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DOCK8 Drives Src-Dependent NK Cell Effector Function

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Mutations in the *dedicator of cytokinesis 8* (*DOCK8*) gene cause an autosomal recessive form of hyper-IgE syndrome, characterized by chronic immunodeficiency with persistent microbial infection and increased incidence of malignancy. These manifestations suggest a defect in cytotoxic lymphocyte function and immune surveillance. However, how DOCK8 regulates NK cell–driven immune responses remains unclear. In this article, we demonstrate that DOCK8 regulates NK cell cytotoxicity and cytokine production in response to target cell engagement or receptor ligation. Genetic ablation of DOCK8 in human NK cells attenuated cytokine transcription and secretion through inhibition of Src family kinase activation, particularly Lck, downstream of target cell engagement or NKp30 ligation. PMA/Ionomycin treatment of DOCK8-deficient NK cells rescued cytokine production, indicating a defect proximal to receptor ligation. Importantly, NK cells from DOCK8-deficient patients had attenuated production of IFN-γ and TNF-α upon NKp30 stimulation. Taken together, we reveal a novel molecular mechanism by which DOCK8 regulates NK cell–driven immunity. *The Journal of Immunology*, **2017, 199: 2118–2127.**

Autosomal recessive hyper-IgE syndrome (AR-HIES) is a rare primary immunodeficiency disease characterized by elevated serum IgE levels; repeated cutaneous viral, bacterial, and fungal infections; and recurrent pneumonia. Other clinical symptoms include eczema and an increased incidence of severe allergies and asthma (1, 2). A major advance in the management of patients with this rare disease was made in 2009 with the discovery that mutations in the *dedicator of cytokinesis 8* (*DOCK8*) gene, located on chromosome 9p, are responsible for most cases of AR-HIES (2, 3).

DOCK8 is a member of the DOCK180-related family of guanine nucleotide exchange factors (GEFs), which promote the activity of Rho GTPases such as Rac and Cdc42 (4), and are involved in diverse cellular processes including cell migration, differentiation, and cell–cell interactions (4). Although DOCK8 is ubiquitously expressed, it is particularly enriched in lymphocytes, suggesting it functions to promote immunity (5). Indeed, recent studies have found that DOCK8 is required for the survival and persistence of $CD8^+$ T cells (6) and NKT cells (7), and for long-term Ab production by B cells (5). DOCK8 also appears to be essential for maintaining the structural integrity of T cells, serving to repress cell death during migration into skin (8), which is essential for the control of herpes virus skin infection (8, 9).

Consistent with the clinical characteristics of AR-HIES, DOCK8 is also implicated in the regulation of the NK cell and $CD8^+$ T cell immune synapse. The immune synapse is a specialized structure that forms between the plasma membrane of two cells, facilitating signaling or the triggering of killer cell effector function (10, 11). Formation of the immune synapse results in rapid signal transduction events that promote optimal cell activation, but also triggers reorganization of the cell cytoskeleton. These changes facilitate the spatial rearrangements of molecules that contribute to the success and quality of the immune synapse (11–13). Upon immune synapse formation in T cells, protein kinase C θ and leukocyte-specific protein tyrosine kinase (Lck) rapidly polarize into a region proximal to the synapse contact site called the central SMAC region (11). These events trigger the TCR-mediated signaling cascades that culminate in T cell activation and acquisition of effector functions. Furthermore, adhesion, costimulatory, and conjugation-promoting molecules, such as LFA-1 and talin, migrate to a ring that surrounds the central SMAC region (called the

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The sequencing data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE101467.

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Abbreviations used in this article: AR-HIES, autosomal recessive hyper-IgE syndrome; *DOCK8*, *dedicator of cytokinesis 8*; GEF, guanine nucleotide exchange factor; GSEA, gene set enrichment analysis; MTOC, microtubule-organizing center; NCR, natural cytotoxicity receptor; RNA-Seq, RNA sequencing; siRNA, small interfering RNA; WASp, Wiskott–Aldrich syndrome protein.

peripheral SMAC), which serves to improve the quality of the immune synapse (10, 11).

DOCK8 was initially identified as a regulator of the B cell immunological synapse, where it was found to be required for long-lived Ab production (5). Later, DOCK8 was found to also regulate the NK cell cytotoxic synapse (14, 15). In this study, it was demonstrated that DOCK8 was essential to promote the polarization of cytotoxic granules to the synaptic cleft via an interaction with Talin and Wiskott–Aldrich syndrome protein (WASp) (14). Similarly, NK cells derived from patients with DOCK8 deficiency were characterized by poor actin accumulation at the synapse contact site (15). However, it remains unclear whether DOCK8 regulates other important NK cell functions, such as the signal transduction events required to promote inflammatory cytokine production.

Mechanistically, DOCK8 links the TCR to the actin cytoskeleton via a bridging interaction between WASp and WASp-interacting protein (16). DOCK8 also functions as an adaptor protein in signaling pathways downstream of TLR9 receptor engagement on B cells essential for B cell proliferation and differentiation via STAT3 (17). Similarly, DOCK8 regulates $CD4^+$ T cell T_H17 polarization through a constitutive association with STAT3 (18, 19). Thus, evidence is emerging that suggests that DOCK8 not only promotes proper synapse formation, but also adopts important roles in the signal transduction events that are required for optimal immunological signaling cascades in diverse cell types.

NK cell function is governed through the relative strength of activatory and inhibitory signals, delivered to the NK cell by ligands expressed on target cells (12, 20). ITAM-bearing receptors, such as natural cytotoxicity receptors (NCRs) and NKG2D, promote NK cell function, including cytotoxic granule delivery and cytokine production, through activation of signaling cascades initiated by Src kinase family members such as Lck and Fyn (20–22). Src kinases phosphorylate the cytoplasmic tails of receptor ITAMS to recruit the "second-line" proteins, such as ZAP70 and SLP-76, which propagate signals leading to activation of MAPKs and transcription factors that promote granule polarization and cytokine transcription (22, 23). Given that the clinical characteristics of DOCK8 deficiency suggest poor NK cell–mediated immunity, we investigated whether DOCK8 regulates NK cell function by promoting the signal transduction events that drive both cytotoxicity and inflammatory cytokine production.

Materials and Methods

Cells and assays

NK cells were cultured in RPMI 1640 supplemented with 10% FCS plus IL-2 (450 U/ml for KHYG1 cells, 100 IU/ml for primary human NK cells). Cytotoxicity was measured using a chromium release assay (24). Cytokine secretion was measured using human Th1/Th2 Cytometric Bead Array (BD Biosciences) and analyzed on a FACSVerse.

Antibodies

Western blot analysis was performed using the following Abs: anti-human DOCK8 (Santa Cruz), phospho-Src, Src, LCK, phospho-LAT, LAT, phospho-ERK, ERK, phospho-P38, P38, phospho-P65, and P65 (Cell Signaling). Phospho-LCK^{Tyr505} (Gentex) and NKp30-PE were used for FACS analysis (BioLegend). Agonistic NKp30 Ab was from R&D Systems. Agonistic NKp44 Ab was from Thermo Fisher.

