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Fyn-ADAP signaling via Carma1-Bcl10-MAP3K7 signalosome exclusively regulates inflammatory cytokine production in NK cells

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Abstract

Inflammation is a critical component of the immune response. However, acute or chronic inflammation can be highly destructive. Uncontrolled inflammation forms the basis for allergy, asthma, and multiple autoimmune disorders. Here, we identify a signaling pathway that is exclusively responsible for inflammatory cytokine production but not for cytotoxicity. Recognition of $H60^+$ or $CD137L^+$ tumor cells by murine NK cells led to efficient cytotoxicity and inflammatory cytokine production. Both of these effector functions required Lck, Fyn, PI(3)K-p85α, PI(3)K-p110δ, and PLC-γ2. However, the complex of Fyn and the adapter ADAP exclusively regulated inflammatory cytokine production but not cytotoxicity in NK cells. This unique function of ADAP required a Carma1-Bcl10-MAP3K7 signaling axis. Our results identify

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AUTHOR CONTRIBUTION

K.R. designed and analyzed cytotoxic potentials and inflammatory cytokine production in fresh and IL-2-cultured NK cells from *Fyn*$^{-/-}$, *PI(3)K-p110δ*(D910A), *Adap*$^{-/-}$, *Carma1*$^{-/-}$, and *Carma1*(ΔCARD). K.R. also performed various NK cell activation assays, prepared cell lysates, performed immunoblotting and immunoprecipitation assays. K.R. purified human primary NK cells and used them for siRNA treatment and functional analyses. P.K. designed siRNA and performed the knockdown of various murine signaling proteins. K.M.S. maintained various animal colonies and prepared fresh and IL-2-cultured NK cells for the studies. E.J.P. provided *Adap*$^{-/-}$ spleens and discussed the data. B.V. provided the *PI(3)K-p110δ*(D910A) mice. E.J.P. and B.V. helped in the final editing of the manuscript. M.S.T. helped in the preparation of the manuscript. S.M. conceived the study, designed experiments, analyzed the data and wrote the manuscript.

molecules that can be targeted to regulate inflammation without compromising NK cell cytotoxicity.

Inflammation is caused by soluble mediators including prostaglandins, histamine, lysosomal granules, and cytokines or chemokines. Lymphocytes, including NK and $CD8^+$ T cells, produce many of these factors and thereby play a central role in causing chronic inflammation. Intracellular signaling molecules that regulate the production of inflammatory mediators in lymphocytes are largely unknown. Therefore, defining unique signaling molecules that are exclusively responsible for inflammatory cytokine production promises a crucial advancement in existing immunotherapy and anti-inflammatory protocols.

The activatory receptor NKG2D is ubiquitously expressed on NK cells and activation via NKG2D results in both target cell cytotoxicity and production of inflammatory cytokines. Upon activation, NKG2D recruits Src family protein tyrosine kinases (PTKs) to initiate multiple signaling pathways[1]. Phosphorylation of the adaptor molecule DAP10 in its Tyr-Ile-Asn-Met (YINM) motif by PTK leads to the recruitment of phosphatidylinositol-3-kinase (PI(3)K)[2]. PTKs also phosphorylate the immunoreceptor tyrosine-based activation motif (ITAM)-containing DAP12 (KARAP), which subsequently triggers Syk and Zap70[3]. Earlier studies have shown that lack of DAP12, Syk or Zap70 significantly reduced NKG2D-mediated cytokine production[3]. CD137 belongs to the tumor necrosis factor (TNF) receptor family and functions as an efficient co-stimulatory receptor in T and B cells[4]. CD137 is not constitutively expressed in NK cells; however, can be abundantly expressed following interleukin-2 (IL-2)-mediated activation[5]. Murine CD137 recruits the PTK $p56^{lck}$ [6]; yet, its functional relevance is not known. When T cells are activated through TCR-CD3 complexes and co-stimulated via CD137, recruitment of TRAF1 and TRAF2 to these acidic clusters is essential for the activation of Erk1/2[7], Jnk1/2, p38[8] and NF-κB. Irrespective of these observations, the identities of signaling molecules downstream of NKG2D or CD137 that exclusively regulate inflammatory cytokine production remain elusive.

Here we demonstrate Lck, Fyn, PI(3)K-p85α-p110δ, and PLC-γ2 were required for both cytotoxicity and inflammatory cytokine production by NKG2D and CD137. However, a unique interaction between Fyn and Adhesion and Degranulation-promoting Adaptor Protein, ADAP (also known as Fyn-binding protein, Fyb or SLAP-130) that links upstream signaling to Carma1 (also known as Card11) and MAP3K7 (also known as TAK1) was exclusively responsible for inflammatory cytokine and chemokine production but not for cytotoxicity in murine and human NK cells. Our results provide a molecular blueprint for targeting unique signaling molecules to reduce the levels of inflammatory cytokines in a wide range of autoimmune diseases and cell-mediated immunotherapy.

RESULTS

Inflammatory cytokine production from NK cells

To determine the role of NKG2D and CD137 in inflammatory cytokine production, we generated stable EL4 cell lines expressing NK cell activatory ligands H60 or CD137L. We

established two stable EL4 cell lines with a low (EL4-H60^{lo}) or high (EL4-H60^{hi}) surface expression of H60[9] (Fig. 1a). Additionally, we generated two clones, EL4-CD137L^{lo} and EL4-CD137L^{hi} with differing amounts of CD137L expression. NK cells derived from wild-type (WT) mice mediated significantly increased cytotoxicity against both H60^{+} and CD137L^{+} targets compared to parental EL4; as expected, EL4 targets with higher amounts of ligand expression were lysed to a greater extent (Fig. 1b). NK cells constitutively express NKG2D; however, expression of CD137 is inducible. *Ex vivo* analyses of splenic NK cells from C57BL/6 (WT) mice revealed only a basal level of CD137 expression. However, culturing with IL-2 induced expression of CD137 in the majority of NK cells (Supplementary Fig. 1a, b). IL-2 (or IL-15) along with IL-12 alone or in combination with IL-18 induced the expression of CD137 in NK cells (Supplementary Fig. 1c). Infection of WT mice with mouse-adapted human influenza virus strain, A/PR/8/34 (PR8, H1N1), increased the *in vivo* expression of CD137 in the lung NK cells that was comparable to that of T cells (Supplementary Fig. 1d).

To confirm the potential of CD137 as an independent activation receptor, we stimulated a mixture of IL-2-cultured NK and T cells with plate-bound anti-CD137, anti-CD3 or in combination, and analyzed production of interferon-γ (IFN-γ) by intracellular staining. Activation via CD3 but not CD137 stimulated the production of IFN-γ in CD3^{+} T cells. In contrast, CD137 but not CD3 induced the production of IFN-γ in CD3^{-}NK1.1^{+} NK cells. As expected, co-stimulation of T cells via CD137 along with CD3 considerably enhanced the production of IFN-γ (Fig. 1c). These results reveal that CD137 functions as an independent activation receptor in NK cells while confirming earlier findings that it is a co-stimulatory receptor in T cells[10]. We co-cultured the H60^{+} or CD137L^{+} target cells with WT NK cells and analyzed their culture supernatants for secretion of the proinflammatory cytokines and chemokines IFN-γ, GM-CSF, CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES). Co-culturing NK cells with EL4-H60^{hi} or EL4-CD137L^{hi} targets but not EL4 resulted in significant increases in these cytokines (Fig. 1d). Parental EL4 cells and the stable transfectants expressed comparable amounts of the MHC class I molecules, H2-K^{b} and H2-D^{b} (data not shown), suggesting that CD137L expression, rather than a preferential loss of MHC class I, was responsible for the efficient lysis of CD137L^{+} targets and production of cytokines by NK cells. Activation of NK cells via NKG2D and CD137 using plate-bound mAbs resulted in abundant phosphorylation of Akt, MEK1/2, Erk1/2, p38, and Jnk1/2 (Supplementary Fig. 2a). These results demonstrate that activation of NK cells via NKG2D or CD137 results in cytotoxicity and proinflammatory cytokine production.

NK cell cytotoxicity and cytokine production require Lck

Activation of Lck constitutes one of the earliest membrane proximal signaling events. Therefore, we first determined the association of Lck with NKG2D and CD137 in NK cells. IL-2-cultured, non-activated NK cells were lysed and immunoprecipitated with anti-NKG2D or anti-CD137 mAbs and analyzed for the presence of Lck (Fig. 2a). Immunoprecipitation revealed the constitutive association of Lck with CD137. Absence of Lck in the immunoprecipitate with anti-NKG2D mAb revealed specific association of Lck with CD137, which can be explained by the presence of the Lck-binding motif (CRCP)[6] in its cytoplasmic tail. Fyn was present in both NKG2D and CD137 immunoprecipitations, albeit

at a higher amount with CD137 (Fig. 2a). Pre-incubation of WT NK cells with Lck-specific inhibitor, C8863 significantly reduced the secretion of IFN-γ, following NKG2D- or CD137- but not IL-12 and IL-18-mediated activation that utilizes a Jak-Tyk-based activation pathway (Fig. 2b). To further confirm its role, we used Lck-specific siRNA to knockdown its expression in IL-2-cultured NK cells. Lck-specific but not scrambled siRNA reduced the expression of Lck (Fig. 2c). Knockdown of Lck significantly reduced the ability of NK cells to mediate cytotoxicity against $H60^+$ and $CD137L^+$ stable cell lines (Fig. 2d). Importantly, reduction in the expression of Lck also significantly reduced the NKG2D- or CD137-mediated cytokine and chemokine production (Fig. 2e). Moreover, knockdown of Lck did not alter the ability of NK cells to produce inflammatory cytokines in response to IL-12 and IL-18 (Fig. 2e). Collectively, these results provide definitive evidence that Lck is critical for both NK cell-mediated cytotoxicity and cytokine production.

Fyn regulates cytotoxicity and cytokine production

Lck forms a complex with and transphosphorylates Fyn in the lipid raft[11]. To define the role of Fyn with regards to Lck, we stimulated NK cells with plate-bound anti-NKG2D or anti-CD137 monoclonal antibodies (mAbs). Upon these activations, phosphorylation of Fyn was increased temporally (Fig. 3a). To further understand its role, we used NK cells from $Fyn^{-/-}$ mice (Supplementary Fig. 2b). IL-2-cultured NK cells from WT and $Fyn^{-/-}$ mice expressed comparable amounts of surface NKG2D and CD137 (Supplementary Fig. 2c). Irrespective of this, cytotoxicity of NK cells from $Fyn^{-/-}$ mice to EL4-$H60^{lo}$ and $CD137L^+$ target cells was significantly reduced (Fig. 3b). However, a similar reduction in cytotoxicity was not observed with EL4-$H60^{hi}$ targets (Fig. 3b).

