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Inhibitory Receptor Signaling via Tyrosine Phosphorylation of the Adaptor Crk

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Supplemental Experimental Procedures

Cell Lines and Reagents

The human NK cell line YTS transfected with KIR2DL1-SHP-1 fusion proteins and trapping mutations KIR2DL1-SHP-1 with Asp to Ala (DA) and Arg to Met (RM) (Burshtyn et al., 2000; Stebbins et al., 2003) were cultured in Iscove's (IMDM) supplemented with glutamine, 12.5% FCS and 50 μM 2-mercapto-ethanol. NK92 cells were grown in Myelocult (Stem Cell Technologies) supplemented with 100 U/ml rIL-2 (Roche). Where indicated, YTS-2DL1 cells were pretreated for 48 hours with 5 μM Gleevec (STI571, Novartis) prior to cytotoxicity assays.

Antibodies

The cyt42/43 antibody, specific for the long cytoplasmic tail of KIR2DL1 and KIR2DL2, was produced in rabbits after immunization with peptide AESRSKVVSCP conjugated to KLH (Wagtmann et al., 1995). Antibodies were affinity purified with the peptide. Directly PE-conjugated anti-CD56 and anti-CD3 were from BD Biosciences. Directly conjugated PE: CD158a (EB6), CD158b (GL183), and CD159a (NKG2A) were from Immunotech. Anti-HLA-E (MEM-E/07) was obtained from EXBIO Praha. Anti-KT3 tag (for the KIR2DL1-SHP-1 fusion proteins) was obtained from Covance.

Transfections

NK92 cells were transfected with the BSRαEN vector encoding KIR2DL1-SHP-1 fusion proteins (Burshtyn et al., 2000; Stebbins et al., 2003) by electroporation, as described (Standeven et al., 2004). Briefly, 5×10^6 NK92 cells were transfected with 5 μg DNA using the Gene Pulsar (Bio-Rad) and were placed under 1 mg/ml G418 selection 2 days later. Bulk populations were subcloned and screened for CD158a expression by FACS.

NK-Target Cell Mixing Experiments

Mixing experiments using YTS or NK92 cells expressing the 2DL1-SHP-1(DA) trapping mutant were carried out with 30×10^6 cells as described, except that the $NaVO_3$ was omitted in the lysis buffer.

RNAi Transfections

YTS-2DL1 cells were transfected with 150-300 picomoles siRNA using Amaxa technology. 2×10^6 YTS-2DL1 cells were resuspended in 100 μl of Amaxa Kit V, RNAi was added and the cells were immediately transfected using program O-017. Cells were incubated at 37° C for 48 hours and then assayed in a Europium based killing assay. The

negative siRNA control was obtained from Dharmacon (Non-Targeting siRNA Pool #1). The CrkII sequence targeted by siRNA was GTCCCTTACGTCGAGAAGTATAGACCT.

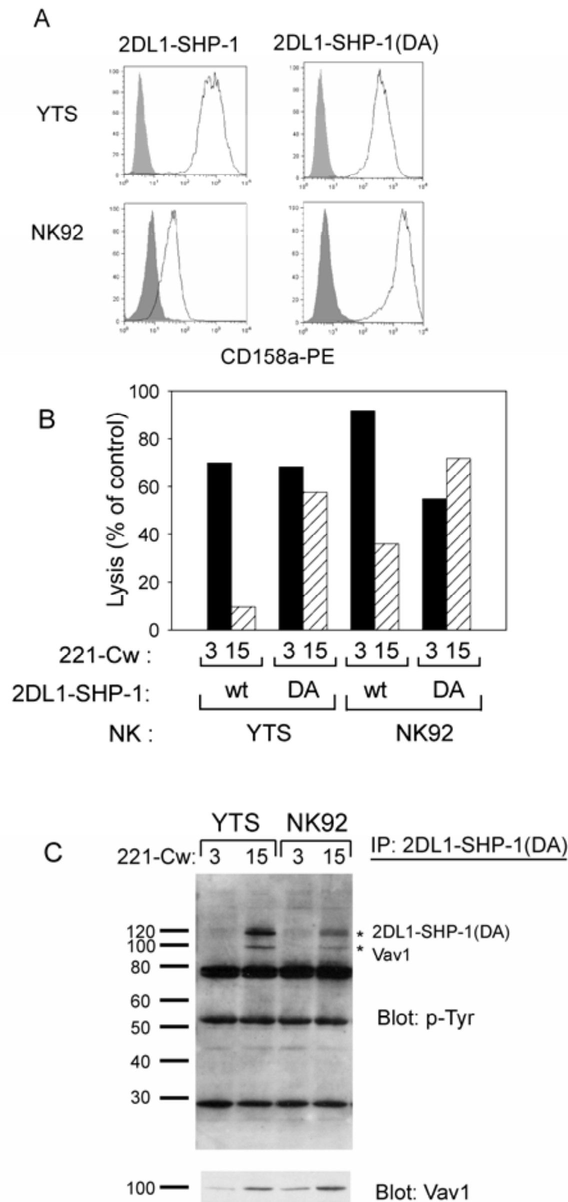
Supplemental References

Burshtyn, D.N., Shin, J., Stebbins, C., and Long, E.O. (2000). Adhesion to target cells is disrupted by the killer cell inhibitory receptor. Curr Biol *10*, 777-780.