Western blotting

To analyze proteins, we used $NaDodSO_4$ PAGE (SDS-PAGE). Samples were prepared in sample 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% glycerol, 2.5% 2-ME, then boiled for 7 min. Lysates were then loaded into 8–12% polyacrylamide gels and electrophoresed at 75 V. Resolved proteins were then transferred onto nitrocellulose membranes at 40 mA overnight. Membranes were blocked for 1 h (5% BSA, 0.05% NaN_3 in PBS, Tween 20), then incubated with primary Ab overnight. Proteins were detected using HRP-conjugated secondary Ab in 0.05% NaN_3 in PBS and Tween 20 containing 5% BSA. After washing, proteins were detected using West Coast SuperSignal.

Conjugation assay

K562 and KHYG1 cells were labeled with CellTrace Violet and CFSE (Molecular Probes), respectively, for 20 min at 37°C. Cells were washed twice, then added to Eppendorf tubes (K562 1×10^5, KHYG1 2×10^5) in a 200 μl final volume. At the indicated time points, cells were vortexed for 2 s, fixed with 4% PFA, and then analyzed on a flow cytometer (BD Fortessa).

Gene knockout/knockdown

For CRISPR, a guide RNA targeting DOCK8 (5′-CAAAGTCCACTGGCTCCACA-3′) was cloned into lentiviral FgH1t-UTG vector with inducible expression of guide RNA and a fluorescent GFP reporter. KHYG1 cells stably expressing Cas9 were transduced and then sorted for GFP positivity. Doxycycline was added for 5 d to induce guide expression. Single cells were then seeded in 96-well plates and expanded. Clones were screened for loss of DOCK8 expression by Western blot, and a single clone was selected for all experiments using the knockout cell line. For RNA interference, KHYG1 or primary human NK cells (cultured in IL-2 for 48 h) were electroporated with 100 nM small interfering RNA (siRNA; Amaxa program X001) to knockdown DOCK8 or Lck. After 48–72 h, cells were treated as indicated. The siRNA sequences were as follows: scramble control: 5′-AUGUUAGUAGCGAUUGUAU-3′; DOCK8: 5′-GGAGAUUAUUUGUGAACUU-3′; and Lck: 5′-UAACCAGGUUGUCUUGCAGUG-3′.

RNA sequencing and analysis

KHYG1 cells were cultured as indicated before stimulation with anti-Nkp30 (duplicate samples). After 90 min, cell pellets were collected and total RNA was extracted using the NucleoSpin RNA extraction kit (Macherey-Nagel). RNA quality was checked on the Agilent 4200 TapeStation, and RNA with RIN values >9 were used for the subsequent analysis. Sequencing libraries were prepared using the QuantSEquation 3′ mRNA-Seq Library Prep Kit (Lexogen) according to the manufacturer's instructions using 200 ng of total RNA input. Single-end 75-bp RNA sequencing (RNA-Seq) was performed on the NextSEquation 500 (Illumina). Demultiplexing of the reads was performed using CASAVAv1.8.2, and low-quality reads $Q < 30$ were removed. Cutadapt (v1.9) was used to trim polyA-derived sequences and biased 3′ reads resulting from random hexamer priming. HISAT2 was used to map the resulting reads to the human reference genome. Read counting was performed using featureCounts, which is part of the subread package (25). Voom-LIMMA workflow was used to normalize data for differential gene expression (26). Gene set enrichment analysis (GSEA) was performed using GSEA2-2.2.2 for identification of enriched signatures obtained from the MSigDB Hallmarks datasets (27).

Accession code

Sequencing data have been deposited into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE101467.

Statistical analyses

Statistical significance was determined using an unpaired Student *t* test. Differences were considered significant when $p < 0.05$.

Results

DOCK8 is required for NK cell cytotoxicity, but not target conjugation

To investigate the proposed (14, 15) role of DOCK8 in human NK cell effector function, we first genetically deleted DOCK8 from the human NK cell line, KHYG1, using CRISPR/Cas9 technology. Loss of DOCK8 significantly attenuated cytotoxicity against both K562 and HeLa target cells (Fig. 1A). To confirm the specificity of this approach, we also demonstrated that siRNA knockdown of DOCK8 reduced NK cell cytotoxicity against K562 and HeLa target cells (Fig. 1B). Thus, DOCK8 appears to be critical for the cytotoxic function of human NK cells. To test whether loss of DOCK8 was impairing cytotoxicity through decreased conjugation to target cells, we performed a FACS-based conjugation assay using K562 cells as targets. DOCK8 knockout NK cells conjugated to targets as efficiently as control NK cells (Fig. 1C); however, confocal analyses of these conjugates confirmed previous reports that DOCK8-deficient

