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Cutting Edge: The Adapters EAT-2A and -2B Are Positive Regulators of CD244- and CD84-Dependent NK Cell Functions in the C57BL/6 Mouse

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EWS/FLI1-activated transcript 2 (EAT-2)A and EAT-2B are single SH2-domain proteins, which bind to phosphorylated tyrosines of signaling lymphocyte activation molecule family receptors in murine NK cells. While EAT-2 is a positive regulator in human cells, a negative regulatory role was attributed to the adapter in NK cells derived from EAT-2A–deficient 129Sv mice. To evaluate whether the genetic background or the presence of a selection marker in the mutant mice could influence the regulatory mode of these adapters, we generated EAT-2A–, EAT-2B–, and EAT-2A/B–deficient mice using C57BL/6 embryonic stem cells. We found that NK cells from EAT-2A– and EAT-2A/B–deficient mice were unable to kill tumor cells in a CD244- or CD84-dependent manner. Furthermore, EAT-2A/B positively regulate phosphorylation of Vav-1, which is known to be implicated in NK cell killing. Thus, as in humans, the EAT-2 adapters act as positive regulators of signaling lymphocyte activation molecule family receptor-specific NK cell functions in C57BL/6 mice. The Journal of Immunology, 2010, 185: 000–000.

A large body of evidence supports the notion that NK cells participate in the defense against infections, in the regulation of immune responses, and in the surveillance of stressed or cancer cells (1). Effector functions of NK cells are regulated by the coordinated interaction of activating and inhibitory receptors (1). Ligation of activating receptors on the surface of NK cells results in cytokine production, cytokinesis, and migration, which are inhibited by the triggering of inhibitory receptors. Well-defined inhibitory receptors include the MHC class I-recognizing members of the murine Ly49 family, human killer Ig-like receptors and CD94/NKG2 in both species (1–3). The inhibitory receptors mediate their effects through one or more ITIMs in their cytoplasmic domains. Established human and mouse NK cell-activating receptors are NKG2D, NKRP1, CD16, DNAM1 (4), activating human killer Ig-like receptors, and activating murine Ly49. As several activating NK cell receptors do not contain cytoplasmic domains, they associate with and signal through adapter molecules such as DAP12, FcR-γ, and CD3ζ, which contain the ITAM (1).

In recent years, there has been accumulating evidence implicating the signaling lymphocyte activation molecule (SLAM) family of receptors (SLAMF1–9) and their specific intracellular adapters in immune regulation (5, 6). SLAMF receptors, which are expressed on hematopoietic cells (6), are self-ligand adhesion molecules with the exception of CD244 and its ligand CD48. After receptor ligation, the tyrosines present on their intracellular domain are phosphorylated, permitting the association to the signaling lymphocyte activation molecule-associated protein (SAP) family of adapters: SAP, EWS/FLI1-activated transcript 2 (EAT-2)A, and, in rodents, EAT-2B (EAT-2–related transcript; see Ref. 7). These adapters are essentially composed of an SH2 domain and a short C-terminal tail, and they are able to trigger biochemical signals that seem crucial for the SLAM-dependent and SLAM-independent functions (5, 6). In human NK cells, SAP and EAT-2 mediate the cytotoxic function of CD244, CD319, and CD352 (6). SAP positively regulates mouse NK cell functions, which are initiated by the SLAMF receptors. However, EAT-2A and EAT-2B play a dual role in regulating the function of the SLAMF receptors in NK cells derived from a 129Sv background (7–9).

Because extensive polymorphisms as well as differences in expression have been found in the SLAMF locus between 129Sv and C57BL/6 (B6) mouse strains (6, 10), we set out to test the hypothesis that the strain background in which the EAT-2A/B mouse strains (6, 10) were generated influences the positive or negative regulatory function of a receptor. To this end, we targeted B6 embryonic stem cells (ES cells) to generate novel EAT-2A–, EAT-2B–, EAT-2A/B–, and EAT-2A/B ×
SAP-deficient mice, as well as CD244-deficient mice without selection cassettes on a B6 background. We found that EAT-2A and EAT-2B positively regulate cytotoxicity mediated by CD244 and CD84 in B6 mouse NK cells.