Activation with plate-bound anti-NKG2D or anti-CD137 mAbs resulted in significantly augmented inflammatory cytokine production in $Fyn^{-/-}$ NK cells (Fig. 3c). This was not due to a generalized hyper-responsiveness of $Fyn^{-/-}$ NK cells, since IL-12 and IL-18-mediated activation resulted in comparable concentration of secreted cytokines and chemokines (Fig. 3c). However, co-culturing $Fyn^{-/-}$ NK cells with EL4-$H60^{hi}$ or EL4-$CD137L^{hi}$ targets resulted in significantly reduced amounts of cytokine and chemokine secretion compared to that of WT (Fig. 3d). This discrepancy between co-culture and plate-bound antibody-mediated activations was only seen with $Fyn^{-/-}$ but not with other knockout NK cells used in this study. Analyses of the translocation of AP-1 and NF-κB in WT and $Fyn^{-/-}$ NK cells following NKG2D- or CD137-mediated activation revealed a substantial failure of c-Jun and p65 nuclear translocation. Although the basal amount of c-Fos in the nucleus was more in NK cells from $Fyn^{-/-}$ compared to WT, its activation-induced nuclear translocation appeared unchanged (Supplementary Fig. 2d). These results demonstrate that Fyn plays a dual role in linking membrane proximal signals to cytotoxic granule release and cytokine production in NK cells.

Lck-Fyn complex links to PI(3)K-p85α-p110δ

The SH3 domains of Lck and Fyn interact with the proline-rich region (PRR) of the regulatory subunit of PI(3)K, p85α[12]. Two PRRs, KRISPPTPKPRPPRPLPVAP and WNEROPAPALPPKPPKPT, between the N-terminal SH3 and SH2 domains of PI(3)K-p85α, have been defined as high affinity binding sites for Lck and Fyn[12]. Therefore, we next

investigated the role of PI(3)K downstream of Lck and Fyn. IL-2-cultured NK cells were activated with anti-NKG2D or anti-CD137 mAbs, lysed, and immunoprecipitated with anti-Lck and anti-Fyn mAbs and analyzed for the presence of PI(3)K-p85α. Our results reveal that activation via CD137 but not NKG2D augmented the recruitment of p85α by Lck. Conversely, the association between p85α and Fyn following NKG2D- or CD137-mediated activation was distinct and strong (Fig. 4a). A reciprocal immunoprecipitation where PI(3)K-p85α was immunoprecipitated and analyzed for its interaction with Lck and Fyn revealed a strong association between Fyn and PI(3)K-p85α after activation through CD137 compared to NKG2D (Supplementary Fig. 3a). Irrespective of this difference, activation via both the receptors resulted in the increased phosphorylation of PI(3)K-p85α (Fig. 4b). However, CD137-mediated PI(3)K-p85α phosphorylation peaked at an earlier time point (5 min) compared to that of NKG2D (20 min).

Successful activation of PI(3)K-p85α facilitates the recruitment of the catalytic PI(3)K-p110δ subunit[13]. Therefore, to confirm the function of PI(3)K, we used gene knockin mice that express a catalytically inactive PI(3)K-p110δ subunit due to a point mutation (*p110δ* D910A/D910A). This mutation, $Asp^{910}\rightarrow$Ala (D910A), produces a complete loss-of-function locus but retains normal amounts of p110δ protein. NKG2D and CD137 expressions were comparable between WT and *p110δ* (D910A) NK cells (Supplementary Fig. 3b); however, lack of functional p110δ significantly reduced the ability of NK cells to lyse both $H60^+$ and $CD137L^+$ targets (Fig. 4c). Next, we analyzed the production of inflammatory cytokines and chemokines. *p110δ* (D910A) NK cells produced significantly less amounts of cytokines and chemokines after NKG2D- or CD137-mediated activation compared to WT (Fig. 4d). To test whether this reduction is due to a generalized hyporesponsiveness, we activated NK cells with IL-12 and IL-18, which induced comparable levels of cytokines and chemokines (Fig. 4d). These findings show that Lck and Fyn recruit PI(3)K-p85α and help to form PI(3)K-p85α-p110δ complex, which regulates both NK cell-mediated cytotoxicity and cytokine production.

NKG2D and CD137 require LAT

PI(3)K activation produces phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which is dephosphorylated to phosphatidylinositol 4,5-biphosphate (PIP_2) by PTEN and SHIP. PIP_2 serves as a substrate for PLC-γ2. Phosphorylation of PLC-γ requires molecular complex comprising the adaptor protein, SLP-76; tyrosine protein kinase, Itk; and the scaffolding protein LAT[14]. We found abundant phosphorylation of PLC-γ2 following NKG2D- or CD137-mediated activation (Fig. 4e). Further, phosphorylation of PKC-θ by diacylglycerol (DAG), a byproduct of PIP_2 hydrolysis by PLC-γ2, was also upregulated (Fig. 4f). Preincubation of WT NK cells with PLC-γ2 (U73122) or PKC (RO-31-8220) inhibitors significantly reduced IFN-γ production, confirming their requisite role in cytokine production (Supplementary Fig. 3c). To determine the role of LAT, NK cells were transfected with specific siRNA, which reduced the quantity of LAT protein (Supplementary Fig. 4a). Activation of specific siRNA-transfected NK cells via NKG2D or CD137 revealed that LAT is more important for cytokine and chemokine production than cytotoxicity (Supplementary Fig. 4b, c).

ADAP connects to CBM signalosome

The Fyn-binding protein ADAP interacts with the C-terminal SH3-MAGUK region of Carma1 and thereby to Bcl10-Malt1[15]. Therefore, we investigated the role of Fyn-ADAP complex in recruiting Carma1 downstream of NKG2D or CD137. NK cells from WT mice were activated with plate-bound anti-NKG2D or anti-CD137 mAbs and Lck was immunoprecipitated and analyzed for Fyn or ADAP (Supplementary Fig. 5a). Although basal amounts of Fyn protein were found in association with Lck in unstimulated NK cells, its quantity was considerably increased following activation. A corresponding increase was also seen with ADAP, indicating that an increase in Fyn association to CD137-Lck leads to increased recruitment of ADAP. This was further validated by the impaired ability of Lck to form a signaling complex with ADAP, and hence with Carma1in *Fyn*$^{-/-}$ NK cells (Supplementary Fig. 5b).

Next, Fyn was immunoprecipitated to further confirm recruitment of ADAP. Lysates from unstimulated WT NK cells contained considerable amounts of preformed Fyn and ADAP complexes Supplementary Fig. 5c. However, activation via NKG2D or CD137 augmented the association between Fyn and ADAP. Carma1 was also present in the preformed Fyn-ADAP complexes in the lysates from unstimulated NK cells. Both NKG2D- and CD137-mediated activation increased the amounts of Carma1 protein association to Fyn-ADAP complexes (Supplementary Fig. 5c). These observations were further confirmed by activation via the receptor NCR1, which exclusively uses ITAM-dependent activation (Supplementary Fig. 5d). Next, we immunoprecipitated Carma1 from NK cell lysates and analyzed for its association with ADAP or PKC-θ. Activation via NKG2D or CD137 increased the association of ADAP to Carma1, compared to unstimulated conditions (Supplementary Fig. 5e). Association between PKC-θ and Carma1 increased after either NKG2D- or CD137-mediated activations. This did not require a physical association with PLC-γ2 (Supplementary Fig. 5e). These results indicate that Lck and Fyn can facilitate the activation of Carma1by recruiting ADAP into their complex.

ADAP regulates inflammatory cytokine production

To validate the functional relevance of the above biochemical findings, we used *Adap* deficient mice (also known as *Fyb*) [16]. Expression of NKG2D and CD137 in NK cells was comparable between WT and *Adap*$^{-/-}$ mice (Supplementary Fig. 5e). NK cells from both WT and *Adap*$^{-/-}$ mice were fully capable of mediating potent cytotoxicity against H60^{+} and CD137L^{+}targets (Fig. 5a). However, both NKG2D- and CD137-mediated activations resulted in significantly reduced amounts of IFN-γ, GM-CSF, CCL3, CCL4, and CCL5 (Fig. 5b). These results reveal that ADAP plays an exclusive role in regulating inflammatory cytokine production. Despite a comparable amount of IFN-γ, GM-CSF, and CCL5 following IL-12 and IL-18-mediated activation of *Adap*$^{-/-}$ NK cells, there was a significant reduction in CCL3 and CCL4 production (Fig. 5b).

To determine that the impairment in cytokine production is NK cell intrinsic and not due to developmental defects, we generated mixed bone marrow chimeras. Equal numbers of bone marrow cells from WT (CD45.1^{+}B6.SJL) and *Adap*$^{-/-}$ (CD45.2^{+}C57BL/6) mice were transferred into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ double knockout mice (Supplementary Fig. 6a). Six to

eight weeks later, fresh splenic or IL-2-cultured NK cells were analyzed for their ability to mediate cytotoxicity (CD107a positivity) or cytokine (IFN-γ) production. There were comparable amounts of CD107a expression between WT and *Adap*$^{-/-}$ NK cells upon NKG2D- or CD137-mediated activation. However, there was a moderate but significant reduction in CD107a^{+} NK cells that lack ADAP when activated via Ly49D receptor similar to a recent finding[17] (Supplementary Fig. 6b). Irrespective of these differences, the percentages of intracellular IFN-γ^{+} cells within *Adap*$^{-/-}$ CD45.2^{+} NK population were significantly reduced compared to that of WT CD45.1^{+} NK cells. Since freshly isolated NK cells express only a minimal amount of surface CD137 (Supplementary Fig. 1a), we did not observe any significant decrease in IFN-γ^{+} cells following anti-CD137 mAb-mediated activation. As expected, this reduction was much more pronounced in IL-2-cultured NK cells (Supplementary Fig. 6c). These results strongly suggest that the impairment in cytokine production caused by the lack of ADAP is cell intrinsic.

To determine whether the defect is at the transcriptional level, we quantified the amounts of *Ifnγ* mRNA after NKG2D- or CD137-mediated activation by RT-qPCR. There was significantly reduced copy number of *Ifng* mRNA in *Adap*$^{-/-}$ NK cells, confirming a defect at the transcriptional level (Fig. 5c). We investigated the activation of AP-1 and NF-κB to determine the molecular mechanism by which ADAP regulates cytokine and chemokine gene transcriptions. Lack of ADAP considerably reduced the nuclear translocation of c-Fos, c-Jun, and the p65 subunit of NF-κB (Fig. 5d). These findings confirm that ADAP plays a critical role in linking Lck-Fyn to CBM signalosome-mediated AP-1 and NF-κB activations.

CBM signalosome and cytokine production

To determine the role of the CBM complex, we first investigated the expression of NKG2D and CD137 in IL-2-cultured NK cells from WT, mice that specifically lack the N-terminal CARD domain of Carma1 (*Carma1*(ΔCARD)), and *Carma1*$^{-/-}$. Lack of a CARD domain in Carma1 interrupts the recruitment of Bcl10 to the CBM signalosome[18]. Our results show that lack of a CARD domain or the full-length Carma1 did not affect the surface expression of NKG2D or CD137 (Supplementary Fig. 6d). *Carma1*(ΔCARD) or *Carma1*$^{-/-}$ mice-derived NK cells consistently showed moderately reduced abilities to lyse H60^{+} or CD137L^{+}targets compared to WT NK cells (Fig. 6a). However, *Carma1*(ΔCARD) or *Carma1*$^{-/-}$ NK cells were significantly impaired in their ability to produce inflammatory cytokines (Fig. 6b).

