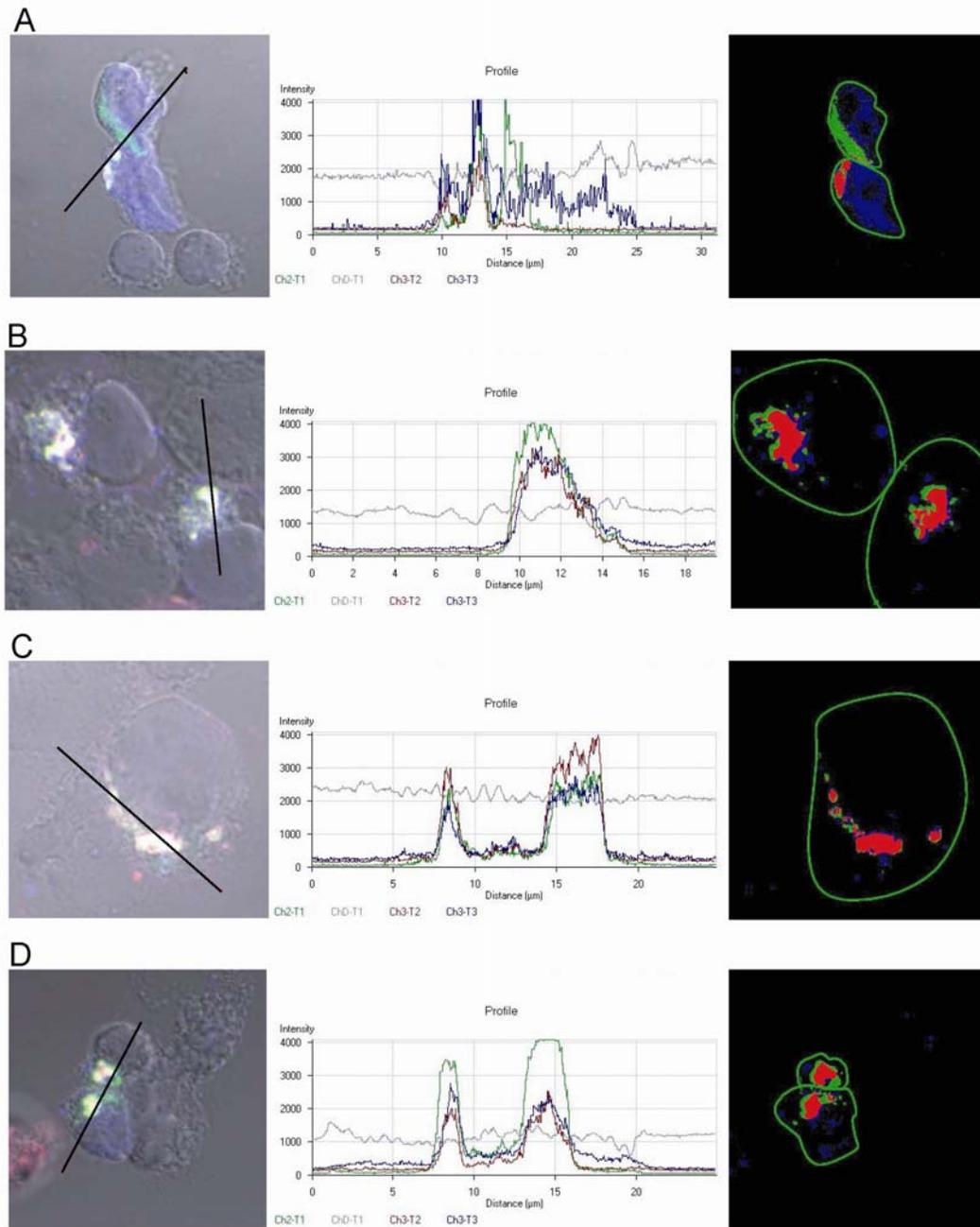


Fig. S6. Colocalization profiles of CD158d, Rab5Q79L, and Akt. Shown are the colocalization profiles of the data presented in Fig. 5C (A), Fig. 5D (B), fig. S5 (C and D). The left panels in each row show merged fluorescence images from the indicated figures with a black line to indicate the axis along which the fluorescence intensities were traced. The middle panels show the graphs of the fluorescence intensity of each fluorophore at each point along the black line versus the relative position of each point along the line. The different tracings represent intensities for CD158d (red), Rab5Q79L (green), Akt (blue), and DIC (gray). The right panels represent a pseudo-colored colocalization analysis. Red represents pixels that are positive for both Akt and Rab5Q79L, green represents pixels that are positive for Rab5Q79L only, and blue represents pixels that are positive for Akt only.

Table S1. Compounds tested for their ability to inhibit CD158d-dependent secretion of IFN- γ by resting NK cells. Resting NK cells were preincubated with the listed inhibitors in the indicated dose ranges for 1 hour prior to the addition of agonist mAb (#33) against CD158d. Cells were incubated at 37°C for 16 hours in the continuing presence of the individual inhibitors. Culture media were tested for the presence of secreted IFN- γ by ELISA. None of the inhibitors listed in the table blocked the secretion of IFN- γ in response to CD158d stimulation.

Name	Target	Dose range
Herbimycin	Src family kinase	0.01 to 1 μ M
PP1	Src family kinase	0.1 to 10 μ M
PP2	Src family kinase	0.1 to 10 μ M
Wortmannin	PI3K	3 to 300 nM
LY294002	PI3K	1 to 30 μ M
Piceatannol	Syk, Zap70	2 to 200 nM
AG490	JAK2, JAK3	1 to 200 μ M
Pertussis toxin	G α i	0.1 to 10 μ g/ml
U73122	PLC	0.1 to 10 μ M
Chelerythrine	PKC	0.1 to 10 μ M
Staurosporine	PKA, PKC, PKG	0.01 to 1 μ M
D609	PC-PLC	0.1 to 10 μ M