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*J Immunol* published online 26 January 2011
http://www.jimmunol.org/content/early/2011/01/26/jimmunol.1000112
Ubiquitylation of an Internalized Killer Cell Ig-Like Receptor by Triad3A Disrupts Sustained NF-κB Signaling

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Killer cell Ig-like receptor (KIR) with two Ig-like domains and a long cytoplasmic domain 4 (2DL4; CD158d) is a structurally and functionally unique activating member of the human KIR family and the most highly conserved KIR among primates (1). The receptor is normally only expressed on the surface of a minor subset of CD56<sup>high</sup> NK cells that elicit mostly cytokine production (2, 3). In contrast to other KIR, surface expression of 2DL4 is upregulated on NK cells by IL-2 stimulation (2, 3). Structurally, 2DL4 contains features of both activating and inhibitory KIR family members. The receptor has a basic arginine residue in the transmembrane domain, which allows association with the FcεRIα transmembrane adaptor protein (4). The cytoplasmic domain of 2DL4 also contains a single ITIM, which has the capacity to recruit SHP-2 protein tyrosine phosphatase, at least in the context of a chimeric receptor construct (5). The role of SHP-2 recruitment on function of the endogenous receptor is currently unclear, however.

The only known ligand of 2DL4 is a soluble form of the non-classical MHC class I molecule, HLA-G, which is normally produced by fetal-derived trophoblast cells in pregnant women (6–8). Because 2DL4 is expressed on uterine NK cells during pregnancy (9), it has been proposed that the interaction of 2DL4 with HLA-G may benefit pregnancy (8). Uterine NK cells have limited cytolytic capacity, but produce cytokines that may promote remodeling of the maternal vasculature to support fetal development in mice and humans (10, 11). Interestingly, 2DL4 ligation stimulates production of several proangiogenic cytokines, suggesting that it may contribute to this process in the decidual tissue during pregnancy (8, 12).

2DL4 transduces activating signals through at least two different modules. Association with the transmembrane adaptor, FcεRIγ (4), provides a cytoplasmic ITAM that recruits Syk/ZAP70 tyrosine kinases. FcεRIγ supports 2DL4-mediated Ca<sup>2+</sup> mobilization, weak target cell killing, and production of numerous cytokines (4, 12). Surprisingly, 2DL4-mediated stimulation of MAPK, NF-κB, and MIP-1α production are largely independent of FcεRIγ association (12). Furthermore, 2DL4 can stimulate production of IL-8 in FcεRIγ-deficient 293T cells, which requires the receptor cytoplasmic domain (8), suggesting that the cytoplasmic domain contains a second signaling module to directly recruit signaling interaction partners. Recent evidence indicates that the cytoplasmic domain of 2DL4 interacts with the catalytic subunit of DNA-dependent protein kinase to stimulate the phosphorylation and activation of Akt (13), which is likely to contribute to FcεRIγ-independent activating function.

2DL4 has further been shown to internalize and accumulate in Rab5-containing early endosomes, where it has been suggested to transduce intracellular signals (8) through the association with DNA-dependent protein kinase at this intracellular site (13). It has been recently recognized that endocytosis of several receptor types can promote sustained intracellular signaling processes, including the activation of MAPK (14, 15). In contrast, roles for receptor-mediated endocytosis in downregulating receptor func-

Received for publication January 15, 2010. Accepted for publication December 15, 2010.

This work was supported by Grant R01-CA100226 (to K.S.C.), Training Grant T32-CA09035 (to S.M.S.M., A.K.P., and D.A.A.-A.), and Cancer Center Support Grant CA06927 (to Fox Chase Cancer Center) from the National Institutes of Health and an appropriation from the Commonwealth of Pennsylvania.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 2DL4, KIR2DL4; DN, dominant-negative; EEA1, early endosomal Ag 1; HA, hemagglutinin; IKK, IκB kinase; KIR, killer cell Ig-like receptor; KIR2DL4, killer cell Ig-like receptor with two Ig-like domains and a long cytoplasmic domain 4; MFI, mean fluorescence intensity; qRT-PCR, quantitative RT-PCR; RT, room temperature; shRNA, short hairpin RNA; TRAF, TNFR-associated factor; Ub, ubiquitin; WT, wild-type.

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tion have been widely appreciated, whereby internalized receptors can be sorted to degradation pathways mediated by lysosomes or the proteasome. Conjugation with ubiquitin (Ub) is a common mechanism for tagging proteins for trafficking, degradation, and signaling (16, 17). Ubiquitylation of transmembrane proteins can promote either the initial step of internalization and/or subsequent sorting from the early endosome to degradation in lysosomes or the proteasome. Ub peptides are covalently attached to lysine residues on target proteins through substrate-specific recognition by distinct Ub-protein ligases, known as E3 subunits.

Using a yeast two-hybrid approach, we have identified the RING-finger-containing E3 Ub ligase, Triad3A, as a 2DL4 cytoplasmic binding partner. We show that Triad3A interacts with 2DL4 and induces ubiquitylation and degradation of the receptor, although surface receptor expression is unchanged. Our data also show that Triad3A suppresses 2DL4-mediated cytokine production, accumulation in early endosomes, and sustained NF-κB activation, but does not affect early signaling events, suggesting that Triad3A is a critical regulator of late-term 2DL4 signaling at an intracellular site.