**Materials and Methods**

**Generation of EAT-2A−, EAT-2B−, and EAT-2A/B−deficient B6 mice**

A B6 bacterial artificial chromosome clone containing the EAT-2A and EAT-2B (82d161 and 82d162) genes was used to construct a targeting vector with a neomycin resistance cassette flanked by two LoxP sites. EAT-2A− or EAT-2B−targeted ES cells clones generated by standard methods were injected into blastocysts, and the chimeric mice were crossed with B6 mice. To delete the neomycin resistance gene from the targeted locus, EAT-2A or EAT-2B heterozygous mice were crossed with B6 Cre deleter mice (11) (Supplemental Figs. 1, 2).

To generate EAT-2A/B double-deficient mice, we used a modified EAT-2B targeting vector to re-target the previously generated EAT-2A mutant ES cell clone (Supplemental Fig. 3). Cotargeting of the two targeting vectors on the same chromosome was assessed by in vitro transfecting-targeted ES cell clones with Cre recombinase expression vector. Deletion of the whole EAT-2 locus was confirmed by PCR (Supplemental Fig. 3). To delete neomycin and hygromycin resistance genes from EAT-2A/B−targeted loci, homozygous EAT2A/B−/− mice were bred with B6 Cre deleter mice (11).

**NK cell isolation**

Splenocytes harvested from wild-type (wt) or mutant B6 mice were processed in PBS with 2% FCS. After RBC lysis, NK cells were isolated from spleen cells using magnetic microbeads according to the manufacturer's recommendations (Miltenyi Biotech, Auburn, CA). Purified NK cells (>92% NK1.1-positive) were cultured in DMEM medium supplemented with 1000 U recombinant human IL-2 (BioLegend, San Diego, CA) for 7 d, as described (12).

**Cell lines**

The cell lines RMAS/CD48+ or RMAS/CD48− (H-2b), P815/CD48+ or P815/CD48− (H-2b), B16, Y2B/0, CHO, and YAC-1 were cultured in supplemented DMEM medium, as described (13). To generate CD84+ stable cells, CD84 cDNA was cloned into the pcDNA3.1 expression vector that was then stably transfected into P815 or B16 tumor cells.

**Cytotoxicity assays**

Specific lysis of targets was determined by using a standard 4-h 51Cr-release assay in 96-well U-bottom plates as previously described (14). Alternative nonradioactive cytotoxicity assay was used to quantitatively measure lactate dehydrogenase (LDH) that is released upon cell lysis (CytoTox 96; Promega, Madison, WI). Redirected killing assays using P815 targets were performed as previously described (13).

**In vivo tumor clearance assay**

Tumor clearance assays were performed as previously described (12). Briefly, the target cells were labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) at 37°C for 10 min. CFSE-labeled target cells were washed three times with cell culture media. Target cells (5 × 107) were injected i.p. in 300 μl of PBS. The mice were sacrificed and peritoneal cells were recovered after 18 h. The residual target cells were counted by FACS.

**Immunoprecipitation and Western blot analysis**

Approximately 10−20 × 106 IL-2−/−/CD84−/− cell lysates were labeled with anti-CD244 mAB for 30 min on ice. Anti-mouse Ab was used for crosslinking at 37°C for 15 min. Cells were lysed and CD244 or Vav-1 was immunoprecipitated. Immunoprecipitation and Western blot analysis were performed as previously described (13).

**Results and Discussion**

**Impaired in vivo killing by EAT-2A/B−/− and SAP−/− NK cells using CD244- and CD84-specific target cells**

To determine whether the genetic background could play a role in the function of EAT-2A and EAT-2B, EAT-2A− and EAT-2B−deficient mice were generated from B6-derived ES cells (Bruce 4) in which the first exon of EAT-2A gene or EAT-2B gene was replaced by the LoxP-flanked selection markers neomycin and/or hygromycin. After breeding the mutant mice with the Cre deleter transgenic mouse (11), none of the mutant mice contained the selection cassettes (Supplemental Figs. 1–3). EAT-2A, EAT-2B, or both transcripts were not detected by RT-PCR in NK cells from the resulting mutant mouse strains (Supplemental Fig. 3).

To evaluate the role of the EAT-2 adapters in CD244-mediated NK cell functions, EAT-2A/B−/−, EAT-2A−/−, and EAT-2B−/− mice were injected i.p. with CFSE-labeled RMAS cells that express CD48, the high-affinity ligand for CD244. As controls, RMAS/CD48− cells were used. After 18 h, the number of RMAS/CD48+ tumor cells was significantly higher in the peritoneal cavity of EAT-2A/B−/− and EAT-2A−/− mice than in B6 mice (Fig. 1A and Supplemental Fig. 4). However, the number of RMAS/CD48+ tumor cells recovered from the peritoneal cavity of EAT-2B−/− mice was comparable to that in wt mice (Supplemental Fig. 4). As expected, without triggering of CD244 by its ligand CD48, the absence of EAT-2A/B in the NK cells had no effect (Fig. 1A).