Standeven, L.J., Carlin, L.M., Borszcz, P., Davis, D.M., and Burshtyn, D.N. (2004). The actin cytoskeleton controls the efficiency of killer Ig-like receptor accumulation at inhibitory NK cell immune synapses. J Immunol *173*, 5617-5625.

Stebbins, C.C., Watzl, C., Billadeau, D.D., Leibson, P.J., Burshtyn, D.N., and Long, E.O. (2003). Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. Mol Cell Biol *23*, 6291-6299.

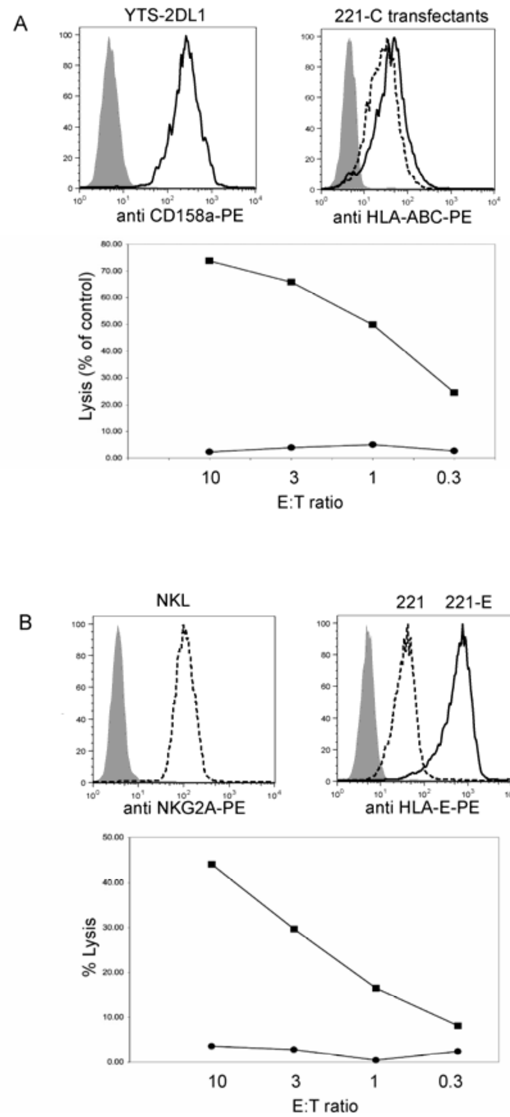
Wagtmann, N., Rajagopalan, S., Winter, C.C., Peruzzi, M., and Long, E.O. (1995). Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. Immunity *3*, 801-809.



Supplemental Fig 1 Peterson and Long

Figure S1. Trapping with KIR2DL1-SHP-1(DA) Gives Similar Results in YTS and NK92 cells

(A) Cell surface expression of KIR2DL1-SHP-1(wt) and catalytically inactive KIR2DL1-SHP-1(DA) on transfected YTS and NK92 cells. Staining with CD158a (open) or secondary antibody alone (shaded). (B) Lysis of 221-Cw3 (filled bars) and 221-Cw15 cells (hatched) by YTS and NK92 cells expressing KIR2DL1-SHP-1(wt) and KIR2DL1-SHP-1(DA). (C) YTS and NK92 cells expressing KIR2DL1-SHP-1(DA) were mixed with 221-Cw3 or 221-Cw15 cells, incubated at 37° C for 5 min and lysed. Lysates were immunoprecipitated with KT3 antibody, and probed with phosphotyrosine Ab 4G10. Asterisks indicate the position of KIR2DL1-SHP-1(DA) and of Vav1. The bottom panel is a re-blot with Vav1 Ab.



Supplemental Figure 2 Peterson and Long

Figure S2. Expression and Function of KIR2DL1 on YTS Cells and of NKG2A on NKL Cells.