Materials and Methods

Cells and culture

The KHYG-1 cell line [obtained from Health Science Research Resources Bank, Japan Health Sciences Foundation; JCRB0156 (18)] was cultured in IL-2–containing α-MEM medium as previously described (5, 12). HEK-293T cells (kindly provided by Dr. Shao Cong-Sun, M.D. Anderson Cancer Center) were cultured in DMEM plus 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The retrovirus producing Phoenix-Ampho cells were cultured as described (19). Sorted primary PBMCs were co-cultured in the presence of 20% human serum for 4 days in IL-2-containing medium. The KHYG-1 cell line [obtained from Health Science Research Resources Bank, Japan Health Sciences Foundation; JCRB0156 (18)] was cultured in IL-2–containing α-MEM medium as previously described (5, 12).

Ab and reagents

Abs included: anti–FLAG mAb (M2) from Sigma-Aldrich (St. Louis, MO); anti-2DL4 mAb (53.1; mouse IgG1) and anti-NKp44 mAb (3.43.13) from Abcam or kindly provided by Dr. T.-H. Chuang (Scripps Institute, La Jolla, CA); HPV-conjugated anti-mouse IgG and anti-goat-rabbit IgG from Jackson ImmunoResearch Laboratories; mouse mAb to GAPDH (a gift of Dr. N. Davis). Other Abs and reagents and Triad3- or GAPDH-specific primers and probes on a 7500 Real-Time PCR system (all from Applied Biosystems, Foster City, CA). The mRNA copy numbers of a particular gene in each sample were normalized to the mRNA copy number of the housekeeping gene GAPDH in that sample.

Assay of JNK activity

JNK was assayed using GST-c-Jun as a substrate to measure the phosphorylation by JNK in an in vitro kinase assay (24). Briefly, GST-c-Jun (aa 1–79) was isolated on glutathione-agarose (Pierce) and used to precipitate JNK from lysates of stimulated NK cells. After immunoprecipitation, JNK-GST–c-Jun complexes were suspended in 40 mM Tris-HCl, pH 7.4, 100 mM sodium chloride, 10 mM β-glycerophosphate, 0.4 mM sodium orthovanadate, and 1 mM EDTA. Incubated for 1 h at 4 °C while mixing the assay components by vortexing, denatured with dithiothreitol (DTT) and loaded onto a 2% polyacrylamide gel. After electrophoresis, protein bands were visualized using Coomassie blue stain. For immunoblotting, the gel was subjected to 20% methanol and 5% acetic acid for 30 min, washed with 100 mM NaOH for 4 min, rinsed with water, and dried. The membrane was incubated overnight at 4 °C with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T). The membrane was then incubated for 1 h with rabbit polyclonal or mouse monoclonal Abs directed against the C-terminal of FLAG (from Sigma-Aldrich) or the C-terminal of GAPDH. After washing, the membrane was incubated for 1 h with HRP-conjugated anti-rabbit or anti-mouse IgG, respectively. Immunoreactive bands were visualized with the appropriate detection reagents (Bio-Rad, Hercules, CA). The membranes were scanned for analysis using ImP Image software (v2.0; Bio-Rad). For quantification, the integrated optical density (IOD) of each band was determined and expressed as a percentage of the IOD of the GAPDH band on each membrane. The mean ± SD of three independent experiments was calculated.

Quantitative RT-PCR

Total RNA was isolated from the RNeasy Kit (Qiagen, Valencia, CA), and quantitative RT-PCR (qRT-PCR) was performed using TaqMan PCR reagents and Triad3- or GAPDH-specific primers and probes on a 7500 Real-Time PCR system (all from Applied Biosystems, Foster City, CA). The mRNA copy numbers of a particular gene in each sample were normalized to the mRNA copy number of the housekeeping gene GAPDH in that sample.

Cellular assays

For cytokine/chemokine assays, KHYG-1 cells transduced with FLAG-2DL4 (20) were cultured for 24 h in a 96-well plate containing plate-bound mAb and culture supernatants were assayed using ELISA kits for IL-8, MIP-1α (Pierce Biotechnology), or IFN-γ (BD Pharmingen) as previously described (12).

KIR2DL4 internalization assay

The internalization assay was based on previously established assays (20, 25, 26). Cells were stained with 10 μg/ml biotinylated anti-FLAG mAb for 1 h on ice, washed twice, kept at 37 °C for the indicated times, stained with PE-conjugated streptavidin for 1 h on ice, washed, and analyzed by FACS. The percent internalization was calculated from mean fluorescence intensity (MFI) of staining at the indicated time point (t) compared with time zero (0) using the calculation: % internalization = 100 t/MFI.

KIR2DL4 turnover assay

2DL4 surface turnover was assayed as previously described (20, 27). Briefly, cells were blocked with 50 μg/ml unlabeled anti-FLAG polyclonal Ab (Sigma-Aldrich) in HBSS plus 1% FBS for 40 min at 4 °C, washed, and
incubated in α-MEM at 37°C. Cell aliquots were stained on ice with biotinylated anti-FLAG mAb and PE-streptavidin, analyzed on an FACSscan (BD Biosciences), and evaluated with FlowJo software (Tree Star). The percent turnover of 2DL4 staining = 100 × (MFI_{exp} – MFI_{blocked})/MFI_{total} – MFI_{blocked}, in which MFI_{exp} is 2DL4 staining at the indicated time, MFI_{total} is staining of untreated cells and MFI_{blocked} is staining cells blocked with unlabeled Ab and kept at 4°C. In some experiments, cells were treated with 50 μg/ml cycloheximide during blocking and throughout the assay (20).