The crucial role played by Carma1 and ADAP is further confirmed by co-culturing NK cells with H60^{+} and CD137L^{+} target cells. Analyses of culture supernatants from *Carma1*$^{-/-}$ and *Adap*$^{-/-}$ NK cells revealed a significant reduction in the production of cytokines and chemokines compared to that of WT (Supplementary Fig. 7). Stimulation of WT, *Carma1*(ΔCARD), and *Carma1*$^{-/-}$-derived NK cells with IL-12 and IL-18 revealed that their responses were comparable (Fig. 6b). Quantification of *Ifng*-encoding mRNA showed significantly less copy numbers in *Carma1*(ΔCARD) and *Carma1*$^{-/-}$ NK cells (Fig. 6c). Mixed bone marrow chimera experiments further confirmed that the reduction in IFN-γ production is NK cell intrinsic (Fig. 6d).

We next analyzed the phosphorylation of PLC-γ2 and MAP3K7 downstream of CD137 (Supplementary Fig. 8a) and NKG2D (data not shown). Lack of Carma1 or the CARD domain of Carma1 did not affect the phosphorylation of PLC-γ2; however, it considerably reduced the CD137-mediated phosphorylation of MAP3K7 (Supplementary Fig. 8a). Among MAPKs, phosphorylation of Erk1/2 and Jnk1/2 were considerably reduced in *Carma1*(ΔCARD) and *Carma1*$^{-/-}$-derived NK cells compared to that of WT. Activation of p38 was not altered (Supplementary Fig. 8a). Phosphorylation of IκBα following CD137-mediated activation was reduced in NK cells from *Carma1*(ΔCARD) and *Carma1*$^{-/-}$ mice indicating an inefficient activation of NF-κB. Further, analyses of c-Fos, c-Jun, and NF-κB p65 in nuclear extracts following NKG2D or CD137-mediated activation revealed that their activation and subsequent nuclear translocations were substantially reduced in *Carma1*$^{-/-}$ compared to WT-derived NK cells (Supplementary Fig. 8b). These results demonstrate that the CBM signalosome plays a moderate and significant role in cytotoxicity and inflammatory cytokine production, respectively.

MAP3K7 links CBM signalosome to cytokine production

To further confirm the role of MAP3K7 (TAK1), we used a conditional knockdown model[19]. Splenic NK cells from poly (I:C)-treated floxed *Map3k7*$^{fx/fx}$ or *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ mice were cultured with IL-2. As expected, MAP3K7 protein expression was considerably reduced in poly (I:C)-treated NK cells obtained from *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ compared to poly (I:C)-treated *Map3k7*$^{fx/fx}$ mice (Fig. 7a). MAP3K7 knockdown resulted in a moderately decreased CD137-mediated cytotoxicity compared to poly (I:C)-treated *Map3k7*$^{fx/fx}$ or untreated *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ mice (Fig. 7b). *Map3k7* knockdown significantly reduced the production of cytokines and chemokines (Fig. 7c). Stimulation of NK cells from poly (I:C)-treated *Map3k7*$^{fx/fx}$ or *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ mice with IL-12 and IL-18 resulted in comparable levels of cytokines and chemokines (Fig. 7c). Collectively, these results demonstrate that MAP3K7 regulates CD137-mediated cytotoxicity and inflammatory cytokine production.

ADAP regulates cytokine production in human NK cells

To determine the role of ADAP in the effector functions of human NK cells, we knocked down its expression using specific siRNA. Human NK cells were isolated from the peripheral blood mononuclear cells (PBMC) of healthy individuals (Fig. 8a), and following siRNA transfection tested for their cytotoxic potential and production of inflammatory cytokines. Use of ADAP-specific siRNA reduced (>40%) the quantity of ADAP protein (Fig. 8b). Knockdown of ADAP did not alter the NK cell-mediated cytotoxicity against K562 targets (Fig. 8c). siRNA-transfected NK cells were also activated with anti-NKG2D and anti-CD137 mAb. Comparable amounts of CD107a expression between scrambled or ADAP siRNA-treated NK cells following anti-NKG2D mAb-mediated activation confirmed that ADAP does not play a critical role in cytotoxicity (Fig. 8c). However, knockdown of ADAP significantly reduced the production of IFN-γ, GM-CSF, CCL3, CCL4, and CCL5 in primary human NK cells (Fig. 8d). Since human CD137 lacks the Lck-recruiting CRCP motif, activation with anti-CD137 mAb did not result in detectable cytotoxicity or cytokine production (data not shown), as also reported earlier[20]. These results confirm that ADAP plays similar function in murine and human NK cells.

DISCUSSION

Activation of NK cells results in target cell lysis and production of inflammatory cytokines. However, the exclusive signaling requirement for each of these effector functions has not been determined. Our current findings demonstrate that an ADAP-mediated signaling cascade that includes a Carma1-based CBM signalosome plays an exclusive and obligatory role in inflammatory cytokine production.

Target cell lysis and the production of inflammatory cytokines via NKG2D and CD137 required Lck and Fyn. We found that CD137, but not NKG2D, constitutively associates with Lck potentially using a 'CRCP' motif[6], while both of them associate with Fyn. This is further validated by an increase in Fyn phosphorylation following NKG2D- or CD137-mediated activations. Activation of Fyn is possibly mediated by Lck, which has been found to be juxtaposed to Fyn in the lipid raft, leading to the trans-phosphorylation of its catalytic domain[21].

Fyn$^{-/-}$ NK cells were impaired in their ability to mediate cytotoxicity against EL4-H60^{lo}, EL4-CD137L^{lo}, or EL4-CD137L^{hi} cells, similar to other target cells reported earlier[22]. However, a higher amount of H60 expression in EL4-H60^{hi} provided a signaling threshold that overcame this impairment. Other Src kinases such as Yes, Lyn, Hck, c-Fgr, and Src are expressed in NK cells[23, 24] and whether an increased activation threshold makes Lck transphosphorylate any of these Src kinases has yet to be determined. When *Fyn*$^{-/-}$ NK cells were co-cultured with EL4-H60^{hi} or EL4-CD137L^{hi} targets, they produced significantly reduced amounts of cytokines, which corroborated earlier observations[22]. However, activation of *Fyn*$^{-/-}$ NK cells with anti-NKG2D or anti-CD137 mAbs significantly augmented the production of inflammatory cytokines. These findings are in line with earlier reports where the cytokine production was either augmented[25] or remained unchanged[26] in immune cells derived from *Fyn*$^{-/-}$ mice. It is also important to note that a difference in cytokine production between co-culture and plate-bound antibody-mediated activation was observed only with *Fyn*$^{-/-}$ NK cells. None of the other knockout NK cells tested showed such a discrepancy.

NKG2D can directly recruit PI(3)K-p85α via DAP10[27]. Unlike NKG2D-DAP10, CD137 does not contain a YXNM motif. However, CD137-bound Lck, along with activated Fyn, can recruit PI(3)K-p85α[28, 29]. Thus, the recruitment of PI(3)K-p85α by Fyn[30] or by Lck-Fyn[28] can facilitate an independent activation via CD137. This is supported by our study as activation through CD137 resulted in increased interaction between Lck and Fyn and phosphorylation of Fyn. Immunoprecipitation of Fyn revealed its association with PI(3)K-p85α. An interesting possibility is that Fyn, and to a lesser extent Lck, are interacting with the two stretches of proline rich regions in PI(3)K-p85α via their SH3 domains[28]. PI(3)K-p110δ is the major isoform required downstream of NKG2D in NK cells[31,32]. *PI(3)K-p110δ* (D910A) mice-derived NK cells had a significant reduction in cytotoxic potentials and in the production of inflammatory cytokines. PI(3)K activates PDK1 that plays an important role in the phosphorylation of PKC-θ, which phosphorylates Carma1 and thereby linking it to the CBM signalosome[33]. Our earlier findings demonstrated that lack of PLC-γ2 that depends on PI(3)K for its substrate, phosphatidylinositol 4,5-bisphosphate, significantly reduced both

cytokine production and cytotoxic potentials of NK cells[34]. Thus, the recruitment of PI(3)K-p85α-p110δ to NKG2D-DAP10 or CD137-Lck (and Fyn) constitutes an important step required for NK cell-mediated effector functions.

Fyn also associates with the adaptor protein, ADAP[35]. NKG2D or CD137-mediated activation increased the interaction between Fyn and ADAP. This is of importance since ADAP can directly interact with either Carma1[15] or MAP3K7[36]. A region between amino acids 426 and 541 of ADAP interacts with the C-terminus of Carma1[15]. A highly conserved C-terminus domain of ADAP between amino acids 691 and 708 was responsible for its interaction with MAP3K7[36]. In our study, lack of ADAP did not alter NK cell cytotoxicity, which is in line with an earlier study that used RMA/S cells[37]. This could be due to the fact that the lack of ADAP did not alter the phosphorylation of Erk1/2 and Jnk1/2[15, 16], which plays a critical role in cytotoxic granule mobilization[38, 39]. However, the production of inflammatory cytokines was significantly reduced in *Adap*$^{-/-}$ NK cells indicating the exclusive requirement of ADAP for their gene transcriptions. The reverse was true in *Vav1*$^{-/-}$, where cytotoxicity but not inflammatory cytokine production was significantly impaired[40]. A considerable reduction in the nuclear translocation of c-Fos, c-Jun, and NF-κB p65 confirms that ADAP is an obligatory linker of upstream signals to transcriptional regulation of cytokine genes. Although Fyn is directly upstream of ADAP, *Fyn*$^{-/-}$ NK cells were impaired in both cytotoxicity and cytokine production. This could be due the dual function played by Fyn in both recruiting PI(3)K-p85α for Erk1/2 activation and ADAP that links to the CBM signalosome.

The requirement of the CBM signalosome for TCR-CD28[41] in the T cell, BCR[18] in the B cell, NKG2D in the NK cell[42] has been well established. Our study shows that PKC-θ is present in a complex along with Lck, Fyn, and ADAP, and Carma1 was recruited by ADAP upon activation. Lack of CARD domain of Carma1, full-length Carma1, or MAP3K7 moderately reduced the cytotoxicity of NK cells. A reduction in the phosphorylation of Erk1/2 and Jnk1/2 in NK cells from *Carma1*$^{-/-}$ and *Carma1*(ΔCARD) mice could be the cause for this reduction. Jnk1/2, regulated by MAP3K7[43], is critical for microtubule organizing center (MTOC) reorientation, polarization and the exocytosis of perforin-granzyme-containing cytotoxic granules[39]. Thus, our findings imply that the CBM signalosome is at least partly responsible for regulating the cytotoxic function of NK cells. Our present study also confirms the role of ADAP in human NK cells.

We found that unlike cytotoxicity, production of inflammatory cytokines was significantly reduced in NK cells that lack Carma1 or Map3k7. The CBM signalosome regulates activation of NF-κB via IKKα-NEMO(IKKγ) and Jnk2 via MAP3K7[43]. AP-1 is made up of c-Jun and c-Fos and the activation of c-Fos requires Erk1/2[44]. MAP3K7 via Jnk2 regulates the activation of c-Jun[45]. We predict that a PI(3)K-p85α-p110δ-Ras-mediated pathway could be responsible for c-Fos activation and, thereby, AP-1 heterodimer formation and nuclear translocation.