(A) Cell surface staining of YTS-2DL1 with CD158a (solid line) or secondary antibody alone (shaded). 221-Cw3 (solid line) and 221-Cw15 (dashed line) were stained with HLA class I mAb W6/32 or secondary antibody alone (shaded). 221-Cw3 (squares) and 221-Cw15 (circles) cells were used as targets in a killing assay with YTS-2DL1. (B) Staining of NKL cells with NKG2A mAb (solid line) or secondary antibody alone (shaded) in the left panel. 221 (dashed line) and 221-HLA-E (solid line) were stained with HLA-E mAb or secondary antibody alone (shaded) in the right panel. 221 (squares) and 221-HLA-E (circles) cells were used as targets in a killing assay with NKL.

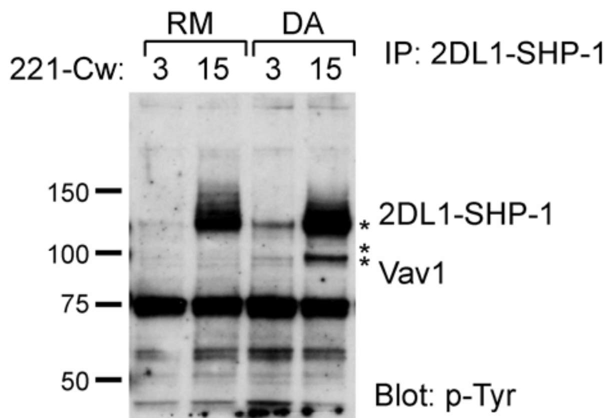


Figure S3. A Protein Co-migrating with c-Cbl is Pulled Down during Trapping of Vav1

YTS-2DL1-SHP-1(DA) and YTS-2DL1-SHP-1(RM) cells were mixed with 221-Cw3 and 221-Cw15 cells, incubated at 37° C for 5 min, and lysed. The KIR2DL1-SHP-1 fusion proteins were immunoprecipitated with KT3 antibody, and probed with phosphotyrosine Ab 4G10. The three asterisks indicate the position of KIR2DL1-SHP-1(DA), of a ~120 kDa protein, and of Vav1. The phosphorylated ~120 kDa protein was detected in nine out of ten experiments in which a strong phospho-Vav1 band was obtained.