EMSA analysis

Nuclear extracts were prepared by using a nuclear extraction kit (Panomics). For EMSA, the NF-κB consensus oligonucleotide 5′-GGGACCTTCCCG-3′ (Santa Cruz Biotechnology) was end-labeled with [γ-^32P]ATP (PerkinElmer) and Tα polynucleotide kinase (Promega) and purified using Microspin G-50 columns (GE Healthcare). Nuclear extracts were mixed with the labeled probe in 20 μl buffer (3.75 mM HEPES [pH 7.6], 1.5% v/v glycerol, 0.5 mM DTT, 0.015 mM EDTA, 0.5 μg polyinosinic:polycytidylic acid [Roche]) and incubated at room temperature (RT) for 30 min. For supershifts, Abs to the p65 subunit of NF-κB (Santa Cruz Biotechnology) were added to extracts for 30 min prior to addition of radiolabeled oligonucleotide. DNA–protein complexes were separated on 5% nondenaturing PAGE and autoradiographed.

Immunostaining and confocal microscopy

Cells were surface stained with primary anti-myc Ab (9E10) and secondary Alexa Fluor 647-conjugated anti-mouse Ig Ab (Invitrogen) on ice, incubated at 37°C for 1 to 2 h to allow receptor internalization, settled onto poly-L-lysine–coated culture slides (Lab Scientific; BD Pharmingen) for 10–30 min at RT, fixed, and permeabilized in 3 to 4% paraformaldehyde/PBS plus 0.1% Triton X-100 for 15 min, stained with anti-Rab5 or anti-early endosomal Ag (EEA1) Ab for 15 min at RT or overnight at 4°C, respectively, and Alexa Fluor 350-conjugated secondary Ab for 30 min at RT in PBSS (0.1% sodium azide). In some experiments, cells were treated with 50 μg/ml cycloheximide during blocking and throughout the assay (20).

FIGURE 1. Identification of Triad3A as a 2DL4-binding partner by yeast two-hybrid screening. The 2DL4 cytoplasmic domain (aa 858–1032) was used to identify interacting proteins in a yeast two-hybrid screen. A, Five positive clones identified in a growth assay, which all encoded the C terminus of Triad3A, also tested positive for β-galactosidase reporter activity. Results are mean fold increase LacZ activity ± SD comparing inducing conditions (galactose/raffinose; Gal/Ref) to uninduced conditions (glucose; activity = 1) from three assays. B, The 2DL4-interacting portion of Triad3A protein identified in all five clones (aa 507–866; bottom panel) includes the Triad domain (two RING domains and IBR domain) from the full protein (top panel). C, Immunoblotting for expression of Triad3 and GAPDH in total lysates of primary NK cells, KHYG-1 NK cell line, NK-92 NK cell line, NKL NK cell line, 293T cells, and 293T cells transfected with Triad3A CDNA. Results are representative of at least three experiments. D, qRT-PCR analysis of Triad3 mRNA expression in KHYG-1, primary NK, and 293T cells. Results from two experiments were normalized to GAPDH mRNA levels in each cell type and expressed as fold difference as compared with the amounts detected in KHYG-1 cells, which were set at a relative value of 1.
other proteins to mediate intracellular signaling. Therefore, we used the yeast two-hybrid system to identify proteins that interact with the 2DL4 cytoplasmic domain. Five positive clones isolated from the library screen growth assay were also found to interact with the cytoplasmic domain in a secondary assay for β-galactosidase production, as shown in Fig. 1A. All five clones contained cDNA encoding the C-terminal sequence of human Triad3 (also known as ZIN or RNF216), which exhibits E3 Ub ligase activity (29, 30). The 2DL4-interacting sequence (aa 507–866) contained the two TRIAD (two RING finger and DRIL) domains and the IBR (in between RING) region (Fig. 1B). Triad3 is one of six mRNA splice variants (designated Triad3 and Triad3A-E), all of which include the common C-terminal domain isolated in our screen. Subsequently, we focused our studies on Triad3A, which is one of the largest and most abundantly expressed forms (29). Immunoblot and qRT-PCR analysis (Fig. 1C, 1D) confirmed that Triad3A is expressed in human NK cell lines (KHYG-1, NKL, and NK-92), primary human NK cells, and 293T cells, and the size of the major endogenous protein in these cells (~130 kDa) is similar to that of transfected Triad3A.

Previous work has shown that Triad3A interacts with several TLRs, promotes their degradation by ubiquitylation, and inhibits their function (29). Triad3A also acts as a negative regulator in TNF-α receptor signaling by interacting with and inducing ubiquitylation and degradation of RIP-1 kinase, as well as degradation of the TIRAP and TRIF adaptors, all of which are important for initiating NF-κB signaling (30). Recently, it has been shown that Triad3A also targets TNFR-associated factor (TRAF) 3 for ubiquitylation and degradation and negatively regulates the RIG-I/MAVS pathway (31). These results suggested that Triad3A might similarly suppress 2DL4 functions.

**Triad3A interacts with 2DL4 in 293T cells to promote polyubiquitylation and degradation**

We tested whether Triad3A interacts with 2DL4 and promotes its degradation by cotransfecting cDNAs encoding both proteins into 293T cells. Cotransfection of Triad3A dramatically diminished the total level of 2DL4 protein detected in 293T cell lysates (Fig. 2A), which is consistent with the previously reported impacts on TLRs (29). To test whether Triad3A associates with 2DL4, we cotransfected 293T cells with increasing amounts of Triad3A cDNA and a constant amount of 2DL4 cDNA. Immunoprecipitation of the receptor showed progressively decreased 2DL4 protein recovery that correlated with increasing amounts of Triad3A protein expression and coordinate increases in the amount of Triad3A coimmunoprecipitating with the receptor (Fig. 2B).