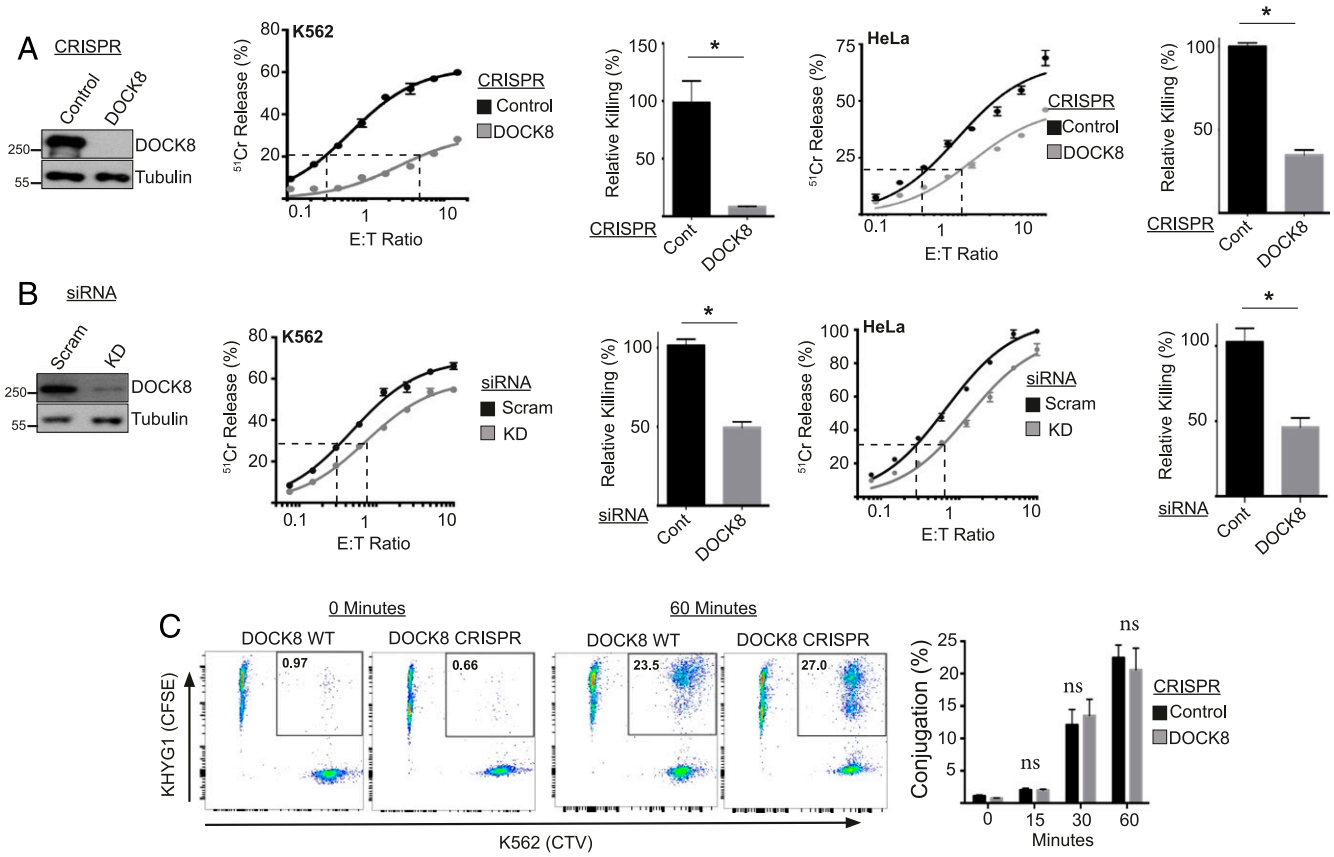

FIGURE 1. DOCK8 is required for NK cell cytotoxicity, but not target conjugation. (**A**) Control or DOCK8 CRISPR knockout KHYG1 cells were used at the indicated E:T ratio in a chromium release assay with K562 or HeLa cells as targets. Killing kinetics are represented as a Michaelis–Menten plot and relative killing on the right. (**B**) Control or DOCK8 knockdown KHYG1 cells were used at the indicated E:T ratio in a chromium release assay with K562 or HeLa cells as targets. Killing kinetics are represented as a Michaelis–Menten plot and relative killing on the right. (**C**) Control or DOCK8 CRISPR knockout KHYG1 cells were subject to a conjugation assay with K562 cells. At the indicated time points, cell conjugates were analyzed by flow cytometry, as described in *Materials and Methods*. Representative FACS plots are displayed. Data are representative of at least three independent experiments. Error bars on the Michaelis–Menten plots represent the mean ± SEM of triplicate determinations from a representative experiment. Error bars on graphs (relative killing) are mean ± SEM pooled from three independent experiments. $*p < 0.05$ by unpaired Student *t* test.

NK cells do not efficiently polarize the microtubule-organizing center (MTOC) and perforin-containing granules to the immune synapse (14, 15) (Supplemental Fig. 1A). These data suggest that DOCK8 regulates NK cell cytotoxicity downstream of NK cell–target conjugation.

DOCK8 regulates inflammatory transcriptional events upon activation through NKp30 engagement

Recognition of targets by NK cells results in receptor-mediated activation of signaling pathways that drive granule polarization to the cytotoxic synapse and gene transcription leading to rapid cytokine synthesis and secretion (23). ITAM-bearing receptors, such as NCRs, deliver activating signals through signaling motifs within their cytoplasmic tails. The NCR, NKp30, is known to trigger NK cell activity against K562 cells upon binding to its ligand B7-H6 (28). To determine the effects of DOCK8 deletion on NK cell activation, we performed genome-wide gene expression analysis using 3′ mRNA-Seq on DOCK8 control and knockout NK cells, untreated or after NKp30 stimulation (Fig. 2).

As expected, DOCK8 expression was reduced in DOCK8 CRISPR knockout NK cells, regardless of treatment (Fig. 2A). Differential gene expression analysis of transcripts regulated by NKp30 in control NK cells demonstrated that stimulation caused an increase in multiple transcripts associated with immune cell activation. The immune activation signature was present in both DOCK8 control and knockout NK cells (Fig. 2B, Supplemental Tables I, II). However, a direct comparison of NKp30-stimulated DOCK8 control and knockout NK cells showed that the magnitude of the response elicited by NKp30 was impaired in DOCK8 knockout cells. DOCK8 loss resulted in reduced expression across the majority of NKp30 target genes, including key inflammatory factors. In accordance with this observation, GSEA on the RNA-seq data revealed that genes involved in inflammation, K-RAS signaling, and the IFN-γ response were significantly reduced in NKp30-stimulated NK cells upon DOCK8 deletion compared with their control counterpart. Importantly, gene signatures associated with distinct signaling pathways such as TGF-β signaling, PI3K/AKT signaling, and protein secretion pathways were unaffected in DOCK8 knockout NK cells (Fig. 2C). Interestingly, upregulation of genes encoding the cytokines IFN-γ, TNF-α, IL-10, IL-8, and IL-2 was attenuated in response to NKp30 stimulation in DOCK8 knockout NK cells (Fig. 2D, 2E), whereas expression of DOCK8 or key signaling mediators such as Lck and NKp30 was unaffected by NKp30 stimulation (Fig. 2E). These data demonstrate that loss of DOCK8 leads to a reduction in cytokine gene transcription, suggesting that DOCK8 deficiency may impair signaling pathways leading to transcriptional regulation and secretion of key NK cell cytokines such as IFN-γ and TNF-α.

DOCK8 is required for cytokine secretion after target cell conjugation or NKp30 engagement

To confirm that DOCK8 is involved in receptor-induced signaling cascades leading to cytokine transcription and production, we