EAT-2A/B−/− and SAP−/− mice were equally impaired in their ability to remove the RMAS/CD48+ cells (Fig. 1B). The striking decrease of CD244-dependent cytotoxicity in EAT-2A/B−/− and SAP−/− mice raised the question of whether NK cell functions would be more severely impaired by the loss of all SAP-related adapters. Indeed, in mice that lack all three adapters (i.e., EAT-2A/B−/− × SAP−/−), clearance of RMAS/CD48+ tumor cells in mice was less than in either EAT-2A/B−/− or SAP−/− mice (Fig. 1B). These studies strongly suggest that in B6 mice, both EAT-2A/B and SAP are positive regulators of CD244-dependent in vivo NK cell killing and that these specific adapters may act synergistically.

In agreement with the notion that both the CD244−EAT-2A/B and CD244−SAP pathways represent an activating receptor−adapter system in B6 mice is our observation that CD244−/− mice are also impaired in the in vivo clearance of RMAS/CD48+ tumor cells (Fig. 1C). When NK cells had been removed by treatment with anti-NK1.1, the EAT-2A/B mutation did not have any effect on the killing of RMAS/CD48+ cells (Supplemental Fig. 5). Thus, as in humans, in B6 mice CD244 and its SAP-related adapters predominately appear to be part of the activating system for NK cell cytotoxicity.

To determine whether NK killing by another SLAMF receptor, which binds EAT-2 (15), is also positively regulated by EAT-2A/B, in vivo killing of CD84-expressing P815 tumor cells was evaluated in EAT-2A/B−/− and EAT-2A/B−/− × SAP−/− mice. Again, in the absence of EAT-2A/B, the number of P815/CD84+ target cells was markedly increased as compared with wt B6 littermate controls (Fig. 1D). Similarly, the number of P815/CD84+ target cells recovered in EAT-2A/B−/− × SAP−/− mice was dramatically higher than in wt littermates (Fig. 1D). Collectively, these data strongly suggest that, similar to SAP, EAT-2A/B positively regulate CD244- and CD84-dependent NK cell functions in vivo. Furthermore, the data suggest that the contribution of EAT-2A is greater than that of EAT-2B.

EAT-2A/B are positive regulators of in vitro CD244- and CD84-dependent NK cell killing

To evaluate whether the in vivo observations correlated with in vitro killing, RMAS, P815, and B16 target cells with or
without CD48 or CD84 were used in either an in vitro [51Cr]-release assay or in an LDH cytotoxicity assay. Compared to wt NK cells, EAT-2A/B−/− NK cells were impaired in their ability to in vitro lyse RMAS/CD48+, but not RMAS/CD48−, target cells (Fig. 2A). Whereas EAT-2A/B−/− and EAT-2A−/− NK cells (Fig. 2B and Supplemental Fig. 6) had lost their ability to kill P815/CD48+ targets, the EAT-2B−/− mutation had no impact. Similarly, P815/CD84+ or B16/CD84+ targets were killed less efficiently by NK cells that lacked the EAT-2A gene (Fig. 2C). In contrast, NK cells derived from EAT-2A, EAT-2B, or EAT-2A/B mutant mice efficiently killed the target cells YB2/0, YAC-1, and CHO (Supplemental Fig. 7A–C). Thus, whereas EAT-2A positively regulates in vitro NK cell killing that is mediated by CD244 and CD84, the effect of the EAT-2B mutation is marginal.

EAT-2A/B−/−, EAT-2A−/−, and EAT-2B−/− NK cells are also defective in anti-CD244-dependent killing of [51Cr]-labeled P815 cells (Fig. 3A and Supplemental Fig. 8A). Similarly, anti-CD84–coated P815 cells were more efficiently killed in a redirected killing assay by wt than by EAT-2A/−/− NK cells (Fig. 3B). A defect in IFN-γ production by EAT-2A/B−/− NK cells was also observed by triggering of CD244 with anti-CD244 mAb (Fig. 3D).