**FIGURE 2.** Triad3A promotes polyubiquitylation and degradation of 2DL4 in 293T cells. A, Coexpression of Triad3A reduces 2DL4 expression in 293T cells. 293T cells were transfected with FLAG-2DL4 + FLAG-Triad3A; lysed 48 h later, separated by SDS-PAGE, and immunoblotted with anti-FLAG mAb. B, Triad3A associates with and induces degradation of 2DL4 protein. 293T cells were cotransfected with indicated amounts of FLAG-Triad3A- and 1 μg FLAG-2DL4-encoding plasmids, lysed 48 h later (lysates in top panel), and 2DL4 immunoprecipitates (IP in bottom panel) were immunoblotted with anti-FLAG mAb. C, Triad3A induces ubiquitylation of 2DL4. 293T cells were transfected with indicated cDNAs, lysed 48 h later, immunoprecipitated with anti-2DL4 mAb, and analyzed for Ub by anti-HA immunoblotting. D, Structure of WT-2DL4 and the 5R-2DL4 constructs. E, Direct polyubiquitylation of lysines in the cytoplasmic domain of 2DL4 by Triad3A. 293T cells were transfected with indicated combinations of cDNAs, lysed 48 h later, immunoprecipitated with anti-2DL4 mAb, and immunoblotted for Ub by anti-HA Ab. F, Analysis of lysine-dependent ubiquitylation of 2DL4 by 2D gel electrophoresis. 293T cells were transfected with FLAG-Triad3A, HA-Ub, and FLAG-WT-2DL4 (left panel) or FLAG-5R-2DL4 (right panel), lysed 48 h later, immunoprecipitated with anti-2DL4 mAb, separated by two-dimensional gel electrophoresis (isoelectric focusing/SDS-PAGE), and immunoblotted for Ub with anti-HA Ab. K, Lys; R, Arg; TM, transmembrane.
We next tested whether Triad3A also induces the ubiquitylation of 2DL4 by transfecting FLAG-tagged Triad3A, FLAG-2DL4, and HA-tagged Ub (HA-Ub) cDNAs into 293T cells. As shown in Fig. 2C, Triad3A induced strong polyubiquitylation of 2DL4 when all three cDNAs were coexpressed. Mutation of the five cytoplasmic lysines in 2DL4 (Fig. 2D) abrogated the polyubiquitylation of 2DL4 (Fig. 2E), indicating direct polyubiquitylation of at least one of these lysines by Triad3A. Two-dimensional gel electrophoresis demonstrated the extensive degree of direct 2DL4 polyubiquitylation that was disrupted by lysine mutation (Fig. 2F).

**Triad3A suppresses KIR2DL4 function in 293T cells**

A previous report showed that 2DL4 transfection into 293T cells resulted in spontaneous IL-8 production (8), suggesting that these cells may contain a ligand that engages the receptor to result in productive signal transduction. Thus, we speculated that 2DL4-mediated IL-8 production might be impacted by cotransfection with Triad3A. As shown in Fig. 3A, 2DL4 transfection indeed promoted spontaneous IL-8 production in 293T cells, and Triad3A cotransfection essentially eliminated this IL-8 production, suggesting that Triad3A-induced ubiquitylation and degradation of 2DL4 profoundly inhibits receptor function.

To demonstrate the direct involvement of Triad3A in regulating 2DL4 function, we used shRNA to knockdown expression in 293T cells. We prepared three shRNA expression constructs targeting different regions of Triad3A. Both shRNA1 and shRNA3 efficiently knocked down the cDNA-driven expression of Triad3A protein by >90% (Fig. 3B), whereas shRNA2 was ineffective. We then tested whether knockdown of Triad3A will reverse Triad3A-mediated 2DL4 ubiquitylation and restore receptor function. As expected, cotransfection of shRNA1 or shRNA3 nearly abolished 2DL4 ubiquitylation (Fig. 3B), whereas shRNA2 had no effect. Furthermore, shRNA1 and shRNA3 efficiently reversed the inhibition of 2DL4-mediated IL-8 production by Triad3A in 293T cells (Fig. 3C), demonstrating direct involvement of **FIGURE 3.** Triad3A suppresses 2DL4-mediated production of IL-8 in 293T cells. **A.** Triad3A coexpression suppresses 2DL4-mediated IL-8 production. 293T cells were transfected with FLAG-2DL4 ± FLAG-Triad3A cDNAs and tested 48 h later for IL-8 secretion by ELISA. Results are mean ± SD of single assays from eight experiments. **B.** Triad3A knockdown reduces 2DL4 ubiquitylation in 293T cells. 293T cells were transfected with cDNAs encoding combinations of FLAG-2DL4, FLAG-Triad3A, HA-Ub, and one of three shRNAs targeting Triad3A. After 48 h, cells were lysed and 2DL4 immunoprecipitates (top panel) or cell lysates (bottom panels) were immunoblotted as indicated. **C.** 2DL4-mediated IL-8 production was restored by Triad3A knockdown. 293T cells were transfected with indicated combinations of FLAG-2DL4, FLAG-Triad3A, and one of the shRNA constructs and tested 48 h later for IL-8 production by ELISA. Results are mean ± SD of single determinations from three experiments. **D.** Knockdown of endogenous Triad3A significantly enhanced 2DL4-mediated IL-8 production in 293T cells. 293T cells were transfected with shRNA3 and stably transfected cells were selected by treating with puromycin (2 μg/ml) for 7 d. The shRNA transfectants and 293T parent cells were then transfected with 2DL4 and assayed 48 h later for IL-8 secretion by ELISA (top panel). Results are mean ± SD of a single assay from three experiments. Lysates from 293T transfectants were immunoblotted with anti-Triad3A and anti-GAPDH to assess knockdown efficiency (bottom panel). Note that 10-fold less lysate was added for the Triad3A transfected sample (lane 3).
Triad3A in regulating 2DL4 function. To further confirm that endogenous levels of Triad3A impact 2DL4 function, we found that knocking down expression levels of endogenous Triad3A in 293T cells with shRNA3 significantly enhanced 2DL4-mediated IL-8 production (Fig. 3D), demonstrating the importance of physiological levels of Triad3A in controlling 2DL4 function.