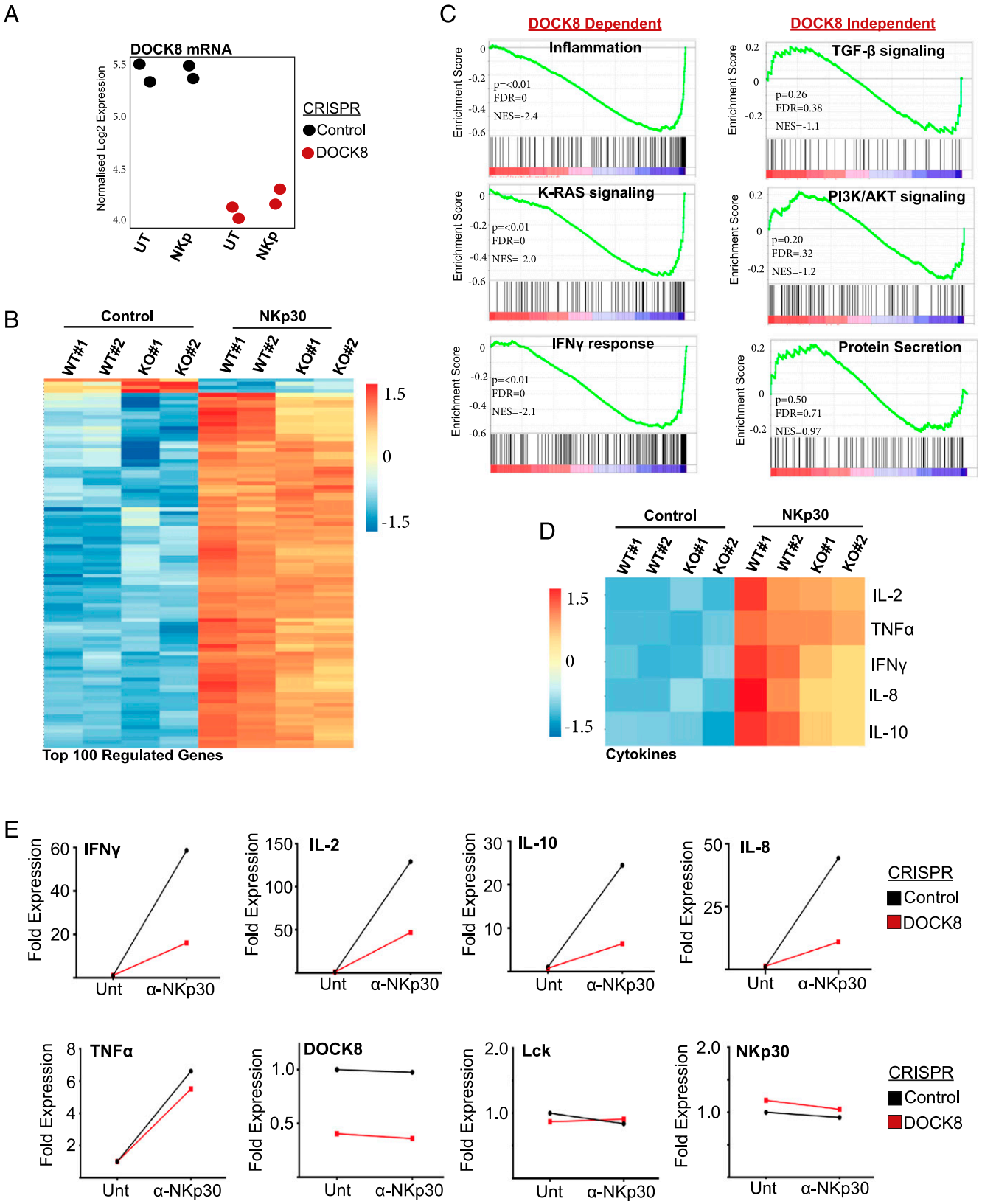

FIGURE 2. DOCK8 regulates inflammatory transcriptional events upon activation. (**A**) Control or DOCK8 CRISPR knockout KHYG1 cells ($n = 2$) were either left untreated or activated with anti-NKp30 (1 μg/ml) for 90 min followed by 3′ mRNA-Seq. Normalized Log2CPM (count per million) DOCK8 expression is depicted. (**B**) Heat map of the RNA-Seq data from (A) depicting the top 100 regulated genes upon Nkp30 stimulation. (**C**) GSEA enrichment score plots from RNA-Seq data showing significant correlation between DOCK8 expression, or not, and genes regulating the indicated cellular processes. (**D**) Heat map of the RNA-Seq data from (A), showing a selection of inflammatory cytokines. (**E**) Fold gene expression from the RNA-Seq data in (A), for a selection of inflammatory cytokine genes and control genes.

exposed DOCK8 control or knockout NK cells to K562 target cells and measured the release of cytokines. Consistent with the RNA-seq data, DOCK8-deficient NK cells secreted significantly less IFN-γ, IL-2, TNF-α, and IL-10 compared with control NK cells (Fig. 3A), demonstrating that DOCK8 drives cytokine secretion upon target cell engagement. Furthermore, when stimulated via the NCR, NKp30, DOCK8 knockout NK cells exhibited reduced cytokine release compared with control cells (Fig. 3B). To demonstrate the specificity of this approach, we repeated the experiment using NK cells with siRNA-mediated knockdown of DOCK8 and found a similar reduction in the secretion of IFN-γ, IL-2, TNF-α, and IL-10 (Fig. 3C).

DOCK8 drives cytokine production through activation of Src kinases, independent of the cytoskeleton

Because DOCK8 acts as a signaling adaptor to promote Src kinase activation upon TLR9 stimulation in B cells (17), we hypothesized that DOCK8 may drive NK cell function by linking ITAM-based signaling to Src kinase activation, which is known to be crucial for NK cell activation (23). We exposed DOCK8 control or knockout NK cells to K562 targets and monitored activation of proximal kinases. Unlike control cells, DOCK8-deficient NK cells largely failed to activate Src kinases, including Lck phosphorylation on the activation motif, Tyr^{505} (Fig. 4A). We next asked whether DOCK8 was required for ITAM-triggered signaling upon NKp30 ligation. In control NK cells, NKp30 stimulation potently triggered activation of Src family kinases, including Lck (Fig. 4B). However, activation of Src family kinases, including Lck, was attenuated in the absence of DOCK8, leading to reduced phosphorylation of ERK and p38 MAPK downstream (Fig. 4B, Supplemental Fig. 2A). DOCK8 knockdown NK cells also failed to activate Src kinases as efficiently as control cells (Fig. 4C, Supplemental Fig. 2B). PMA/Ionomycin treatment activated ERK and p38 MAPK equivalently in both DOCK8 control and knockout NK cells (Fig. 4D), and we could rescue the defect in cytokine production using nonspecific PMA/Ionomycin treatment (Fig. 4E), indicating that loss of DOCK8 attenuates NKp30-induced signaling pathways, but does not invoke a global inability to trigger cytokine synthesis. There was no significant difference in cytokine production when DOCK8 control or knockout NK cells were stimulated through engagement of another NK cell NCR, NKp44 (Fig. 4F), even though DOCK8 control and knockout NK cells expressed equivalent levels of both NKp30 and NKp44 (Supplemental Fig. 1B). These data suggest that DOCK8 may be a specific downstream effector of NKp30 engagement.