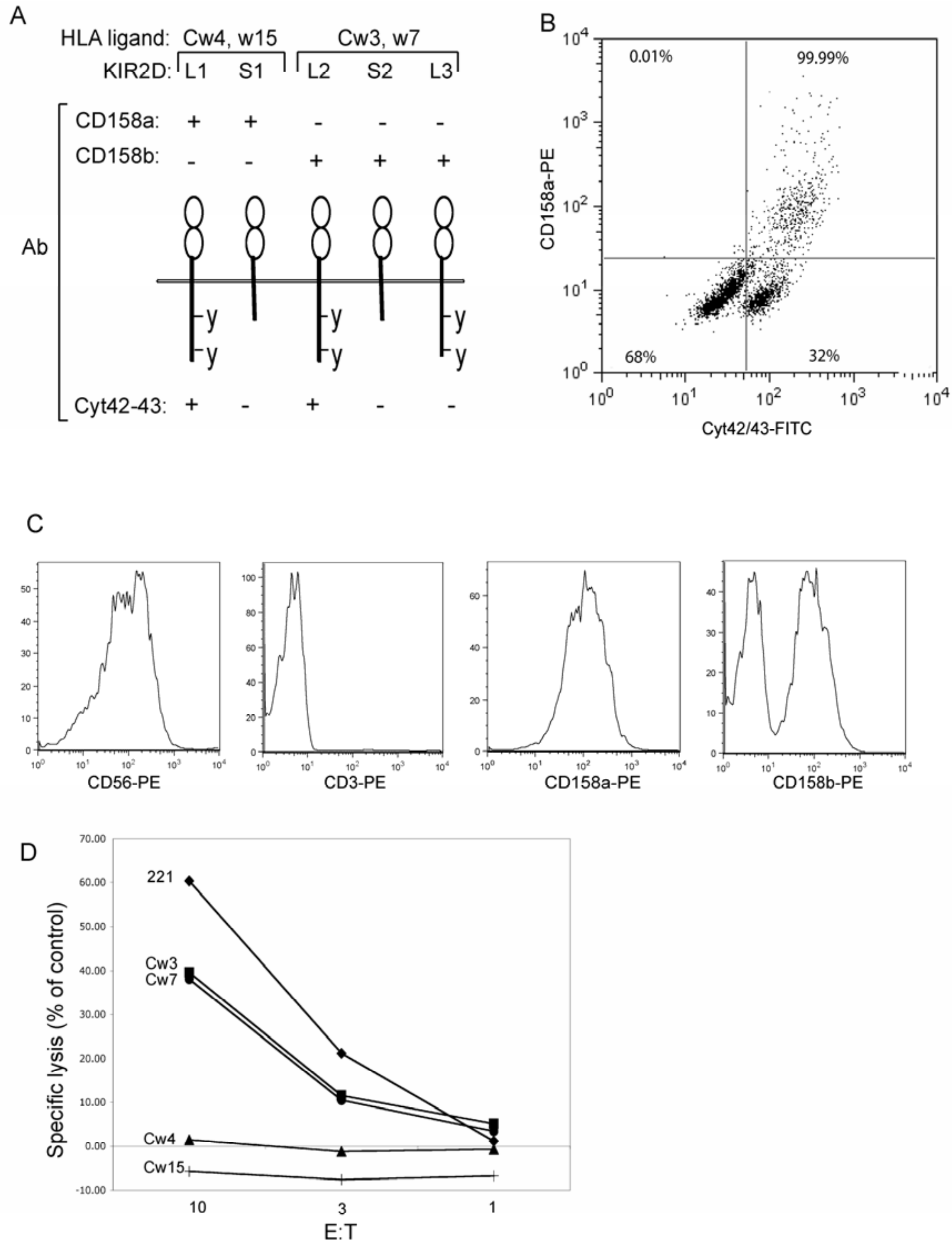


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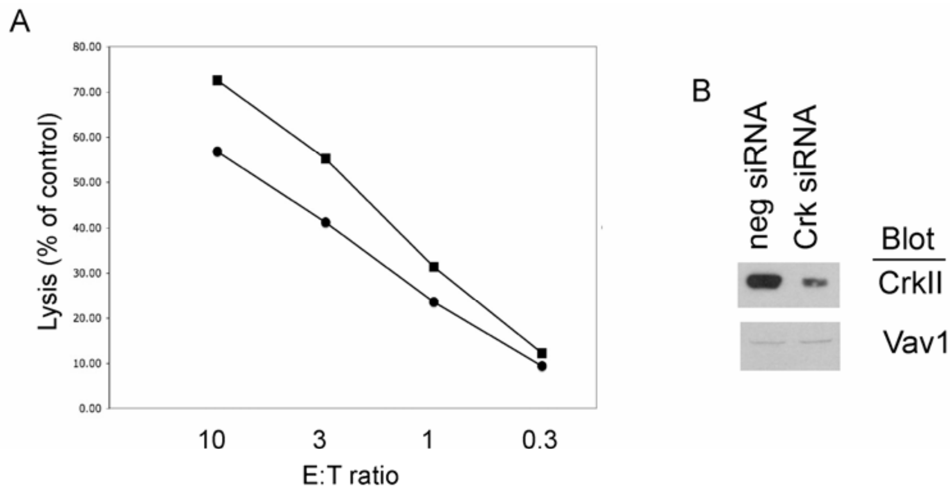
Figure S4. Isolation and Function of KIR2DL1^{+} NK Cells

(A) Reactivity of Abs CD158a, CD158b, and rabbit antiserum cyt42/43, specific for the cytoplasmic tail of KIR2DL1 and KIR2DL2, with several KIR2D receptors. Y indicates tyrosines within ITIMs. The short-tailed KIR2DS1 and KIR2DS2 have no ITIM. The cytoplasmic tail of KIR2DL3 is shorter than the tail of KIR2DL1 and KIR2DL2. (B)

Staining of NK cells with CD158a and cyt42/43. Double-positive cells express KIR2DL1 but no KIR2DS1. Note that KIR2DS1 is not expressed in this NK cell population. (C) Staining of sorted $KIR2DL1^+$ NK cells for CD56, CD3, CD158a, and CD158b. Note that double-positive $KIR2DL1^+$ and $KIR2DL2^+$ NK cells are not uncommon. (D) $KIR2DL1^+$ NK cells are inhibited by HLA-Cw4 and HLA-Cw15. Partial inhibition is seen with 221-Cw3 and 221-Cw7 cells, which express ligands of KIR2DL2.



Supplemental Figure 5 Peterson and Long

Figure S5. Reduced Cytotoxicity of YTS-2DL1 Cells after CrkII Knockdown

YTS-2DL1 cells were transfected with 300 picomoles of either scrambled negative siRNA or CrkII siRNA. (A) Lysis of 221 cells by YTS-2DL1 cells 48 hours after transfection with scrambled negative (squares) or CrkII (circles) siRNAs. (B) Knockdown of CrkII protein. Total lysates of YTS-2DL1 cells after transfection with the indicated siRNAs were immunoblotted for CrkII and Vav1. This is a representative experiment out of 6 independent transfections, in which the average decrease in killing was 14% ± 5.6% ($p = 0.002$).