2DL4-mediated cytokine production is suppressed by Triad3A in an NK cell line

We and others have previously reported that engagement of 2DL4 with plate-bound Ab stimulates the production of cytokines and chemokines in NK cell culture supernatants (2, 9, 12). To test the effect of Triad3A expression on the 2DL4-mediated induction of cytokine production, we engineered the KHYG-1 NK cell line to stably express FLAG-2DL4 alone or in combination with Triad3A by retroviral transduction. As in 293T cells, total 2DL4 protein expression in NK cell lysates was significantly suppressed by Triad3A coexpression, indicating that 2DL4 is degraded by ubiquitylation (Fig. 4A). We were surprised to find, however, that 2DL4 surface expression on the NK cell line was unaffected by Triad3A coexpression (Fig. 4B), indicating that Triad3A decreases the intracellular pool of 2DL4 while not impacting the level of surface receptor. Next, we tested the effect of Triad3A coexpression on the 2DL4-mediated activation of cytokine production in NK cells. KHYG-1 cells expressing FLAG-2DL4 alone or in combination with Triad3A were either left unstimulated or stimulated with plate-bound anti-FLAG mAb or anti-NKp44 mAb to ligate another NK cell activating receptor. Consistent with results in 293T cells, Triad3A coexpression significantly suppressed 2DL4-mediated production of IL-8, MIP-1α, and IFN-γ by the NK cell line, whereas NKp44-mediated cytokine production was unaffected (Fig. 4C–E). Our data demonstrate that expression of Triad3A selectively and efficiently suppresses 2DL4 function in NK cells, and suppression correlates with substantially decreased total levels of intracellular 2DL4, whereas surface receptor expression levels are unaffected.

Triad3A suppresses 2DL4-mediated late-term NF-κB activation without affecting early signaling

In addition to stimulating cytokine production, 2DL4 cross-linking activates MAPK (phosphorylation of JNK, ERK, and p38) and NF-κB (phosphorylation of IKKB and IκBα, and degradation of IκBα) (12). We also previously showed that all of these pathways are critical for downstream cytokine production in NK cells, and stimulation of p38 and ERK are upstream of NF-κB activation (12). Therefore, we tested whether exogenous Triad3A expression in KHYG-1 cells regulates 2DL4-mediated activation of MAPK and NF-κB. To our surprise, Triad3A expression did not suppress 2DL4-mediated phosphorylation of ERK or p38 (Fig. 5A), and the activation of JNK was unaffected (Fig. 5B). In addition, Triad3A expression did not affect early phosphorylation of Iκκα/β, which is an upstream kinase of the NF-κB activation cascade (Fig. 6A). The canonical NF-κB pathway culminates in translocation of the p65 subunit to the nucleus, where it binds to specific DNA sequences to regulate gene transcription. Therefore, we further tested the effect of Triad3A coexpression on 2DL4-mediated activation of NF-κB by EMSA. Our results show that expression of Triad3A in KHYG-1 cells consistently diminished the maintenance of p65 DNA binding activity in response to 2DL4 engagement, especially at later time points (Figs. 6B, 6C). To further confirm the negative impact of Triad3A on sustained NF-κB activation, we measured activity in 293T cells 48 h posttransfection with 2DL4 ± Triad3A using a luciferase reporter assay. Our data demonstrated that Triad3A expression nearly abolishes 2DL4-mediated activation of NF-κB (Fig. 6D). In conclusion, Triad3A does not affect the 2DL4-mediated early activation of MAPK and IKKα/β, but suppresses sustained activation of NF-κB, demonstrating that only late-term signaling is disrupted by Triad3A.

**FIGURE 4.** Triad3A significantly reduces total 2DL4 levels and suppresses 2DL4-mediated cytokine production without impacting surface receptor expression in the KHYG-1 NK cell line. A, Triad3A expression reduced the total level of 2DL4 protein in KHYG-1 cell lysates. KHYG-1 cells were transduced with FLAG-2DL4 ± FLAG-Triad3A and cell lysates were analyzed by immunoblotting with anti-Triad3A (top panel; top band is nonspecific), anti-FLAG (middle panel), and anti-GAPDH (bottom panel). B, Surface expression of 2DL4 was not affected after cotransduction of Triad3A in KHYG-1 cells. KHYG-1 cells (assayed 2 d after IL-2 stimulation) that had been stably transduced with FLAG-2DL4 alone (shaded histograms) or in combination with FLAG-Triad3A (dashed line histograms) were stained for surface expression with anti-FLAG mAb plus PE-conjugated anti-FLAG Ab or secondary alone (2ndary) by FACS. C–E, KHYG-1 cells stably transduced with FLAG-2DL4 ± FLAG-Triad3A were harvested on day 2 after IL-2 stimulation, cultured for 24 h (without IL-2) either with or without (NS) plate-bound anti-FLAG mAb (M2) or anti-NKp44 mAb (3.43.13), and analyzed by ELISA for secretion of IL-8 (C), IFN-γ (D), and MIP1-α (E). Results are representative mean ± SD from at least three experiments.
Triad3A inhibits 2DL4 turnover without affecting receptor internalization

Endocytosis is a critical event influencing the turnover, signal transduction, and biological functions of cell-surface receptors. Internalized receptors generally enter Rab5+ early endosomes, where some receptors are sorted to lysosome and proteasome compartments for degradation, whereas others can be recycled back to the cell surface (15, 32).