DOCK8 has previously been shown to play an important role in establishing the cytoskeletal networks required for efficient synapse formation and granule delivery leading to cytotoxic activity in NK cells (14, 15). To determine whether the attenuation in cytokine production in the absence of DOCK8 was dependent on the cytoskeleton, we treated the cells with nocodazole, which disrupts the tubule network and impairs translocation of the microtubule-organizing network (29). Although treatment of the NK cells with nocodazole significantly impaired cytotoxic activity, we found no effect on the secretion of either IFN-γ or TNF-α (Fig. 4G, 4H). These data demonstrate that, under these conditions, NK cell

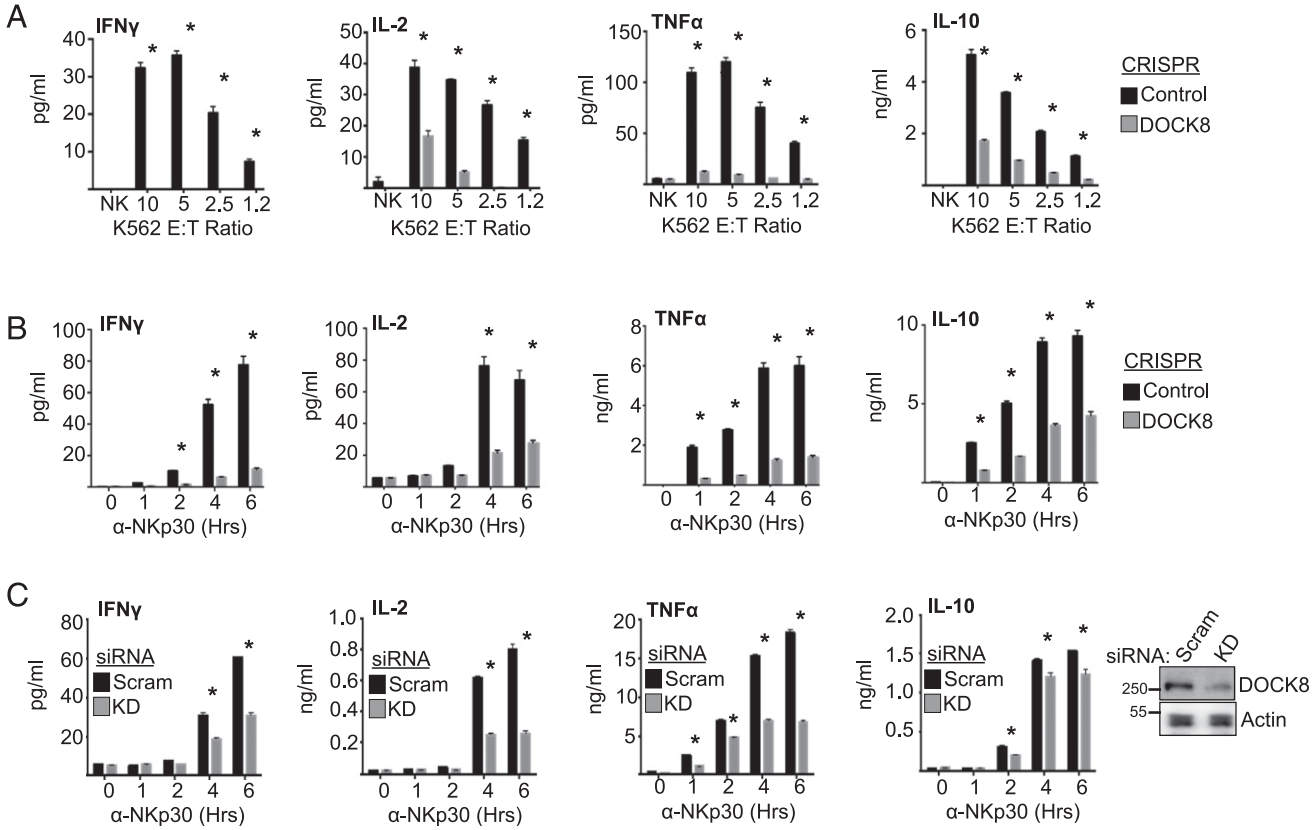

FIGURE 3. DOCK8 is required for NK cell cytokine production. (**A**) Control or DOCK8 CRISPR knockout KHYG1 cells were exposed to K562 cells at the indicated E:T ratios. After 4 h, cytokines in the supernatants were analyzed by cytometric bead array (CBA). (**B**) Control or DOCK8 CRISPR knockout KHYG1 were stimulated with anti-NKp30 (1 μg/ml). At the indicated time points, cytokines in the supernatants were analyzed by CBA. (**C**) Control or DOCK8 knockdown KHYG1 cells were stimulated with anti-NKp30 (1 μg/ml). At the indicated time points, cytokines in the supernatants were analyzed by CBA. Knockdown efficiency is shown on the right. Data are representative of at least three independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. *$p < 0.05$ by unpaired Student t test.