To exclude the possibility that the defective redirected cytotoxicity and IFN-γ production by EAT-2A/B−/− NK cells was caused by a global dysfunction of NK cells, mAbs directed against the activating NK cell receptors NK2D and Ly49D were used. EAT-2A/B−/−, EAT-2A−/−, or EAT-2B−/− NK cells lysed [51Cr]-labeled P815 cells coated with anti-Ly49D or anti-NKG2D equally efficiently as did wt B6 NK cells (Fig. 3C and Supplemental Fig. 8B, 8C). Thus, consistent with the absence of immunoreceptor tyrosine-based switch motifs in the cytoplasmic portions of these NK receptors, the absence of EAT-2A and -2B did not affect their functions. This was confirmed by the finding that IFN-γ production induced by anti-Ly49D or anti-NKG2D was comparable in EAT-2A−/−, EAT-2B−/−, or EAT-2A/B−/− and B6 NK cells (Fig. 3E and Supplemental Fig. 9).

Taken together, the outcomes of these experiments demonstrate that the lytic functions and IFN-γ production of EAT-2A−/− and EAT-2A/B−/− NK cells are defective in a CD244- and CD84-mediated manner. Our data, therefore, contrast with a previous report, in which J29 background EAT-2A−/− and EAT-2B−/− NK cells were found to have enhanced ability to kill xenogeneic target cells and also increase IFN-γ production upon triggering not only by CD244, but also by other NK cell-activating receptors NKG2D and Ly49D (7). These differences may be due to different genetic background between J29 and B6 mice and to the absence of selection markers in the targeted allele. Whether and how the mouse genetic background affects EAT-2A and EAT-2B regulation of NK cell function require further investigation.

Absence of EAT-2A and EAT-2B affects CD244-mediated phosphorylation of Vav

The precise mechanisms by which EAT-2A and EAT-2B are involved in the CD244-mediated signaling pathways are not well understood. Upon engagement of CD244 by anti-CD244- or CD48-expressing target cells, the receptor is recruited to lipid rafts, where the tyrosines of the immunoreceptor tyrosine-based switch motifs are phosphorylated, leading to recruitment and activation of several downstream signaling molecules. In addition to SAP and EAT-2, these include Vav-1, SHIP, PI3K, Csk, PLCγ, SH2 domain-
containing tyrosine phosphatase-1, SH2 domain-containing tyrosine phosphatase-2, and linker for activation of T cells (6, 16).

To assess whether the absence of EAT-2A/B would affect a downstream signaling molecule that could be responsible for the impaired CD244-mediated cytotoxicity, we focused on the guanine nucleotide exchange factor Vav-1 (17). To this end, wt- and EAT-2A/B–cultured NK cells were stimulated with anti-CD244 mAb, and NK cell lysates were used for immunoprecipitation with anti–Vav-1. Tyrosine phosphorylation of Vav-1 was significantly reduced in EAT-2A/B–cultured NK cells compared with wt NK cells (Fig. 4A). Decreased phosphorylation of Vav-1 was also observed with EAT-2A/B–cultured NK cells triggered by CD48-expressing targets (Fig. 4B). As the guanine nucleotide exchange factor activity of Vav proteins is activated by tyrosine phosphorylation and the phosphorylated Vav proteins are able to positively regulate NK cell-mediated killing, the reduced phosphorylation of Vav most likely contributes to impaired lysis in the EAT-2A/B–cultured NK cells.

This defect of Vav phosphorylation is not dependent of phosphorylation of the receptor itself, because tyrosine phosphorylation of CD244 was not affected in EAT-2A/B–cultured NK cells (Fig. 4C). As we know that phosphorylation of CD244 precedes SAP and EAT-2A/B binding to its cytoplasmic tail (18), and because SLAMF receptors can be phosphorylated in the absence of the adapters (6), the role of CD244 phosphorylation in the pathway toward Vav-1 is not immediately clear. In general, NK cell activation requires synergizing receptors (e.g., NKG2D and CD244), which is regulated at the level of Vav-1 by a hierarchy of mechanisms. Phosphorylation of phospholipase PLC-γ2, Ca2+ mobilization, and degranulation are involved. It is likely that c-Cbl plays an inhibitory role (19). Thus, as the pathway from CD244 to Vav will, as in humans, undoubtedly involve a number of factors (e.g., SHIP; see Ref. 20) that are recruited to the NK cell synapse by CD244, this requires a more detailed study.