Previous work showed that vesicle-mediated endocytosis is required to transport 2DL4 to the Rab5+ early endosomal compartment, because expression of a dominant-negative (DN) form of dynamin inhibited 2DL4 internalization to that location (8). Dynamins are large GTPases that facilitate vesicle invagination and scission during endocytic processes, especially involving clathrin-coated vesicles or caveolae (33). In view of the DN-dynamin impact and our observations that Triad3A expression in KHYG-1 cells selectively abolished 2DL4-mediated cytokine production without altering surface expression or early signaling events, we speculated that the E3 Ub ligase might be disrupting intracellular receptor trafficking to prevent sustained NF-κB signaling and achieve cytokine production. Therefore, we examined whether Triad3A expression affects 2DL4 endocytosis, turnover, and intracellular localization in response to receptor ligation.

First, we found that Triad3A expression does not affect receptor endocytosis over time in KHYG-1 cells (Fig. 7A). Consistent with our previous findings, these data further demonstrate that Triad3A does not impact 2DL4 at the cell surface. Next, we tested whether 2DL4 receptor turnover back to the cell surface was affected by Triad3A expression. Although 2DL4 was efficiently turning over in KHYG-1 cells expressing the receptor alone, Triad3A expression significantly suppressed receptor turnover (Fig. 7B). This result was consistent when performed in the presence of the protein synthesis inhibitor cycloheximide (data not shown), indicating that Triad3A expression is not suppressing 2DL4 surface expression derived from de novo synthesis, but is instead suppressing the recycling of internalized 2DL4 back to the cell surface. Therefore, we hypothesize that Triad3A expression promotes the ubiquitylation of the majority of internalized 2DL4, which is shuttled to the proteasome or lysosome for degradation, thereby preventing recycling back to the cell surface.

Triad3A disrupts 2DL4 localization with Rab5+ early endosomes

We next determined whether vesicle-mediated endocytosis is necessary for 2DL4 to stimulate the production of IL-8 in 293T cells. As shown in Fig. 7C, cotransfection of 2DL4 in combination with DN-dynamin nearly abolished 2DL4-mediated IL-8 production, indicating a requirement for receptor internalization to trigger cytokine production. We further observed that the pharmacological dynamin inhibitor, dynasore (34), similarly blocked 2DL4-mediated IL-8 production in 293T cells in a dose-dependent manner (Fig. 7D). Although DN-dynamin expression was toxic to KHYG-1 cells, we found that dynasore also suppressed IL-8 production in response to engaging 2DL4 in the NK cell line without affecting production of the cytokine upon engaging NKp44 (Fig. 7E). Our results demonstrate that 2DL4-mediated cytokine production requires receptor internalization, which is consistent with recent work by Rajagopalan et al. (13).

Previous evidence indicates that 2DL4 localization into the Rab5+ early endosomes is a critical step to induce cytokine production (8, 13), so we tested whether Triad3A expression alters the localization of 2DL4 to early endosomes after 2DL4 ligation in KHYG-1 cells. Cells expressing myc-tagged 2DL4 either with or
Triad3A expression suppresses sustained activation of NF-κB in response to 2DL4 ligation. A, KHYG-1 cells transduced with FLAG-2DL4 ± FLAG-Triad3A (day 2 after IL-2 culture) were stimulated with soluble anti-FLAG mAb (M2; 10 μg/ml) for the indicated time or PMA (P; 10 nM) + ionomycin (I; 1 μM) (10 min). Cell lysates were immunoblotted with anti–phospho-IKKα/β (pIKKα/β) and anti-GAPDH. B, 2DL4 ligation-induced nuclear DNA binding activity of NF-κB–p65 complexes. KHYG-1 cells expressing FLAG-2DL4 ± FLAG-Triad3A were stimulated with soluble anti-FLAG mAb (10 μg/ml) for indicated time or PMA and ionomycin (P/I) for 120 min. Nuclear extracts were preincubated with radiolabeled dsNF-κB oligonucleotide probe and analyzed on nondenaturing PAGE. The last lane shows anti-p65 Ab supershift (ss) analysis of a PMA/ionomycin-stimulated extract. Results are representative of at least three experiments. C, The p65 DNA binding band in B was quantified with ImageJ software (National Institutes of Health), and plot shows percent binding activity compared with unstimulated control at time zero (= 100%) from B. D, Triad3A expression suppressed 2DL4-stimulated NF-κB luciferase reporter activity. 293T cells were transfected with NF-κB renilla luciferase reporter plasmid in combination with FLAG-2DL4 ± FLAG-Triad3A cDNAs and luciferase activity in lysates was assessed 48 h later. Results are mean ± SD of relative units NF-κB activity from six experiments. NS, nonspecific band.

without Triad3A were surface stained with anti-myc Ab and fluorophore-conjugated secondary Ab, incubated at 37°C for 1 to 2 h to allow for receptor internalization, fixed, permeabilized, and stained for the early endosomal markers Rab5 (Fig. 8A; 8B, Supplemental Fig. 1) or EEA1 (Fig. 8C; 8D, and Supplemental Fig. 2). When 2DL4 was expressed alone, ~15% of the receptor entered the Rab5/EEA1 compartment under these cross-linking conditions (Fig. 8, Supplemental Figs. 1, 2), although the fraction accumulating in the early endosomes with our cellular system was significantly lower than shown in previous work by Rajagopal et al. (8, 13). Nonetheless, when cells coexpressed Triad3A, the 2DL4 appeared to be distributed more at the periphery of the cells or in cellular extensions, and only ~5% of the receptor colocalized with Rab5 or EEA1 (Fig. 8, Supplemental Figs. 1, 2). Taken together, our data demonstrate that Ab-engaged 2DL4 is endocytosed, and some localizes to Rab5+/EEA1+ early endosomes.