In summary, we demonstrate that downstream of NKG2D and CD137, Lck and Fyn initiate at least two distinct pathways. First, Lck and Fyn recruit PI(3)K-p85α, which is a critical for Ras-GTP-dependent PAK1→C-Raf→MEK1/2→Erk1/2 activation that predominantly plays

a role in cytotoxicity. Second, Fyn binds to ADAP leading to the recruitment of the CBM complex, which is primarily responsible for inflammatory cytokine production. Since ADAP also interacts with Fyn, Carma1 and MAP3K7 in T cells[15, 35], we predict the existence of a conserved signaling pathway. These results for the first time identify potential therapeutic targets to exclusively minimize inflammatory cytokine production without compromising the cytotoxic potentials of NK cells.

METHODS

Mice and stable cell lines

C57BL/6 mice (WT), *Fyn*$^{-/-}$ (B6;129S7-*Fyn*^{tm1sor}/J, B6;129SF2/J were obtained from Jackson Laboratory (Bar Harbor, ME). *PI(3)K-p110δ* (D910A/D910A) (C57BL/6) has been described[46]. *Adap*$^{-/-}$ mice were generated and backcrossed with C57BL/6 mice for at least 6 generations[16]. *Adap*$^{-/-}$ mice were also a kind gift from Dr. Kim E. Nichols, Children's Hospital, Philadelphia, PA. *Carma1*(ΔCARD) and *Carma1*$^{-/-}$ mice (C57BL/6) were described earlier[47,18]. Spleens from interferon-inducible *Map3k7* knockdown mice[48] and *Map3k7*$^{fx/fx}$ mice[19] were a kind gift from Drs Jiwang Zhang and Yechen Xiao, Loyola University Chicago Stritch School of Medicine. All mice were maintained in pathogen-free conditions at the Biological Resource Center (BRC) at the Medical College of Wisconsin (MCW), Milwaukee, WI; and Center for Immunology, University of Minnesota, Minneapolis, MN. Female and male mice between the ages of 6 to 12 weeks were used. All animal protocols and human PBMC usage were approved by the respective institutional IACUC and IRB committees. Interferon-inducible *Map3k7* knockdown *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ mice and control *Map3k7*$^{fx/fx}$ mice were injected with 5 μg/g body weight of poly (I:C) on day 1 and day 3 to induce *Map3k7* knockdown. Spleens of treated mice were collected on day 4[48]. EL4 and K562 cells were purchased from ATCC (Rockville, MD) and maintained in RPMI-1640 medium containing 10% heat-inactivated FBS (Life Technologies, Grand Island, NY). These cell lines were periodically tested to exclude mycoplasma contamination. Generation of *H60*-expressing EL4 stable cell lines has been described[9]. To generate *CD137L*-expressing EL4 stable cell lines, full-length murine cDNA-encoding *CD137L* was amplified using *CD137L*-F: 5′ *cgc gga tcc atg gac cag cac aca ctt gat g* 3′ and *CD137L*-R: 5′ *cta gtc tag aaa aac ata gca gct tga gg* 3′ primers. This cDNA was cloned into pCDNA3 between BamHI and XbaI and used to transfect EL4 cells by electroporation (300 v, 25μF) (GenePulser, BioRad, Richmond, CA). Transfected cells were maintained in RPMI-1640 medium with 10 % FBS. After 12 h, fresh medium containing 10 mg/ml G418 (Cellgro, Manassas, VA) was added and stable cell clones were analyzed for CD137L expression by flow cytometry and PCR. CD137L-positive clones were maintained in 1 mg/ml G418.

Antibodies

Antibodies for NK1.1 (PK136), CD3ε (17A2, 145-2C11), NKG2D (A10), CD137 (17b5), CD137L (TKS-1), anti-human IFN-g (MG1.2), and anti-human CD107a (1D4B) were obtained from e-Bioscience (San Diego, CA). Anti-Ly49D (4E5) was obtained from BD Pharmingen (San Jose, CA). Antibodies for H60a, total JNK1/2 (MAB2076), and NCR1 (MAB2225) were obtained from R&D Systems (Minneapolis, MN). Antibodies for TAK1

(07-263) and β-actin (C4) were obtained from Millipore (Lake Placid, NY). Phospho-TAK1 (Thr184/187, 90C7), phospho-JNK1/2 (T183/Y185, 98-F2), ERK1/2 (137F5), phospho-ERK1/2 (T202/Y204, D13.14.4E), p38 (9212), phospho-p38 (Thr180/Tyr182, 3D7), PKCθ (2059), phospho-PKCθ (Thr538), PLC-γ2 (3872), phospho-PLC-γ2 (Tyr1217, 3871), phospho-PI(3)K-p85α/p55α (Tyr458, Tyr199, 4228), IκBα (L35A5), phospho-IκBα (Ser32, 14D4), NF-κB-p65 (D14E12), c-Fos (9F6), c-Jun (60A8), and Carma1 (1D12) were purchased from Cell Signaling Technologies (Boston, MA). Antibodies for ADAP (07-546) and PI(3)K-p85α (06-497) were obtained from Upstate (Lake Placid, NY). Antibodies for phospho-AKT (Thr308, SC-16646-R), total AKT (c20, SC1618), YY1 (H-10, SC-7341), Fyn (FYN3, SC-16-G), Lck (3A5, SC-433), and Carma1 (SC-20458) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho Fyn (p-Y530, ab53690) was obtained from Abcam (Cambridge, MA). Antibodies to Fyn (Fyn-59), anti-CD45.1 (A20), and CD45.2 (#104), were obtained from BioLegend (San Diego, CA).

NK cell preparation

NK cells were purified as described[35]. Briefly, single cell suspensions from spleen were passed through nylon wool columns to deplete adherent populations consisting of B cells and macrophages. Nylon wool-non-adherent cells were cultured with 1000U/ml of IL-2 (NCI-BRB-Preclinical Repository, Maryland, MD). Purity of the NK cultures was checked and preparations with more than 95 % of $NK1.1^+$ cells were used on day seven.

Transfections

NK cells were transfected with specific or scrambled (NC1) siRNAs (IDT DNA Technologies, Coralville, IA) using Amaxa mouse T cell Nucleofector medium (VZB-1001, Lonza, Basel, Switzerland) and Amaxa 96-well shuttle Nucleofector (Lonza, Basel, Switzerland). Human NK cell nucleofection was performed using the Amaxa human NK Nucleofector kit (VPA-1005, Lonza, Cologne, Germany). Three million IL-2-cultured NK cells were resuspended in 90 μl of Nucleofector solution was electroporated with a final siRNA concentration of 0.02 μM using the Amaxa Nucleofector (Lonza, Cologne, Germany). Murine NK cells were treated with Lck (N001162432.12.3, 5′ *ggu ucu uca aga auc uga gcc gua a* 3′; 3′ *aac caa gaa guu cuu aga cuc ggc auu* 5′) and LAT (N010689.12.1, 5′ *aga auc uac agg agc uua acu gaa a* 3′; 3′ *acu cuu aga ugu ccu cga auu gac uuu* 5′)-specific siRNA (Integrated DNA technologies, Coralville, Iowa, USA). Human NK cells were treated with ADAP-specific or scrambled (NC1) siRNAs, cultured for 16 h in 10% RPMI medium supplemented with 1000 U/ml IL-2 (NCI-BRB-Preclinical Repository, Maryland, MD), and used for functional studies. The nucleotide sequences of the siRNA duplexes are listed in Supplementary Table 1.

Flow cytometry

IL-2-cultured NK cells, stable cell lines or single cell preparations from spleen or lung were stained with fluorescent-labeled monoclonal antibodies (mAbs)in 1% FCS-PBS as described[9]. One million events were analyzed for each sample. Standard flow cytometry analyses were performed in LSR-II and analyzed with FACSDiva software (BD, Franklin Lakes, NJ) or FlowJo (Ashland, OR).

Cytotoxicity assays

NK-mediated cytotoxicity against EL4, EL4 stably expressing H60 or CD137L and K562 was quantified using ^{51}Chromium (^{51}Cr)-release assays at varied Effector : Target ratio (E:T Ratio)[49]. Percent specific lysis was calculated using amounts of absolute, spontaneous and experimental ^{51}Cr-release from target cells.

Co-culture assays. IL-2-cultured NK cells (1×10^5) were cultured with equal numbers of EL4, EL4$^{CD137L\text{-}Hi}$ or EL4$^{H60\text{-}Hi}$ cell lines and cultured in RPMI-1640 medium supplemented with 10 % FBS for 18 h. Culture supernatants were analyzed for cytokines and chemokines using Bioplex assays.

Quantification of cytokines, chemokines

IL-2-cultured, Fc-blocked (anti-CD16/CD32 mAb 2.4G2, BD, Franklin Lakes, NJ) NK cells were activated with plate-bound mitogenic antibodies against CD137 (17B5), NKG2D (A10) or Ly49D (4E5) for 18 h. Culture supernatants were analyzed in a Bioplex assay (Bio-Rad, Richmond, CA). Intracellular IFN-γ was quantified as previously described[50]. Briefly, Fc-blocked NK cells were activated with plate-bound mAbs in the presence of Brefeldin A. After 12 h, cells were stained for surface CD3ε and NK1.1, fixed, permeabilized and stained with PE-cy7-conjugated anti-IFN-γ mAb (XMG1.2). For inhibition assays, NK cells were incubated for 1 h with varying concentrations of C8863 (Lck), U73122 (PLC-γ), and Rottlerin (PKC), washed and added to anti-NKG2D or anti-CD137 mAb-coated plates. After 18 h, IFN-γ was quantified in the culture supernatants by ELISA. Wherever necessary, IL-2-cultured NK cells were treated with IL-12; 1 ng/ml (R&D Systems, Minneapolis, MN) and IL-18; 10 ng/ml (MBL, Des Plaines, IL), and the supernatants were analyzed similarly. For *IFN-γ*-encoding mRNA quantification, NK cells were activated for 6 hours, lysed and total RNA was purified using RNeasy Mini Kit (Qiagen, Valencia, CA). Real-time PCR was performed using SYBR green protocol with an ABI7900 HT thermal cycler. Transcript in each sample was assayed in triplicates and the mean cycle threshold was used to calculate the *x*-fold change and control changes for each gene. Housekeeping gene *GAPDH* was used for global normalization in each experiment. Primer sequences for *IFN-γ* were 5′ *gactgtgattgcggggttgt* 3′ (sense) and 5′ *ggcccggagtgtagacatct* 3′ (anti-sense).