Taken together, the outcomes of our studies demonstrate that EAT-2A/B and EAT-2A, similar to SAP, positively regulate CD244-mediated NK cell functions in B6 mice, which is different from the model that these adapters have dual roles in 129 NK cells. The diversity of EAT-2A and EAT-2B functions in NK cells may result from extensive polymorphism of SLAM family members between two mouse strains, influence of the presence of selection markers in gene targeted loci, a strain-dependent gender effect (21), or different environment conditions of animal facilities. Moreover, our studies also suggest that EAT-2A and EAT-2B are involved in phosphorylation of downstream effector molecule Vav-1, which plays a critical role in natural cytotoxicity. The notion that EAT-2 is a positive regulatory molecule was first discovered using human cells (15) is consistent with SLAMF receptors functioning as positive regulators on human NK cells (6).
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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental figure legends

Figure S1. Generation of EAT-2A-/- mice
A. The organization of EAT-2A [Sh2d1b1] genomic locus is shown (top). Exons are filled boxes. In the targeting vector (middle), LoxP flanking Neo replaced exon 1. The thymidine kinase gene (TK) lies external to the 3' genomic DNA fragment. The structure of the targeted allele is shown (bottom). B. DNA from EAT-2A+/+, EAT-2A-/+ and EAT-2A-/- mice was subjected to southern blot with external probe, which is green box. C. The deletion of Neo in pups from EAT-2A-/+ x Cre deleter transgenic mice was confirmed by PCR with two sets of primer pairs (P1F - P1R and P2F-P2R).

Figure S2. Generation of EAT-2B-/- mice
A. The organization of EAT-2B genomic locus is shown (top). The targeting vector was generated using the pKO-Scrambler LoxP vector and replacing a 1-kb region encompassing exon 1 of EAT-2B with the neomycin resistance gene (Neo). The recombination vector contained 3 kb and 7.6 kb of homology upstream and downstream, respectively, of LoxP-Neo. B. Southern blot analysis of EAT-2B+/+, EAT-2B-/+ and EAT-2B-/- mice after DNA digestion withSacI. Map location of external probe is shown in green box (See S2A). C. As shown in S1, the deletion of Neo in pups from EAT-2B-/+ x Cre deleter transgenic mice was confirmed by PCR with two sets of primer pairs (P1F-P1R and P2F-P2R).

Figure S3. Generation of mice lacking both EAT-2A and EAT-2B.
A. Schematic outline of the EAT-2A and EAT-2B targeting.
To generate EAT-2A and EAT-2B double mutant ES cell clones, the first exon of EAT-2A was replaced by the Neo flanked by two LoxP sites in the first targeting vector. The second targeting vector with LoxP-Hygromycin cassette was used to delete the first exon of EAT-2B in one of EAT-2A-targeted ES clones. ES cell clones that carried both targeted mutants on the same chromosome were identified by Cre-mediated recombination in which the genomic DNA fragment flanked by two Lox P sites was excised. The arrows indicate the location of two sets of primer pairs (P1F-P1R and P2F-P2R) for PCR detection of deletion. 600 bp or 650 bp PCR
amplicons were amplified from two hygromycin susceptible ES cell clones (1D5H and 2E2A), but not from hygromycin resistant clones (1C3B and 2G1F).

B. Expression analysis.
RNA was isolated from B6, EAT-2A−/−, EAT-2B−/− and EAT-2A/B−/− NK cells. The expression of EAT-2A, EAT-2B or both transcripts was determined by RT-PCR.

Figure S4. EAT-2A−/− NK cells, but not EAT-2B−/− NK cells, are unable to eliminate RMA-S/CD48+ tumor cells.
3x10⁶ CFSE-labeled RMAS/CD48+ or RMAS/CD48− tumor cells were injected into the peritoneum of B6, EAT-2A−/− or EAT-2B−/− mice. After 18 hours, the cells were recovered from peritoneum and the number of tumor cells was determined based on the percentage of CFSE+ cells by flow cytometry.

Figure S5. Impaired clearance of RMAS/CD48+ tumor cells in NK cell-depleted EAT-2A/B−/− and B6 mice.
NK cell-depletion of EAT-2A/B−/− and wt B6 mice was accomplished by injecting 100 µg anti-NK1.1 mAb i.p. on days –2 and day 0. CFSE-labeled RMAS/CD48+ cells were injected i.p. in EAT-2A/B−/− or wt mice. As described in Fig. S4, the total number of tumor cells was calculated based on the percentage of CFSE+ cells.