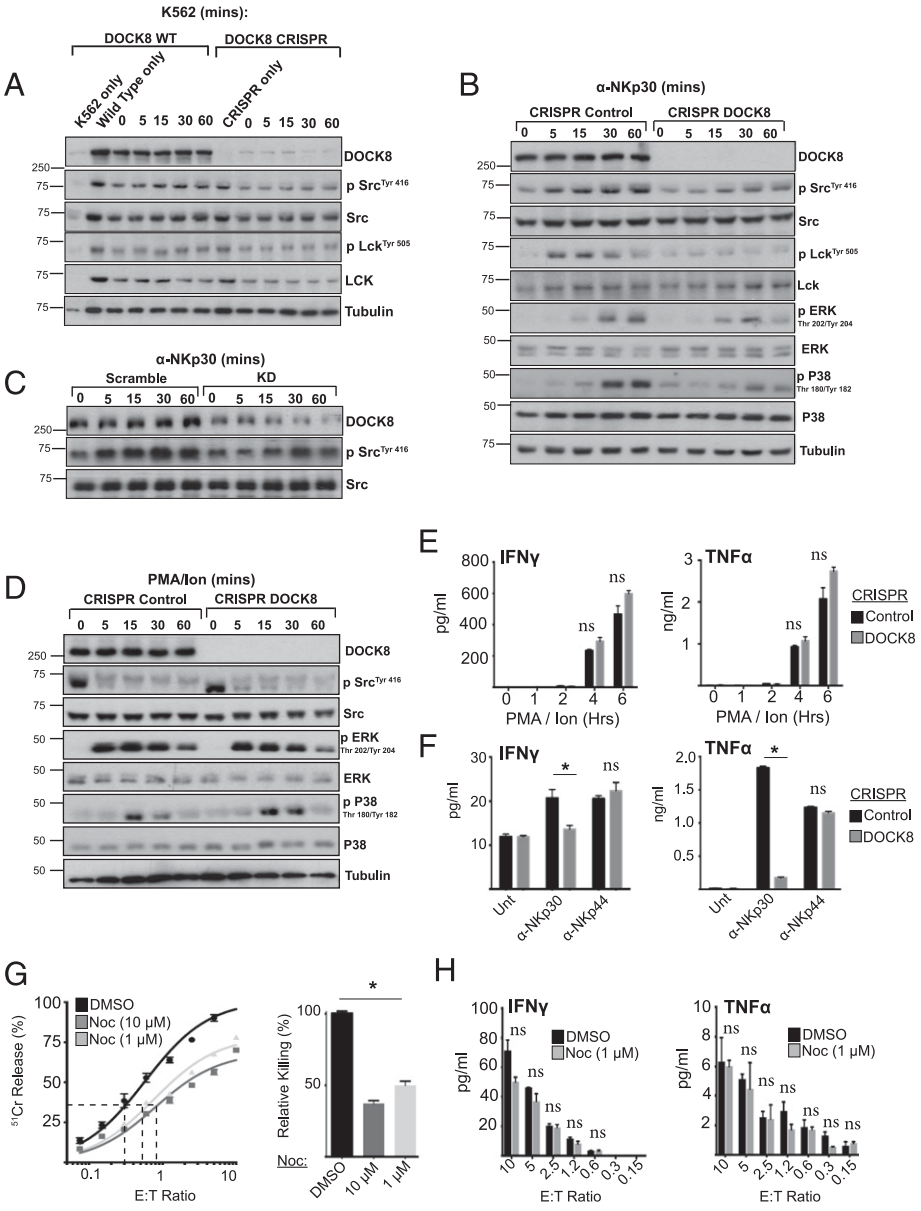

FIGURE 4. DOCK8 is required for optimal Src kinase activation. (**A**) Control or DOCK8 knockout KHYG1 cells were exposed to K562 cells (10:1 E:T ratio). At the indicated time points, cell lysates were analyzed for the indicated proteins by Western blotting. (**B**) Control or DOCK8 knockout KHYG1 cells were stimulated with anti-NKp30 (10 μg/ml). At the indicated time points, cell lysates were analyzed for the indicated proteins by Western blotting. (**C**) KHYG1 cells were electroporated with scramble or DOCK8 siRNA. After 72 h, cells were stimulated with anti-NKp30 (10 μg/ml). Cell lysates were then probed for Src kinase activation. (**D**) Control or DOCK8 knockout KHYG1 cells were treated with PMA (10 ng/ml) and Ionomycin (1 μg/ml). At the indicated time points, cell lysates were analyzed by Western blotting. (**E**) Control or DOCK8 knockout KHYG1 cells were treated with PMA (10 ng/ml) and Ionomycin (1 μg/ml); then cytokines in the supernatants were analyzed by cytometric bead array (CBA) at the indicated time points. (**F**) Control or DOCK8 knockout KHYG1 cells were treated with anti-NKp30 (1 μg/ml) or anti-NKp44 (1 μg/ml). After 4 h, cytokines in the supernatants were analyzed by CBA. (**G**) KHYG1 cells were pretreated with DMSO or the indicated concentration of nocodazole for 30 min, then subjected to a killing assay using K562 cells as targets. Nocodazole concentration was maintained throughout the assay. (**H**) Supernatants from (G) were analyzed by CBA. Data are representative of at least three independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. Error bars on graph of relative killing (G) are mean ± SEM pooled from three independent experiments. *$p < 0.05$ by unpaired Student t test.

cytotoxicity and cytokine secretion are uncoupled, and suggest that the role of DOCK8 is more complex than simply acting as a scaffolding protein to facilitate cytoskeletal changes required for cytotoxicity.

The Src kinase, Lck, and ERK are required for NK cell cytotoxicity and cytokine production

Src kinases, including Lck and Fyn, play a crucial role in the activation of NK cells (23). To determine whether DOCK8 deficiency might attenuate NK cell cytotoxicity and cytokine production through the failure to optimally activate Src, we used the specific Src kinase inhibitor, PP2. As expected, PP2 potently inhibited NKp30-induced activation of Src kinases, including Lck (Fig. 5A), which translated into attenuated ERK activation downstream of Src. Importantly, inhibition of Src-mediated signaling entirely abolished the cytotoxicity of KHYG1 cells against K562 targets (Fig. 5B). Src inhibition also inhibited NKp30-induced secretion of IFN-γ, TNF-α, and IL-2 (Fig. 5B), thus suggesting that DOCK8 is required for NK cell function via activation of Src kinases and downstream MAPKs.