Immunoprecipitation

Unstimulated or plate-bound antibody-activated NK cells were lysed using IP lysis buffer-containing Tris (pH 7.5, 20mM), NaCl (150 mM), EDTA (1 mM), EGTA (1 mM), Triton X100 (1%), sodium pyrophosphate (2.5 mM), beta-glycerophosphate (1 mM), sodium orthovanadate (1 mM), leupeptin (1 ug/ml) and PMSF (1 mM). The lysate was centrifuged to remove debris. For immunoprecipitation, 300–500 μg of the lysate was incubated for 1 h with 2 μg of the indicated antibody at 4° C. 20 μl of Protein G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added and incubated overnight at 4° C. Following centrifugation, the supernatant was aspirated and the beads were washed with the IP lysis buffer. SDS-sample buffer (6X, reducing, BP-111R, Boston BioProducts, Boston, MA) diluted to 1x concentration with the IP lysis buffer was added to the bead pellet and denatured at 95° C for 10 m. The samples were electrophoresed using 10% SDS-PAGE gel.

Immunoblotting

Whole cell lysate (15–20 μg) or nuclear protein extracts (10 μg) isolated using NE-PER reagent (Pierce Inc., Rockford, IL) were resolved using 10% SDS-PAGE gels, transferred to PVDF membranes, and probed with indicated antibodies. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, Massachusetts). The band intensities of phospho-protein were normalized against the respective total protein. The fold changes in phosphorylation following 5, 20, or 60 min of activation were calculated using these normalized values. For the immunoprecipitation experiments, the fold induction of the protein following activation was calculated based on the basal level of that protein in the unstimulated condition, after normalizing with the protein that was immunoprecipitated using ImageJ software.

Adoptive transfers

Bone marrow cells were isolated from CD45.1$^+$ B6.SJL (4007, Taconic, Germantown, NY), CD45.2$^+$ *Adap*$^{-/-}$ and CD45.2$^+$ *Carma1*$^{-/-}$ mice. B10;B6-*Rag2*^{tm1Fwa}*Il2r*γ^{tm1Wjl} (*Rag2*$^{-/-}/_c\gamma^{-/-}$), (4111, Taconic, Germantown, NY) were used as recipients. 1×10^6 bone marrow cells from the CD45.1$^+$ WT and CD45.2$^+$ mice were mixed and retro-orbitally injected into the sub-lethally irradiated (800 cGy) *Rag2*$^{-/-}/_c\gamma^{-/-}$ mice. After four weeks, NK cells from the spleen of these reconstituted mice were enriched through nylon wool as previously described[34] and were either cultured overnight or for 7 days with 1000 U/ml IL-2. These NK cells were activated with plate-bound anti-NKG2D, anti-CD137 or anti-Ly49D mAbs and analyzed by flow cytometry for CD107a surface expression or for intracellular IFN-γ. Donor origin or NK cells were determined based on the cell surface expression of CD45.1 or CD45.2. Percent CD107a$^+$ and IFN-γ^+ NK cells from the CD45.1$^+$ WT and CD45.2$^+$/knockout NK cells following plate-bound antibody-mediated activation were compared.

Human NK cell isolation, activation and functional analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from 25–30 ml of human buffy coat obtained from the donor center at the BloodCenter of Wisconsin with informed consents from all subjects, using Ficoll-Paque™ Plus (17-1440-02, GE Healthcare, Uppsala, Sweden). All samples were identified only with their respective unique patient number (UPN). NK cells were negatively selected from peripheral blood mononuclear cells (PBMC), using EasySep™ Human NK Cell Enrichment Kit (19055, Stemcell technologies, Vancouver, Canada). Human NK cells were activated with plate-bound anti-NKG2D (1D11, eBioscience, San Diego, CA) or anti-CD137 (4B4-1, BioLegend, San Diego, CA) antibodies. For analyzing the NK cell degranulation, 100,000 NK cells were activated for 4 h with plate-bound anti-NKG2D or anti-CD137 antibodies in 10% RPMI culture medium containing PE-conjugated anti-human CD107a antibody (H4A3, BioLegend, San Diego, CA). NK cells were stained using anti-human CD56 mAb (#301040, R&D, Minneapolis, MN). Residual T cells were gated out using Alexa fluor 700-conjugated anti-human CD3 mAb (UCHT1, eBioscience, San Diego, CA). For the analyses of cytokine production, 100,000 human NK cells were activated with plate-bound anti-NKG2D or anti-CD137

mAbs for 18 h in 200 μL 10% RPMI culture medium. The quantity of indicated cytokines and chemokines were assayed using a multiplex kit (BioRad, Richmond, CA).

Experimental data and statistical analysis

Total sample numbers were determined based on previous studies that used similar transgenic mouse models with comparable functional defects. Randomization method or blinding of investigators to group allocation was not used in this study. Statistical analyses were performed using paired, two sample equal or unequal variance Student's t-test depending on the type of data. *P* values of ≤ 0.05 were considered significant. Normal distribution of sample variance was assumed on the basis of earlier studies with data sets similar to ours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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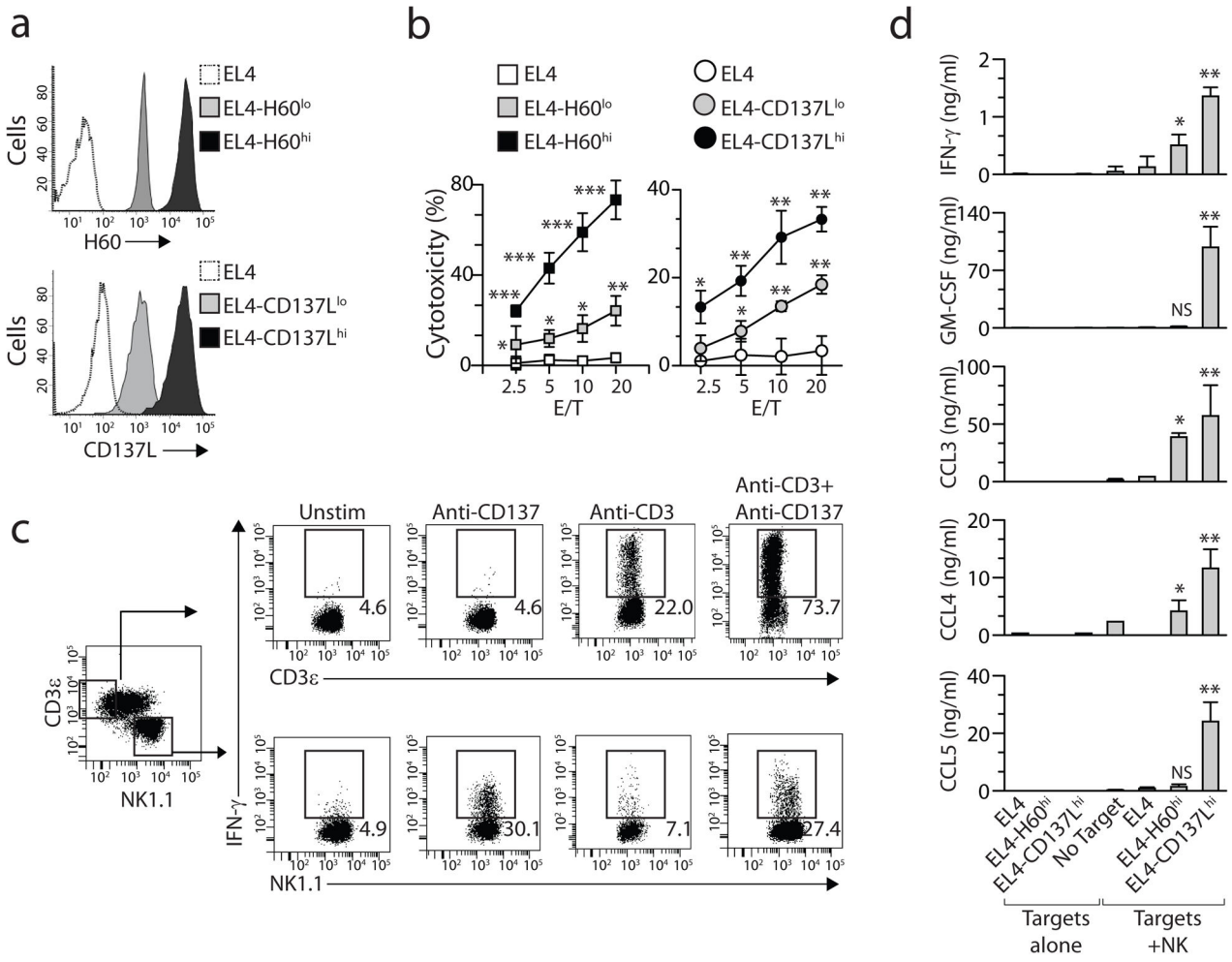

Figure 1. CD137 functions as an independent activation receptor in NK cells

(**a**) Flow cytometry analyses of H60 (top) and CD137L (bottom) expression in stably-transfected EL4 cells. Open histogram: background expression of H60 or CD137L in parental EL4 cells. Grey histogram: ligand expression in EL4-H60^{lo} or EL4-CD137L^{lo}. Black histogram: ligand expression in EL4-H60^{hi} or EL4-CD137L^{hi}. (**b**) Mean percent cytotoxicity with standard deviation of IL-2-cultured WT NK cells following co-culture with indicated target cells. E/T denotes Effector to Target ratio. (**c**) Intracellular IFN-γ staining in IL-2-cultured CD3^{+}NK1.1$^{−}$ T and CD3$^{−}$NK1.1^{+} NK cells from WT mice either left unstimulated or stimulated with plate-bound anti-CD137, anti-CD3 mAb alone or in combination for 12 h. (**d**) Quantitative analyses of IFN-γ, GM-CSF, CCL3, CCL4, and CCL5 from WT NK cells following 18 h co-culture with indicated target cells using bioplex assays. *P<0.05, **P<0.01, ***P<0.001; NS, not significant (Student's t-test; two-tailed, two sample equal variance (**b, d**). Data in **a** is a representative of three independent experiments. Data in **b** and **d** are from five and three WT mice, respectively (mean ± s.d. in **b** and **d**).