Furthermore, expression of Triad3A suppressed 2DL4 recycling to the cell surface and promoted trafficking of internalized 2DL4 away from the early endosome, which was previously shown to be a site of intracellular 2DL4 accumulation and signaling (8, 13). Importantly, we also showed that function of another NK cell activating receptor, NKp44, was not impacted by expression of Triad3A in the same NK cells. Therefore, we conclude that Triad3A selectively controls intracellular 2DL4 function.

It is well established that polyubiquitylation of lysine residues can tag transmembrane, cytosolic, and nuclear proteins for sorting to multivesicular bodies and subsequent transport to proteasomal or lysosomal degradation (16, 35). Several shuttle factors, such as RAD23, Dsk2/Ub, and DDI1, contain both a Ub-binding domain and a proteasome-targeting motif, which bind to polyubiquitylated cargo proteins and direct their transport to the proteasome, respectively (36). Evidence is accumulating that attachment of different ubiquitylation branching arrays to proteins results in distinct functional outcomes: 1) polyubiquitylation chains linked at the Lys48 (K48) residue of the Ub molecules generally targets proteins to the proteasome for degradation; 2) K63-linked polyubiquitylation initiates intracellular signaling or trafficking; and 3) monoubiquitylation can alter protein activity, promote endocytosis, or target to lysosomal degradation (16). Our observation that polyubiquitylation of 2DL4 by Triad3A target only internalized receptors away from the early endosomes to a degradation pathway is consistent with K48-linked branching. Furthermore, a recent report demonstrated that Triad3A

Discussion

We have identified the E3 Ub ligase Triad3A (29) as an important negative regulator of 2DL4 function. This is the first report, to our knowledge, describing the regulation of KIR by ubiquitylation. Our data demonstrate that Triad3A recruitment promotes the direct polyubiquitylation and degradation of 2DL4, and expression of the E3 ligase dramatically reduces total expression of the receptor in cell lysates. However, Triad3A expression did not influence 2DL4 surface expression, endocytosis, or early signaling events triggered through the receptor. Nonetheless, Triad3A expression significantly suppressed cytokine production and blunted sustained NF-κB signaling in response to 2DL4 engagement. Furthermore, expression of Triad3A suppressed 2DL4 recycling to the cell surface and promoted trafficking of internalized 2DL4 away from the early endosome, which was previously shown to be a site of intracellular 2DL4 accumulation and signaling (8, 13). Importantly, we also showed that function of another NK cell activating receptor, NKp44, was not impacted by expression of Triad3A in the same NK cells. Therefore, we conclude that Triad3A selectively controls intracellular 2DL4 function.

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can incorporate K48-linked polyubiquitin chains (31). Other examples of ubiquitylation-mediated trafficking of proteins away from early endosomes include the diubiquitylation of Ig-β of the B cell–Ag receptor complex by Itch (37) and the monoubiquitylation of Lst2 protein (38), although neither of these proteins are targeted to the proteasome.

**FIGURE 7.** Triad3A alters 2DL4 turnover and vesicle-mediated internalization is required for cytokine production. A, 2DL4 internalization was not affected in KHYG-1 cells cotransduced with Triad3A. KHYG-1 cells transduced with FLAG-2DL4 ± Triad3A were assayed for 2DL4 internalization as in Materials and Methods. Results are mean ± SD of eight experiments. B, 2DL4 turnover was suppressed in KHYG-1 cells cotransduced with Triad3A. KHYG-1 cells expressing FLAG-2DL4 ± Triad3A were assayed for 2DL4 turnover as in Materials and Methods. Results are mean ± SD from three experiments. C, DN dynamin blocks 2DL4-mediated IL-8 production in 293T cells. 293T cells were not transfected (NT) or transfected with FLAG-2DL4 ± DN-dynamin and tested 48 h later for IL-8 production by ELISA. D, Dynasore inhibits 2DL4-mediated IL-8 production in 293T cells. 293T cells were transfected with FLAG-2DL4 and cultured with indicated concentrations of dynasore for 48 h and tested for IL-8 secretion by ELISA. E, 2DL4-mediated IL-8 production in KHYG-1 cells was suppressed by dynasore. KHYG-1 cells pretreated with 40 μM dynasore for 30 min were stimulated with either plate-bound anti-FLAG mAb (M2) or anti-NKp44 (3.43.13) and tested 24 h later for IL-8 secretion by ELISA.