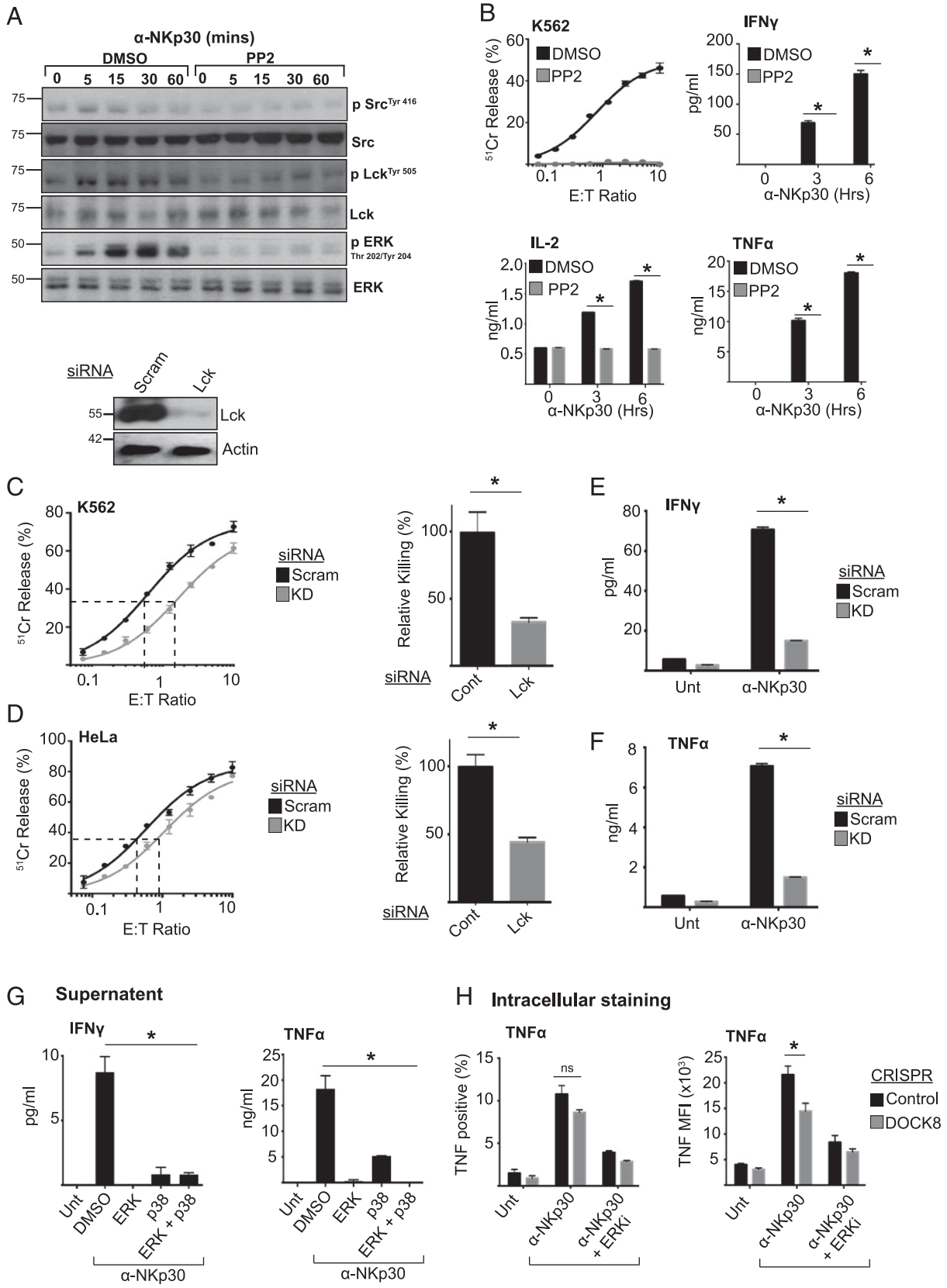

FIGURE 5. Src kinase Lck and ERK are required for NK cell cytokine production. (**A**) KHYG1 cells were treated with DMSO or PP2 (5 μM) for 15 min, then stimulated with anti-NKp30 (10 μg/ml). At the indicated time points, cell lysates were analyzed by Western blotting. (**B**) KHYG1 cells were treated with DMSO or PP2 (5 μM) for 15 min, then subjected to a chromium release assay using K562 cells as targets. KHYG1 cells were treated with DMSO or PP2 (5 μM) for 15 min, then stimulated with anti-NKp30 (1 μg/ml). At the indicated time points, cytokines in the supernatants were analyzed by cytometric bead array (CBA). (**C** and **D**) Control or Lck knockdown KHYG1 cells were used at the indicated E:T ratio in a chromium release assay with K562 or HeLa cells as targets. Killing kinetics are represented as a Michaelis–Menten plot and relative killing on the right. (**E** and **F**) Control or Lck knockdown KHYG1 cells were stimulated with anti-NKp30 (1 μg/ml). After 6 h, cytokines in the supernatants were analyzed by CBA. (**G**) KHYG1 (*Figure legend continues*)

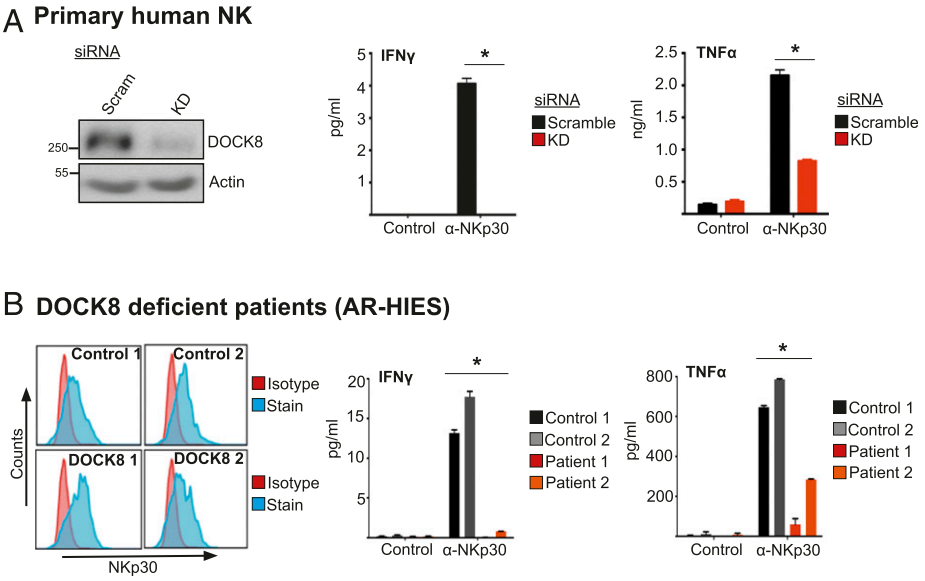

FIGURE 6. NK cells from DOCK8-deficient patients fail to respond to NKp30 stimulation. (**A**) NK cells were isolated from PBMCs from a healthy donor using negative selection, then incubated overnight in IL-2 (100 IU/ml). NK cells were then electroporated with the indicated siRNAs. Seventy-two hours later, cells were collected for Western blot analysis and treated with anti-NKp30 (1 μg/ml) for 4 h. Cytokines in the supernatants were analyzed by cytometric bead array (CBA). Data are representative of results obtained from four individual donors. (**B**) NK cells were isolated from PBMCs from control or DOCK8-deficient patients ($n = 2$) using negative selection, then incubated overnight in IL-2 (100 IU/ml). The cells were then analyzed for NKp30 expression by FACS or stimulated with anti-NKp30 (1 μg/ml). After 4 h, cytokines in the supernatants were analyzed by CBA. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment; $*p < 0.05$ by unpaired Student t test.

To determine whether the Src kinase, Lck, was the key signaling molecule affected by DOCK8 deficiency, as suggested by the Western blots (Fig. 4A, 4B), we used siRNA to specifically knock down Lck in KHYG1 cells. Knockdown of Lck reduced cytotoxicity of NK cells against both K562 and HeLa target cells (Fig. 5C, 5D, respectively). Knockdown of Lck also attenuated the secretion of both IFN-γ and TNF-α upon NKp30 stimulation (Fig. 5E, 5F, respectively), demonstrating that Lck is essential for NK cell cytotoxicity and cytokine production. Furthermore, inhibition of both ERK and p38 significantly reduced IFN-γ and TNF secretion (Fig. 5G), and ERK inhibition reduced intracellular levels of TNF-α in both control and DOCK8 knockout NK cells (Fig. 5H), demonstrating the importance of these downstream signaling molecules in the regulation of these cytokines.

NK cells from DOCK8-deficient patients fail to respond to NKp30 stimulation

To investigate the role of DOCK8 in the function of primary NK cells, we isolated NK cells from the peripheral blood of healthy individuals and used siRNA to knock down DOCK8 (Fig. 6A). Importantly, DOCK8 knockdown NK cells failed to produce IFN-γ and had attenuated secretion of TNF-α in response to NKp30 ligation (Fig. 6A), supporting our previous data with KHYG1 cells.

We next investigated NK cells isolated from the peripheral blood of two patients suffering from AR-HIES as a result of genetic loss of *DOCK8* (Table I). DOCK8-deficient patient NK cells expressed NKp30 to comparable levels as healthy donor-derived NK cells (Fig. 6B), but failed to produce IFN-γ and TNF-α in response to NKp30 ligation, whereas control NK cells produced abundant amounts of these cytokines (Fig. 6B). Thus, DOCK8 regulates cytokine production in human NK cells, suggesting that DOCK8 deficiency in humans likely results in poor NK cell–driven immunity through suboptimal cytokine production.