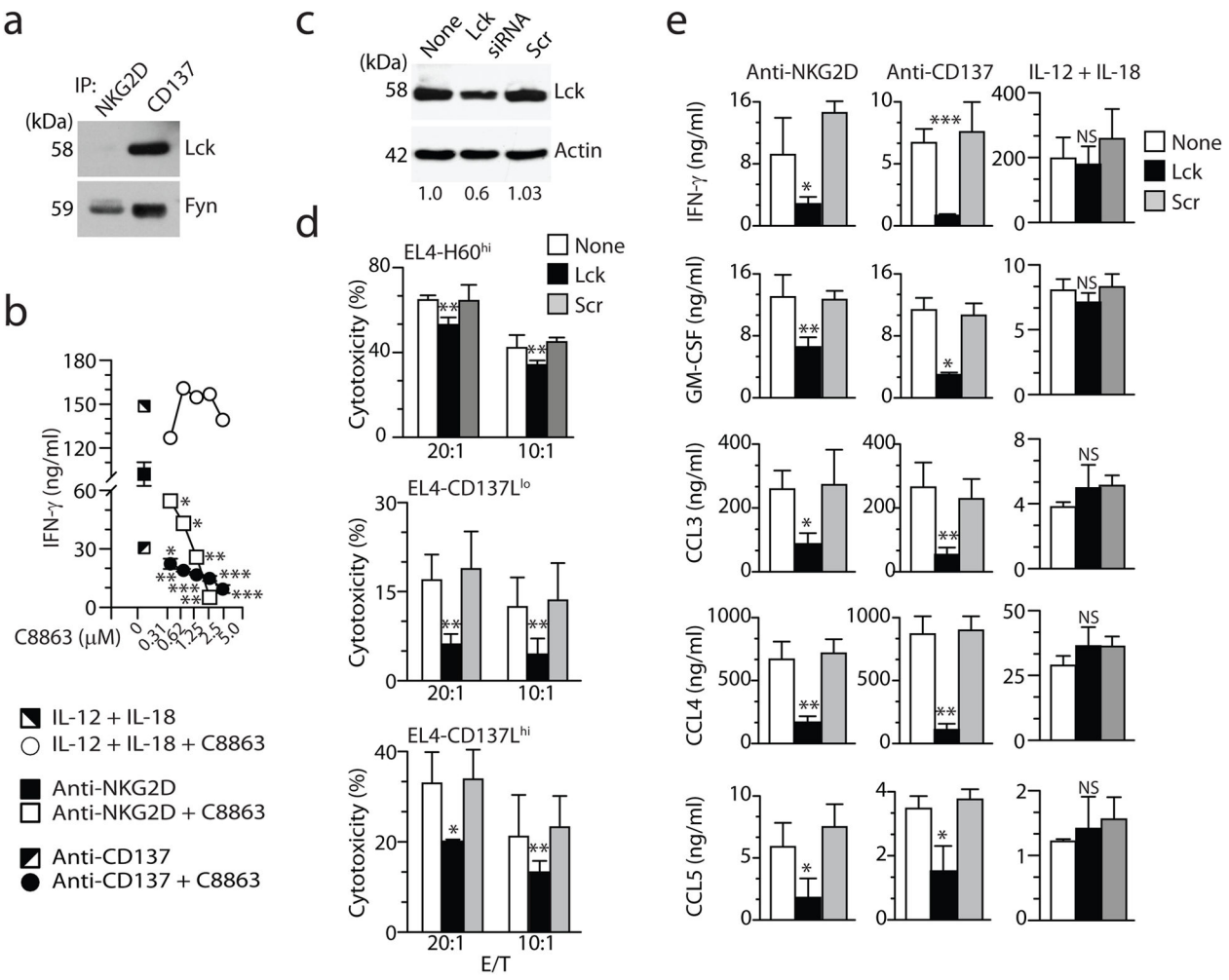

Figure 2. Lck is crucial in CD137-mediated signaling

(**a**) Immunoblot analyses of Lck and Fyn following immunoprecipitation of NKG2D and CD137 receptors in unstimulated, IL-2-expanded WT NK cells. (**b**) Quantitative analysis of IFN-γ production from NK cells after pre-incubation with Lck inhibitor, C8863. Open squares: C8863-treated NK cells with anti-NKG2D mAb activation; filled circles: C8863-treated NK cells with anti-CD137 mAb activation; open circles: C8863-treated NK cells with IL-12 and IL-18 activation. Cytokine production in the vehicle (DMSO)-treated NK cells in response to anti-NKG2D, anti-CD137 mAbs or IL-12 and IL-18 are indicated as closed or half-filled squares. (**c**) Immunoblot for Lck in NK cells following mock, Lck-specific and scrambled siRNA transfections. Fold change in Lck expression was determined by densitometry following normalization with actin. (**d**) Bar diagram represents the mean percent cytotoxicity of NK cells transfected with mock (open), Lck-specific siRNA (black) or scrambled siRNA (grey) against indicated target cells. E/T denotes Effector to Target ratio. (**e**) Quantitative analyses of cytokine and chemokine production, following activation with anti-NKG2D (left), anti-CD137 (middle) mAbs, and IL-12 and IL-18 (right) in WT NK cells transfected with mock, Lck-specific or scrambled siRNA. **P*<0.05, ***P*<0.01, ****P*<0.001; NS, not significant (Student's t-test; two-tailed, two sample equal variance (**b**);

two-tailed, paired (**d, e**). Data in **a** and **c** are representative of three independent experiments. Data in **b**, **d,** and **e** are mean values from three independent experiments consisting of three mice in each (mean ± s.d. in **b**, **d, e**).

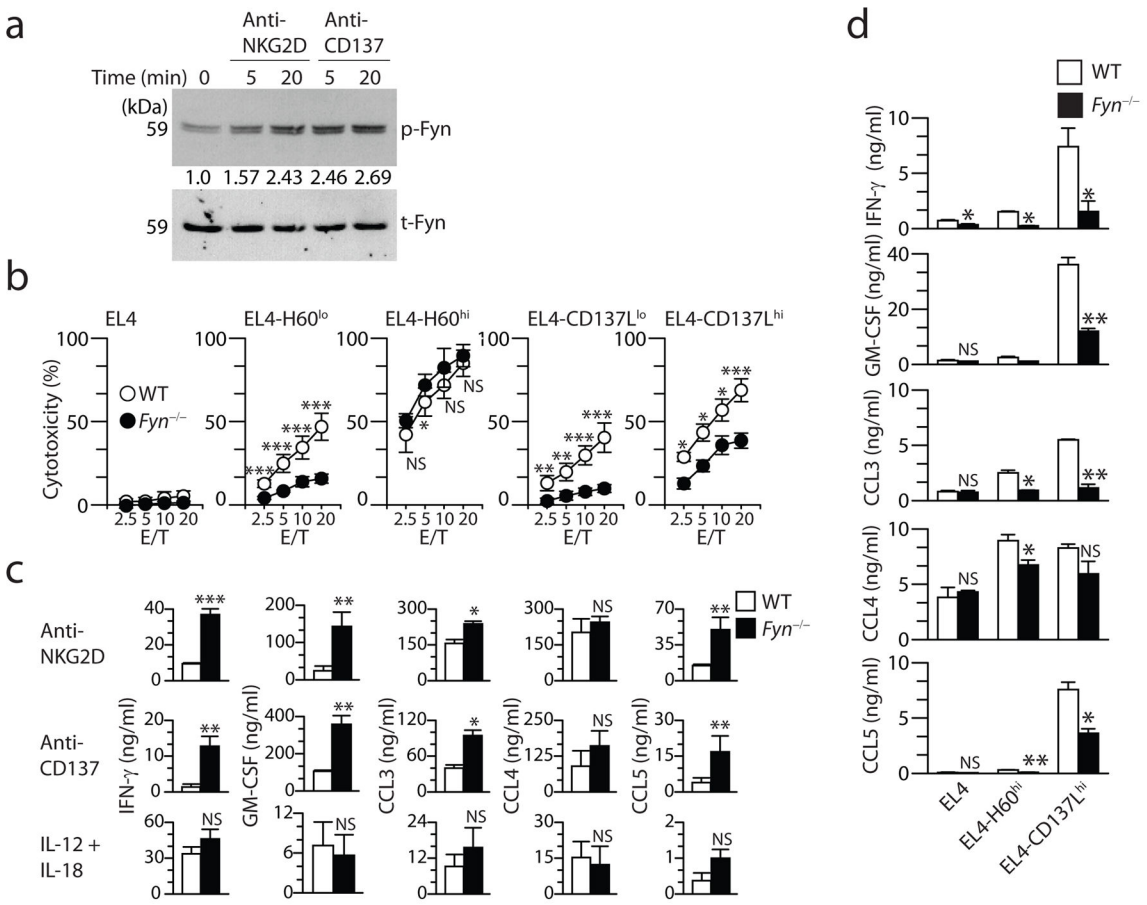

Figure 3. Fyn plays a critical role in mediating NK cell effector functions

(**a**) Immunoblot analyses of phosphorylated (pTyr530) and total Fyn in NK cells that were left unstimulated or stimulated with plate-bound anti-NKG2D or anti-CD137 mAbs. Fold change in tyrosine phosphorylation is shown. (**b**) Cytotoxicity of WT (open circles) and *Fyn*$^{-/-}$ (closed circles) NK cells to H60^{+} and CD137L^{+} target cells. E/T denotes Effector to Target ratio. (**c**) Quantitative analyses of cytokines and chemokines following activation with plate-bound anti-NKG2D or anti-CD137 mAbs or with a combination of IL-12 and IL-18 for 18 h. (**d**) Quantitative analyses of cytokines and chemokines from WT and *Fyn*$^{-/-}$ NK cells following co-culture with EL4, EL4-H60^{hi} or EL4-CD137L^{hi} target cells for 18 h. **P*<0.05, ***P*<0.01, ****P*<0.001; NS, not significant (Student's t-test; two-tailed, two sample equal variance (**b–d**). Data in **a** is a representative of three independent experiments. Data in **b**, **c** and **d** are from seven, five and two mice per genotype from four, four, and two independent experiments, respectively (mean ± s.d. in **b**, **c** and **d**).

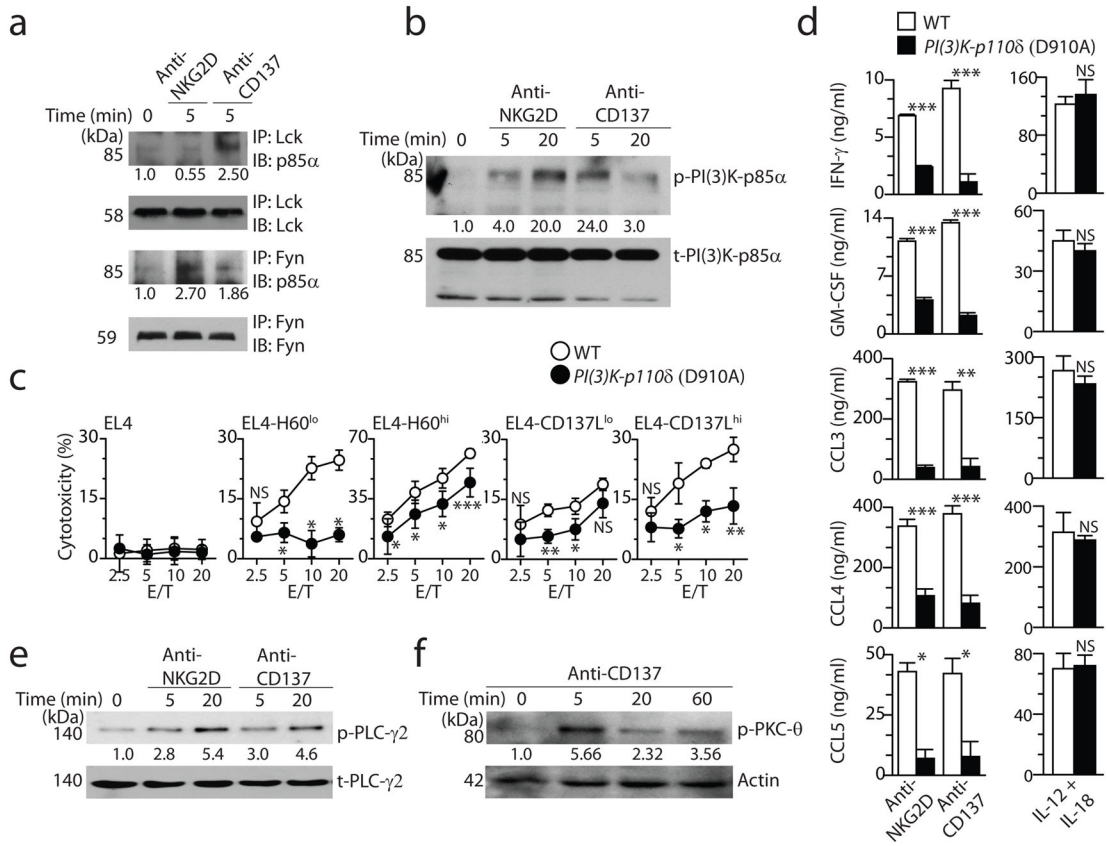

Figure 4. PI(3)K-p85α-p110δ is essential for cytotoxicity and cytokine production in NK cells