**FIGURE 8.** Intracellular trafficking of engaged 2DL4 to early endosomes is reduced by Triad3A. KHYG-1 cells transduced to express myc-2DL4 ± Triad3A were stained with anti-myc Ab + AlexaFluor 647-labeled secondary Ab on ice, cultured at 37°C for 2 h (A, B) or 1 h (C, D), settled onto poly-L-lysine–coated slides, fixed, permeabilized, and stained with polyclonal Ab to early endosomal markers (Rab5, A, B; or EEA1, C, D) plus secondary Ab conjugated with Alexa Fluor 350. Cells were imaged by bright field and immunofluorescence microscopy for myc (2DL4; red) and Rab5 or EEA1 (green). A, Bright field and overlaid max-projected fluorescence images of all z-stack sections from three representative cells stained for Rab5 from each condition (± Triad3A expression). B, Mean ± SE percentage area of 2DL4 compartmentalized with Rab5 was quantified using Meta Vue software from max-projected images of z-stacks of 12–15 cells for each bar. C, Bright field and overlaid max-projected fluorescence images of all z-stack sections stained with EEA1 from three representative cells from each condition. D, Mean ± SE percentage area of 2DL4 compartmentalized with EEA1 was quantified using NIS-Elements software from all of the individual z-sections from 15 cells for each bar (∼200 data points/bar). Percentage values were compared using the two-tailed Mann–Whitney U test, and resulting p values are listed in B and D. Original magnification ×60.
We previously reported that 2DL4-mediated cytokine production requires activation of MAPK and downstream activation of NF-kB (12). In this study, we have found that Triad3A overexpression in the NK cell line KHYG-1 does not affect 2DL4-mediated early activation profiles of ERK, JNK, p38, and IKK-α/β. We conclude that these 2DL4-mediated signaling events occur at the cell surface, and the surface expression and membrane-proximal function of 2DL4 were unchanged in Triad3A-expressing cells. The reduction of total 2DL4 protein levels by Triad3A was most likely due to ubiquitylation-mediated degradation of the intracellular pool of receptor, and we observed a coordinate reduction in sustained NF-kB activation, which we conclude to be critical for transducing intracellular signals leading to cytokine release. In this model, 2DL4 signals sequentially, first from the cell surface and subsequently from an intracellular endosomal compartment. Taken together, our data demonstrate that both early MAPK stimulation and sustained activation of NF-kB are necessary for the induction of 2DL4-mediated cytokine production, and Triad3A disrupts the latter signal.

The Triad3A-mediated reduction in sustained activation of NF-kB is consistent with growing evidence that the E3 ligase targets the NF-kB pathway, particularly in innate immune responses. Chuang et al. (29) reported that Triad3A interacts with distinct TLRs (TLR4, -5, and -9, but not TLR2) and downregulates their function. Importantly, the same report demonstrated that Triad3A inhibited the capacity of these TLRs to stimulate NF-kB reporters by promoting receptor ubiquitylation and degradation via the proteasome. Triad3A has also been shown to act as a negative regulator of TNF-α- and IL-1–mediated activation of NF-kB by associating with and promoting the degradation of the NF-kB signaling proteins RIP-1, TIRAP, and TRIF (30, 39). Recently, Triad3A was also shown to interact with and induce ubiquitylation and degradation of TRAF3 (31). Triad3A was further shown to negatively regulate RIG-I/MAVS-mediated NF-kB activation through targeting TRAF3, thereby modulating innate immune receptor responses toward several invading pathogens (31). In view of the impacts of Triad3A on the receptor-proximal NF-kB signaling pathway, we cannot rule out that direct recruitment of the E3 ligase to 2DL4 may similarly promote the ubiquitylation and degradation of receptor-associated signaling proteins within the NF-kB pathway. Our data, however, are to our knowledge the first to demonstrate that Triad3A specifically influences late-term NF-kB signaling at an intracellular site, but does not disrupt membrane proximal events.

Six different alternative spliced forms of Triad3 have been reported, named Triad3 and Triad3A–E (29), and all of these contain the C-terminal domain found to interact with 2DL4 in our yeast screen. The major protein form that we have identified in human NK cells migrates nearly identical to that of Triad3A in SDS-polyacrylamide gels. The major protein form that we have identified in human NK cells migrates nearly identical to that of Triad3A in SDS-PAGE. Although our studies have therefore focused on the impacts of the common Triad3A form, it is possible that other differentially spliced variants also influence 2DL4 function.

Our data indicate that 2DL4 ubiquitylation by Triad3A is occurring subsequent to internalization, and this prevents accumulation of the receptor in Rab5+/EEA1+ early endosomes to disrupt function. It has been shown that 2DL4 is internalized in a dynamin-dependent manner to early endosomes after receptor ligation in NK cells or 293T cells (8). Consistent with a recent report (13), we show that DN-dynamin and the dynamin inhibitor dynasore suppress 2DL4-induced production of IL-8 in both 293T cells and NK cells, indicating a major role for internalized receptor in cytokine production. Although it is believed that ligand-induced endocytosis is a mechanism of receptor inactivation, numerous studies have shown that receptors can remain active within the endosomal compartment (14, 15), and the temporal dynamics of receptor signaling can dramatically influence functional outcomes (40). Our results indicate that the cytokine-producing function of 2DL4 requires intracellular shuttling of the receptor to early endosomes to stimulate prolonged signaling, which is suppressed by Triad3A. In this way, polyubiquitylation of 2DL4 by Triad3A prevents surface recycling and shuttles the receptor away from the early endosomes, thereby negatively regulating 2DL4 function by limiting the lifetime of its active signaling state at this intracellular site.

Acknowledgments

We thank Drs. Dietmar Kappes and Siddharth Balachandran for constructive advice on the manuscript, Dr. Suresh Basagoudanavar for technical assistance, Dr. Edna Cukierman for assistance in analyzing the immunofluorescence microscopy data, Dr. Xin-Ming Li for two-dimensional gel electrophoresis, and Drs. Erica Golemis, Roger Brent, Brent Passerin, and Ilya J. Serebriiskii for advice and reagents during the yeast two-hybrid screening. We also thank Drs. Marco Colonna, Garry Nolan, Lynn Heasley, Tung-Hsein Chuang, Keiji Tanaka, Shao Cong-Sun, Kiyonao Sada, and Masato Yagita for reagents, and special thanks also to the DNA Synthesis, DNA Sequencing, Flow Cytometry, Biochemistry and Biotechnology, and Cell Culture Facilities at Fox Chase Cancer Center.

Disclosures

The authors have no financial conflicts of interest.

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