Discussion

DOCK8 has recently emerged as a key regulator of lymphocyte function, both in the context of proper immune synapse formation and through contributing to signal transduction events that promote immune cell function (30). In this article, we have demonstrated that DOCK8 regulates the signal transduction events that are required for NK cell effector function acquisition, by promoting the activation of the Src kinase Lck, downstream of target cell recognition or NKp30 stimulation.

NK cells are innate immune effector cells, which are often the first responders to microbial infection or malignant transformation. Upon recognition of infected or transformed cells, NK cells rapidly acquire cytotoxic effector function and secrete inflammatory cytokines such as IFN-γ and TNF. TNF is a highly proinflammatory apical cytokine, which acts upon diverse tissue types to amplify inflammation. Importantly, TNF triggers the secretion of numerous downstream cytokine and chemokines that promote immune cell infiltration into the localized site of infection (31). Furthermore, TNF acts directly on immune cells, enhancing their activation status, and facilitates Ag cross-presentation by promoting macrophage and dendritic cell maturation (32). Indeed, neutralization of TNF is often used to treat inflammatory disorders such as

cells were pretreated with ERK inhibitor (cobimetinib, 125 nM) or p38 inhibitor (SB203580, 125 nM) for 15 min, followed by stimulation with anti-NKp30. After 6 h, cytokines in the supernatants were analyzed by CBA. (**H**) KHYG1 cells were treated with DMSO or cobimetinib (125 nM) for 15 min in the presence of GolgiPlug, then stimulated with anti-NKp30 (1 μg/ml). After 2 h, intracellular accumulation of TNF was measured by FACS staining. Data are representative of at least three independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. Error bars on graphs of relative killing (C and D) are mean ± SEM from pooled from three independent experiments. $*p < 0.05$ by unpaired Student t test.

Table I. Characteristics of DOCK8-deficient patients (AR-HIES)

DOCK8-Deficient Patients	Mutation	Sex	Age (y)	IgE (IU/ml)	Infections	Allergies
Patient 1	Unidentified: lack protein expression[13]	Male	5	17,300	HSV, *Streptococcus pyogenes*, *Haemophilus influenzae*, *Candida albicans*, adenovirus, norovirus, HHV6, EBV, CMV, VZV, *Aspergillus niger*, *Cladosporium*	Food allergies (milk, egg, cashew, pistachio, beef, lamb) Eczema Asthma Bronchiectasis
Patient 2	C.5748insC(p.T1917HfsX30)	Male	13	1,716	*Molluscum contagiosum*, recurrent viral and bacterial respiratory infection	Severe eczema Food, environmental, and drug allergies Asthma Bronchiectasis

Clinical analysis of AR-HIES patients from Fig. 6.
HHV6, human herpesvirus 6; VZV, varicella zoster virus.

Crohn's disease and arthritis. However, these patients become vulnerable to opportunistic bacterial infections, highlighting the importance of TNF in driving both innate and adaptive immunity (33). Similarly, IFN-γ is rapidly produced upon NK cell activation, where it serves to promote the effector status of cytotoxic lymphocytes and prime APCs to drive initiation of adaptive immunity (34). IFN-γ is also critical for promoting antitumor immunity (35, 36), by elevating MHC class I expression on tumor cells, invoking tumor cell cytostasis and increasing effector cell cytotoxic activity. Our data suggest that DOCK8 deficiency in patients is likely to impair NK cell–driven immune responses because of a defect in cytotoxicity and in the secretion of inflammatory cytokines, particularly TNF and IFN-γ, which are required for efficient mobilization of the immune system and subsequent clearance of infections. Reduced NK cell function may also lead to increased susceptibility to malignancies in DOCK8-deficient patients because of inefficient immune surveillance.

NK cell activation results in a rapid cascade of signaling events that culminate in translocation of lytic granules via MTOC polarization in the cytotoxic synapse (12). Early studies into the role of DOCK8 in NK cells identified a defect in cytotoxicity against tumor cell targets (14, 15). This was found to occur through a failure to polarize key molecules, accumulate actin, and translocate the MTOC to the immune synapse. However, it was unclear whether DOCK8 was directly involved in these cytoskeletal changes, or whether DOCK8 was involved in transducing the signals that promote these events, or indeed both. Since these discoveries, several lines of evidence have pointed to a role for DOCK8 in regulating signal transduction events (30). Indeed, recent studies have identified DOCK8 as a critical cell-intrinsic regulator of $CD4^+$ T cell polarization. In this study, DOCK8 was found to constitutively associate with STAT3 in a GEF-independent manner, but promote STAT3 activation using GEF activity upon cytokine stimulation (18, 19). Similarly, DOCK8 also regulates the production of IL-31 in $CD4^+$ T cells by controlling the transcription factor EPAS1 (37). DOCK8 also acts in a nonenzymatic role upon TLR9 stimulation in B cells. In this study, DOCK8 bridges the interaction of the Src kinase, Lyn, with Syk, facilitating STAT3 translocation to the nucleus to drive transcription (17). Importantly, this study demonstrated that DOCK8 constitutively associates with Src kinases, which is enhanced upon receptor triggering. These observations are consistent with our data that demonstrate that DOCK8 plays a crucial role in linking the NKp30 receptor to rapid and efficient activation of the Src kinase, Lck, which is required for downstream signaling that triggers lytic activity and cytokine production in a transcriptional-dependent manner. Interestingly, we did not observe a similar decrease in cytokine production after activation through the NKp44 receptor, suggesting that the DOCK8 may be a specific adaptor for the NKp30 receptor. However, we cannot rule out a role for DOCK8 in mediating signaling through other NK cell–activating receptors without further investigation.

Another outstanding question in the field is the relative contribution of DOCK8 in promoting the cytoskeletal rearrangements that facilitate signal transduction events, versus a more direct role in signal initiation/transduction. Indeed, evidence suggests that DOCK8 is involved in the physical reorientation of the MTOC through its activity as a GEF-dependent activator of Cdc42 (14, 16). We have addressed this question by destabilizing microtubules, which are required for MTOC polarization. As expected, nocodazole attenuated lytic activity; however, under these conditions, target cell synapse-mediated cytokine production was unaffected. These data suggest that rapid, DOCK8-dependent signal transduction events proceed efficiently and independently of a reorganized cytoskeleton. The rapid activation kinetics of Src kinases we have monitored by Western blotting (within 5 min) strongly support this concept, where signaling to cytokine transcription/translation precedes granule delivery to the synaptic cleft. It is also worth noting that this would be consistent with many other receptors that promote inflammatory cytokine production. For example, ligation of the TNFR results in rapid formation of a signaling complex composed of nonenzymatic adaptors, such as TRADD, that serve as platforms for activation of kinases such as RIPK1, triggering chemokine transcription within minutes (38). Further studies will be required to determine the exact kinetic relationship between gene transcription, cytoskeletal rearrangements caused by cytotoxic synapse formation, and the molecular pathways involved in these processes.

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Disclosures

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