Figure S6. Defective killing of CD48+ P815 cells by EAT-2A−/- but not by EAT-2B−/− NK cells.
NK cells were isolated from B6, EAT-2A−/- or EAT-2B−/- mice and cultured, as described in Materials and Methods. Cytolytic activity was determined in a 4 h [⁵¹Cr] -release assay.
Figure S7. Cytotoxicity directed against YB2/0, Yac1 and CHO targets is not impaired in EAT-2A-/-, EAT-2B-/- or EAT-2A/B-/- mice.

EAT-2A-/-, EAT-2B-/-, EAT-2A/B-/- or B6 NK cells were cultured in IL-2 containing medium for 7 days before use in a 4 h killing assay against:

A YB2/0 cells ([51]Cr-release assay)
B YAC-1 cells ([51]Cr-release assay)
C CHO cells (LDH release assay)

Figure S8. Impaired cytotoxic activity mediated by αCD244 antibody, but not by αLy49D and αNKG2D, in EAT-2A-/- and EAT-2B-/- mice.

IL-2-activated NK cells from EAT-2A-/-, EAT-2B-/- or B6 mice were tested for antibody redirected cytotoxic activity against P815 tumor cells in a 4h [51]Cr-release assay.

A αCD244.
B αLy49D.
C αNKG2D.

Figure S9. Defective CD244-mediated IFN-γ production in EAT-2A-/- NK cells.

NK cells isolated from EAT-2A-/-, EAT-2B-/- or B6 mice were cultured, as described in Materials and Methods. The NK cells were stimulated with αNKG2D or αLy49D mAb for 24 hours. The supernatant were measured using ELISA.
Supplemental Figure S1

A. EcoRV

Wild type Allele

Targeting vector

Neo TK

Mutant Allele

8Kb EcoRV

B. Breeding with Cre deleter Tg mice

C. P1F+P1R  P2F+P2R

+/- -/-  +/ +/ +/ +/ +/ +  500bp

12kb EcoRV

8kb EcoRV

500bp
Supplemental Figure S2

A.  

**Wild type Allele**  

- 10.8kb SacI  
- SacI I PstI  
- SacI II III IV  

**Mutant Allele**  

- 6.7kb SacI  
- SacI I PstI  
- SacI II III IV  

Breeding with Cre deleter Tg mice

B.  

- +/- +/– –/– +/– +/+  

C.  

- P1F+P1R  
- P2F+P2R  

- 10.8kb SacI  
- 6.7kb SacI  

300bp
Supplemental Figure S3

A.

EAT-2B EAT-2A

hygro Neo

25kb

EAT-2B EAT-2A

P1F P1R

P2F P2R

1C3B 2G1F 1D5H 2E3A

1C3B 2G1F 1D5H 2E3A

600bp 650bp

B.

EAT-2A EAT-2B GAPDH
Supplemental Figure S4

Number of residual tumor cells (x 10^6)

RMA-S / CD48^+

RMA-S/CD48^−

p < 0.05

B6  EAT-2A  EAT-2B  B6  EAT-2A  EAT-2B
Supplemental Figure S5

Number of residual tumor cells (x 10^6)

RMA-S / CD48^+

B6    FAT-2A/B−/−
Supplemental Figure S6

![Graph showing % Cytotoxicity for P815 CD48− and P815 CD48+ in B6, EAT-2A−/−, and EAT-2B−/− models.](image-url)
Supplemental Figure S7A
Supplemental Figure S7B

E:T

% Cytotoxicity

Yac-1

B6

EAT-2A−/−

EAT-2B−/−

EAT-2A/B−/−
Supplemental Figure S7C

![Graph showing Cytotoxicity (%)](image)

- CHO
- B6
- EAT-2A/B-/-

E:T

Cytotoxicity (%)

- 40
- 30
- 20
- 10
- 0

25:1, 12:1, 3:1
Supplemental Figure S8

A

B

C

% Cytotoxicity

% Cytotoxicity

% Cytotoxicity

B6  EAT-2A /-  EAT-2B /-  B6  EAT-2A /-  EAT-2B /-  B6  EAT-2A /-  EAT-2B /-

Isotype  αCD244  Isotype  αLy49D  Isotype  αNKG2D

0  5  10  15  20  25  30  35  40  45  50  55

0  10  20  30  40  50  60  70

0  10  20  30  40  50  60  70

0  5  10  15  20  25  30  35  40  45  50  55

0  10  20  30  40  50  60  70
Supplemental Figure S9

![Graph showing IFN-γ levels in B6, EAT-2A−/−, and EAT-2B−/− mice with NKG2D+IL-2 and Ly49D+IL-2 conditions.](image-url)