(**a**) Whole cell lysates from WT NK cells, unstimulated or activated with plate-bound anti-NKG2D and anti-CD137 mAbs were immunoprecipitated for Lck or Fyn and probed with anti-PI(3)K-p85α antibody. Fyn and Lck were also analyzed following their respective immunoprecipitation. Fold induction was determined by densitometry following normalization to the respective protein that was immunoprecipitated. (**b**) Immunoblot analysis of phosphorylated and total PI(3)K-p85α following activation of WT NK cells with plate-bound anti-NKG2D or anti-CD137 mAb. Fold induction was determined by densitometry, following normalization to total PI(3)K-p85α. (**c**) Mean percent cytotoxicity of NK cells from WT (open) or *PI(3)K-p110δ* (D910A) (filled) against indicated target cells. E/T denotes Effector to Target ratio. (**d**) Quantitative analyses of cytokines and chemokines produced by WT (open) or *PI(3)K-p110δ* (D910A) (filled) NK cells following activation with plate-bound antibodies to NKG2D and CD137 receptors. Cytokines and chemokines produced by NK cells in response to IL-12 and IL-18 stimulation are shown (right). Immunoblot indicating the (**e**) phosphorylated and total PLC-γ2 or (**f**) phosphorylated PKC-θ following activation of WT NK cells. Fold induction in (**e**) and (**f**) was determined by densitometry, following normalization to total PLC-γ2 and actin, respectively. **P*<0.05, ***P*<0.01, ****P*<0.001; NS, not significant (Student's t-test; two-tailed, two sample equal variance (**c, d**). Data in **c** and **d** are from four and five mice per genotype from three independent experiments, respectively (mean ± s.d. in **c** and **d**). Data in **a**, **b**, **e**, **and f** are representative of three independent experiments

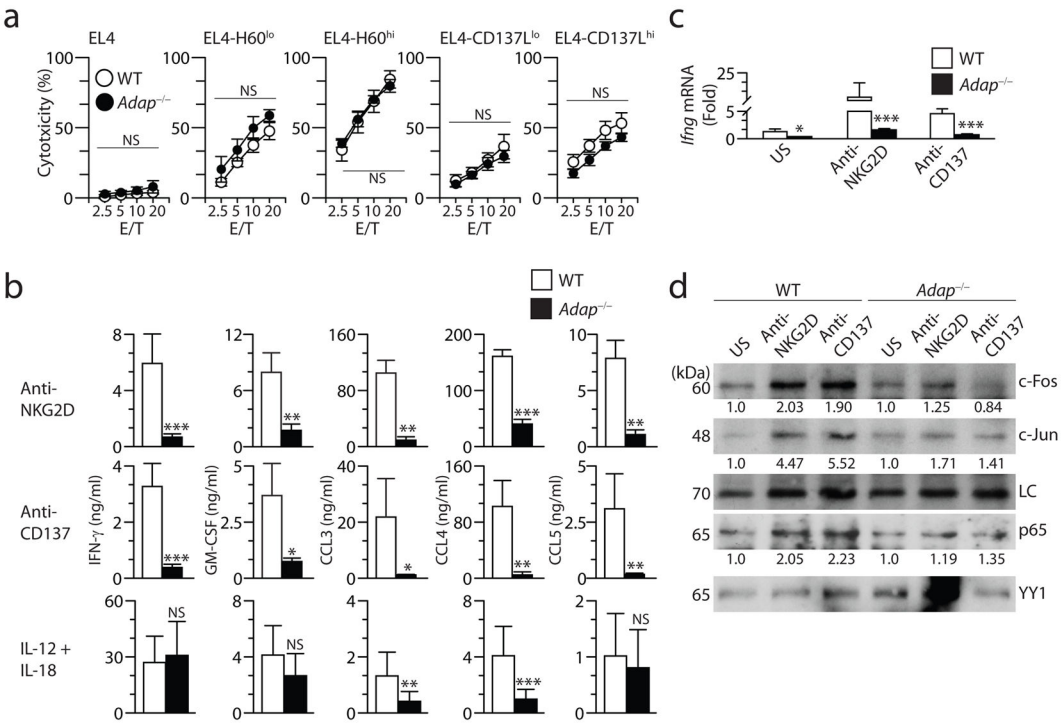

Figure 5. ADAP is essential for cytokine production but not for cytotoxicity in NK cells

(**a**) Mean percent cytotoxicity with standard deviation of NK cells from WT (open circles) or *Adap*$^{-/-}$ (filled circles) mice. E/T denotes Effector to Target ratio. (**b**) Quantitative analyses of cytokines and chemokines produced by NK cells obtained from WT (open) or *Adap*$^{-/-}$ (filled) mice following activation. Cytokines and chemokines produced by NK cells in response to IL-12 and IL-18 stimulation are also shown. (**c**) Relative expression of *Ifng*-encoding mRNA compared to *Gapdh* in WT (Open) and *Adap*$^{-/-}$ (filled) NK cells. (**d**) Immunoblot analyses of c-Fos, c-Jun, and NF-κB p65 in the nuclear extracts isolated from WT and *Adap*$^{-/-}$ NK cells stimulated under indicated conditions. YY1 and a non-specific band obtained in the immunoblot when probed for c-Fos are shown as loading controls. Fold change in the nuclear c-Fos, c-Jun, and NF-κB p65 in the WT and *Adap*$^{-/-}$ NK cells following activation were calculated against their respective unstimulated controls. **P*<0.05, ***P*<0.01, ****P*<0.001; NS, not significant (Student's t-test; two-tailed, two sample equal variance (**b–d**). Data in **a–c** are from four, five and three mice per genotype from three, four, and three independent experiments, respectively (mean ± s.d. in **a**, **b**, and **c**). Data in **d** is a representative of three independent experiments.

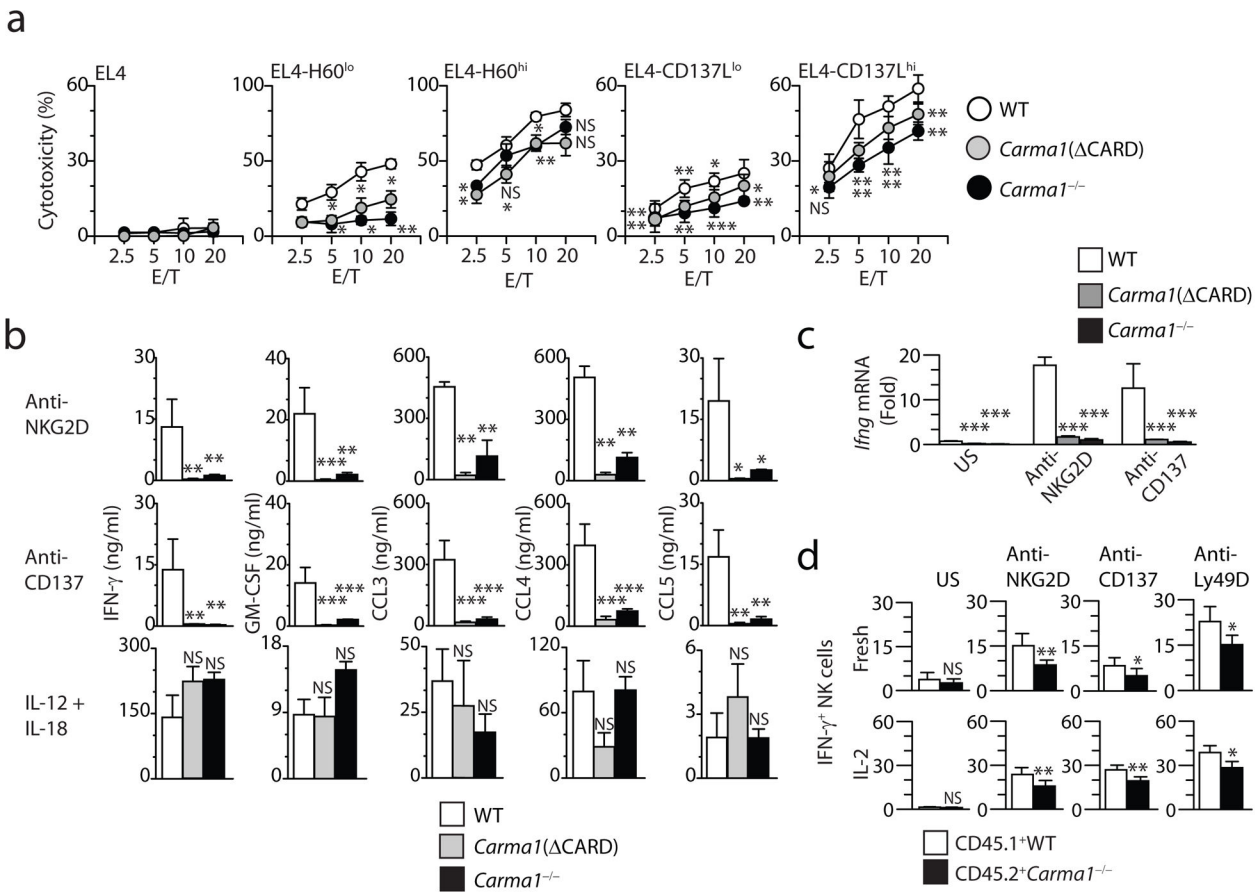

Figure 6. Carma1 is essential for cytotoxicity and cytokine production in NK cells

(**a**) Cytotoxic potentials of NK cells from WT (open circles), *Carma1*(ΔCARD) (grey circles), or *Carma1*$^{-/-}$ (black circles) mice. E/T denotes Effector to Target ratio. (**b**) Quantitative analyses of cytokines and chemokines produced by NK cells obtained from WT (open), *Carma1*(ΔCARD) (grey), or *Carma1*$^{-/-}$ (black) mice following activation. Cytokines and chemokines produced by NK cells in response to IL-12 and IL-18-mediated stimulation are shown. (**c**) Relative expression of *Ifng*-encoding mRNA compared to *Gapdh* in WT (Open), *Carma1*(ΔCARD) (grey), or *Carma1*$^{-/-}$ (black) NK cells left unstimulated or following activation. (**d**) Percent IFN-γ^{+} fresh NK cells (top panel) or IL-2-cultured NK cells (bottom panel) in WT (CD45.1^{+}, open) and *Carma1*$^{-/-}$ (CD45.2^{+}, filled) mice obtained from the spleen of irradiated and reconstituted *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, one month after adoptive transfer of an equal mixture of WT and Carma1$^{-/-}$ bone marrow cells. **P*<0.05, ***P*<0.01, ****P*<0.001; NS, not significant versus WT (Student's t-test; two-tailed, two sample unequal variance (**a, b**); two-tailed, two sample equal variance (**c, d**). Data in **a** is from five WT, three *Carma1*$^{-/-}$ and five *Carma1*(ΔCARD) mice and is from two independent experiments (mean ± s.d.). Data in **b** is from seven WT, three *Carma1*$^{-/-}$ and seven *Carma1*(ΔCARD) mice and is from three independent experiments (mean ± s.d.). Data in **c** and **d** are from four and three mice per genotype from three independent experiments, respectively (mean ± s.d.).

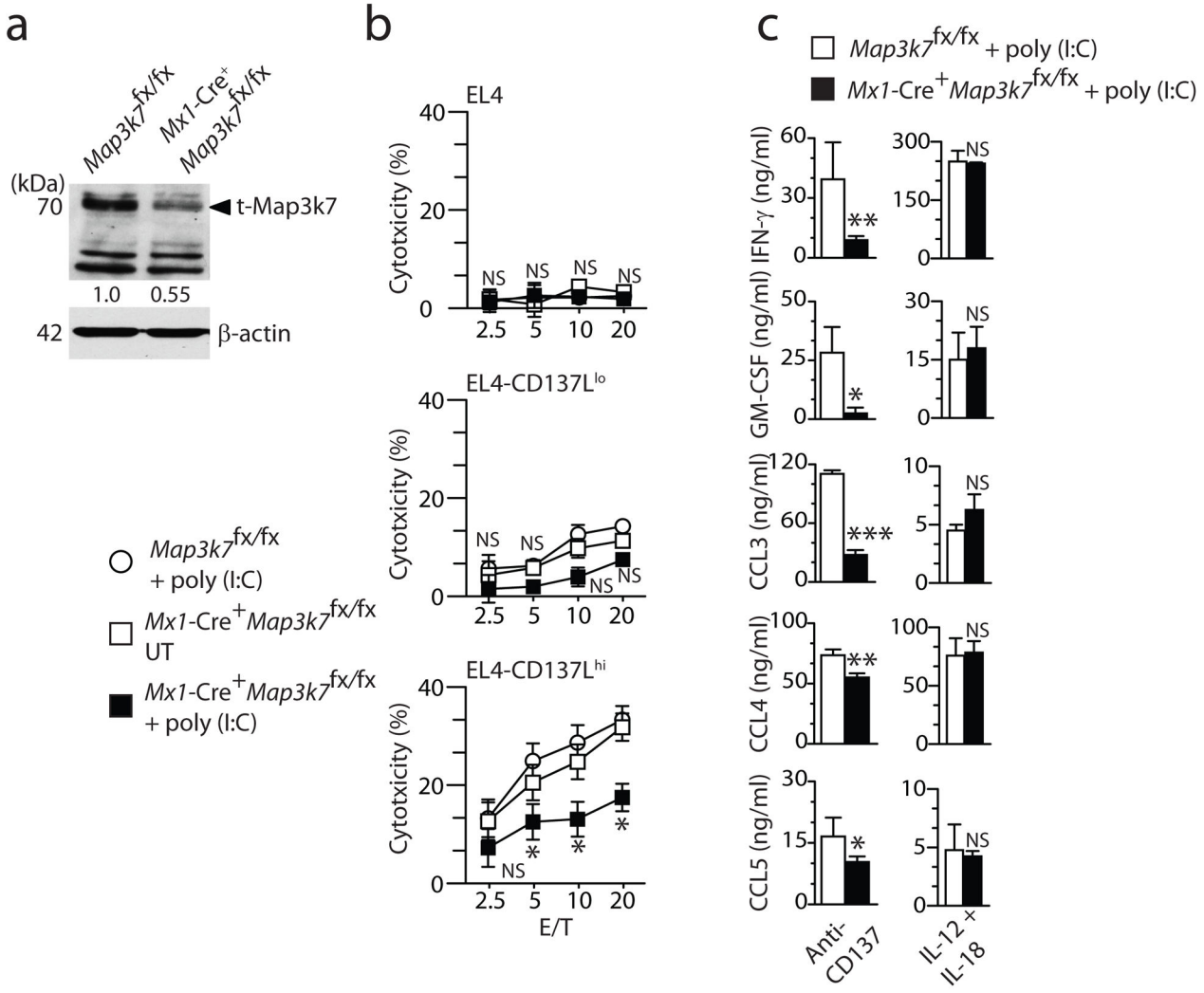

Figure 7. MAP3K7 links Carma1 to the CD137-mediated effector functions in NK cells

(**a**) Immunoblot showing the MAP3K7 expression in IL-2-cultured NK cells obtained from poly (I:C)-treated floxed *Map3k7*$^{fx/fx}$ and *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ mice. Fold change in MAP3K7 expression was determined by densitometry following normalization with actin. (**b**) Mean percent cytotoxicity with standard deviation of NK cells obtained from poly I:C-treated *Map3k7*$^{fx/fx}$ mice (open circle) or from poly (I:C) untreated (open square) and treated (filled square) *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ mice against the indicated targets. E/T denotes Effector to Target ratio. (**c**) Quantitative analyses of cytokines and chemokines produced by NK cells derived from poly (I:C)-treated *Map3k7*$^{fx/fx}$ (open) and *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ (filled) mice following 18 h of activation with plate-bound anti-CD137 mAb (left) or with IL-12 and IL-18 (right). **P*<0.05, ***P*<0.01, ****P*<0.001; NS, not significant versus WT (Student's t-test; two-tailed, two sample unequal variance versus NK cells from *Mx1-Cre*$^{+}$*Map3k7*$^{fx/fx}$ mice that were untreated with poly (I:C) (**b**); two-tailed, two sample equal variance versus NK cells from *Map3k7*$^{fx/fx}$ mice that were treated with poly (I:C) (**c**). Data in **a** is a representative of three independent experiments. Data in **b, c** are from three mice per genotype from three independent experiments (mean ± s.d.).

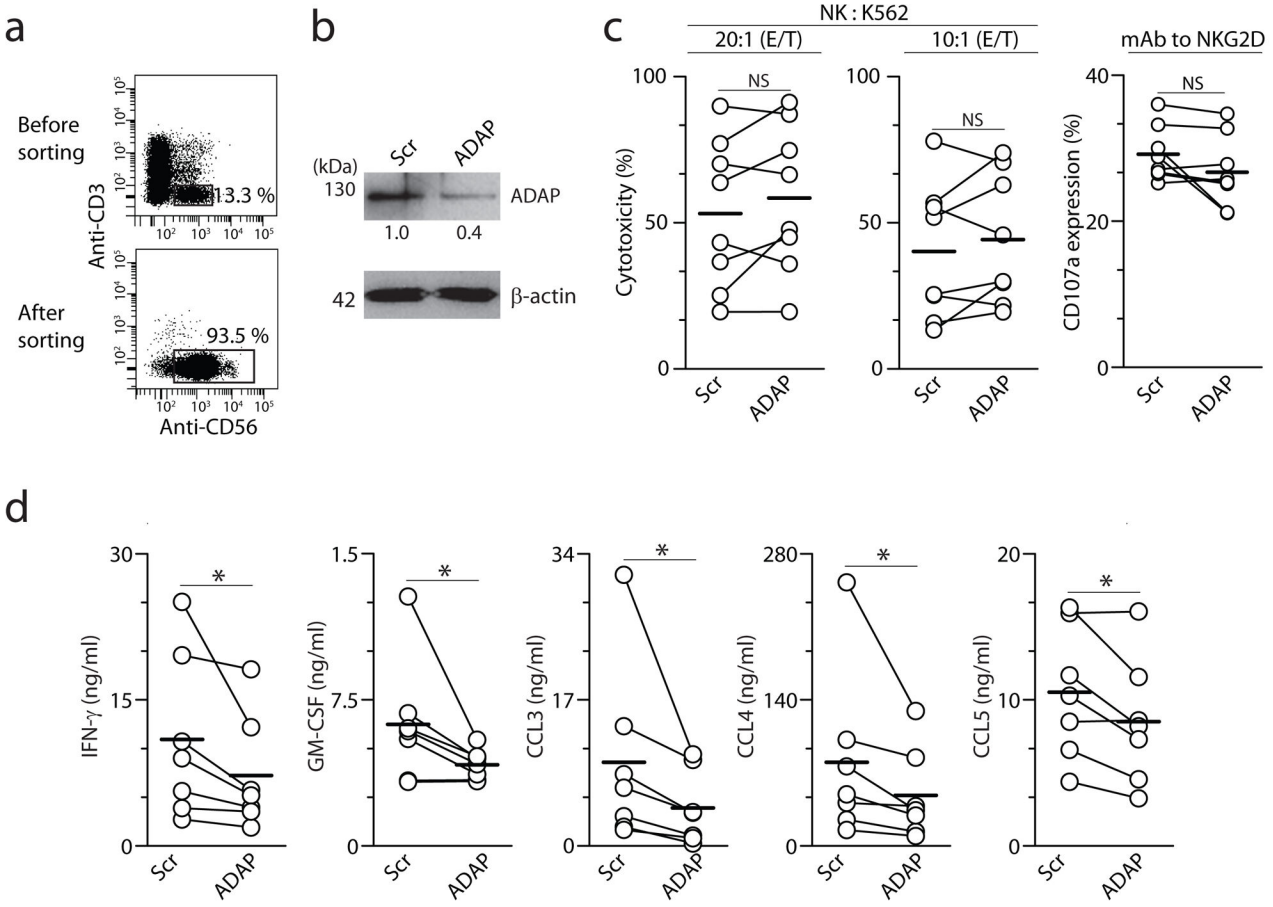

Figure 8. ADAP is essential for cytokine production but not for cytotoxicity in human NK cells

(**a**) Flow cytometry indicating the percentage $CD3^{-}CD56^{+}$ NK cells in total human PBMC (top) and in the cell fraction (bottom) obtained following negative selection for $CD56^{+}$ NK cells. Purified $CD56^{+}$ human NK cells were transfected with scrambled or ADAP-specific siRNA. (**b**) Immunoblot for ADAP in human NK cells that were transfected with scrambled or ADAP-specific siRNA. Fold change in ADAP expression was determined by densitometry following normalization with actin. (**c**) Dot plot represents the mean and percent cytotoxicity against K562 (left panels) and percent $CD56^{+}CD107a^{+}$ NK cells following plate-bound anti-NKG2D-mediated activation (right) of scrambled or ADAP-specific siRNA-transfected human NK cells. E/T denotes Effector to Target ratio. (**d**) Quantitative analyses of cytokines and chemokines produced by scrambled or ADAP-specific siRNA-transfected human NK cells following anti-NKG2D mAb-mediated activation. **P*<0.05; NS, not significant versus NK cells treated with scrambled siRNA (Student's t-test; two-tailed, paired (**c**, **d**). Data in **a** and **b** are representative of three independent experiments. Data in **c** and **d** are from eight and seven different human NK cells from five independent experiments. Horizontal bar in **c** and **d** represent the mean of the representative data set.