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The AP-2 Clathrin Adaptor Mediates Endocytosis of an Inhibitory Killer Cell Ig-like Receptor in Human NK Cells

Amanda K. Purdy,* Diana A. Alvarez Arias,*,† Jennifer Oshinsky,* Ashley M. James,* Ilya Serebriiskii,‡,§ and Kerry S. Campbell*

Stable surface expression of human inhibitory killer cell Ig-like receptors (KIRs) is critical for controlling NK cell function and maintaining NK cell tolerance toward normal MHC class I* cells. Our recent experiments, however, have found that Ab-bound KIR3DL1 (3DL1) readily leaves the cell surface and undergoes endocytosis to early/recycling endosomes and subsequently to late endosomes. We found that 3DL1 internalization is at least partially mediated by an interaction between the μ2 subunit of the AP-2 clathrin adaptor complex and ITIM tyrosine residues in the cytoplasmic domain of 3DL1. Disruption of the 3DL1/μ2 interaction, either by mutation of the ITIM tyrosines in 3DL1 or mutation of μ2, significantly diminished endocytosis and increased surface expression of 3DL1 in human primary NK cells and cell lines. Furthermore, we found that the 3DL1/AP-2 interaction is diminished upon Ab engagement with the receptor, as compared with untreated cells. Thus, we have identified AP-2–mediated endocytosis as a mechanism regulating the surface levels of inhibitory KIRs through their ITIM domains. Based on our results, we propose a model in which nonengaged KIRs are internalized by this mechanism, whereas engagement with MHC class I ligand would diminish AP-2 binding, thereby prolonging stable receptor surface expression and promoting inhibitory function. Furthermore, this ITIM-mediated mechanism may similarly regulate the surface expression of other inhibitory immune receptors.

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atural killer cells selectively recognize and kill virus-infected and transformed cells while remaining tolerant of normal cells (1, 2). Their activation is controlled by a balance of signals from activating (αNKRs), adhesion, and inhibitory (iNKRs) surface receptors (3). Activation is dominantly suppressed upon engagement of iNKRs (especially the human killer cell Ig-like receptors [KIRs]) with MHC class I (MHC-I) expressed on normal cells. With few exceptions, normal cells elicit NK cell tolerance through their high expression of MHC-I and low expression of ligands for αNKRs (4). However, following genotoxic stress (5) or virus infection (6), αNKRs can be upregulated and/or MHC-I downregulated on target cells to tip the balance toward NK cell activation and targeted cytotoxicity.

KIR inhibitory function centers around their cytoplasmic ITIMs [(I/Y)Vxx(L/V)] (3). KIR engagement with MHC-I ligands results in 1) phosphorylation of ITIM tyrosine residues with subsequent recruitment of SHP-1 and SHP-2 protein tyrosine phosphatases that dominantly suppress αNKR signaling pathways, and 2) induced tyrosine phosphorylation of the adaptor Crk, which relocalizes from activating to inhibitory complexes (7–9). These events terminate early NK cell activation signaling and establish tolerance toward normal MHC-I–expressing cells.

The surface levels of KIRs or their cognate ligands can directly impact the activation thresholds of NK cells (10, 11), but little is known regarding the mechanisms regulating the surface expression of KIRs. Generally, receptor surface expression can be controlled by de novo protein synthesis, endocytosis, recycling back to the cell surface, and protein degradation. With respect to KIRs, both KIR3DL2 and KIR2DL4 can relocalize from the cell surface to endosomes to mediate intracellular functions (12, 13). Furthermore, polymorphic sequence variants of KIRs can exhibit wide disparities in surface expression (14, 15). Protein kinase C–dependent phosphorylation of Ser298 also appears to stabilize the surface expression of KIR3DL1 (3DL1), and other sequence motifs, including the first ITIM tyrosine have been implicated in regulating surface expression (16, 17). These reports demonstrate a need for better mechanistic understanding of KIR endocytosis and intracellular trafficking.

Mammalian cells can internalize receptors constitutively or in response to specific stimuli via either clathrin-dependent or -independent endocytosis (18–20). Clathrin forms a triskelion structure that drives endocytic vesicle formation but requires adaptors to bind surface receptors. The AP-2 clathrin adaptor is directly implicated in the internalization of many receptors, including transferrin receptor (TIR), low-density lipoprotein receptor, and epidermal growth factor receptor (21–23). AP-2 is a heterotetrmeric complex composed of α- and β-adaptin that interact with clathrin and the plasma membrane, μ2, which associates with cargo containing tyrosine-based motifs, and σ2, which is involved in binding cargo-containing dileucine-based motifs.
(19, 21). Although the mechanism of KIR endocytosis is unknown, the CD94/NKG2A iKIR is reportedly internalized by a macropinocytosis-like pathway, although the sequence elements involved remain undefined (24).

In this study, we demonstrate that the ITIM sequences of iKIRs, in addition to their role in negative signaling, also provide a handle for 3DL1 internalization. This internalization occurs through interaction with μ2 of the AP-2 clathrin adaptor complex. Our data also suggest that AP-2 association may occur more readily when KIRs are not engaged with MHC-I ligand, whereas interaction with MHC-I ligand may reduce AP-2 association, which would promote stable KIR surface expression to prolong inhibitory function.

### Materials and Methods

**Cells and culture**

KHYG-1, NKL, Jurkat, HEK293T and LentiX 293T cells (Clontech, Mountain View, CA) were cultured as described (8, 25, 26). Healthy volunteer blood donors (N ≥ 10) consented as approved by the Fox Chase Cancer Center Institutional Review Board. Primary CD56+ CD3+ KIR3DL1+/− NK cells were sorted by FACS and cultured in RPMI 1640 medium plus 5% human serum, 10% FBS, and 500–1000 U/ml recombinant human IL-2 (Roche, provided by the National Cancer Institute Biologic Resources Branch, Frederick, MD). Some primary NK cells were restimulated with either irradiated RPMI 8666 cells or irradiated allogeneic PBMCs as described (27).

**Microscopy**

Following attachment to prewarmed poly-l-lysine slides (BD Pharmingen, San Jose, CA), NK cells expressing 3DL1-cherry and EYFP-Rab4 or EGFP-Rab7 (1 d after passage and stimulation with IL-2) were cooled on a Nikon Eclipse Ti-U microscope (Nikon, San Diego, CA) for 30 min. Slides were subsequently washed three times in room temperature PBS to remove excess mAb, warmed to 37˚C for 30 min, and washed three times in PBS at room temperature again. Cells were fixed in prewarmed PBS containing 3% paraformaldehyde for 15 min and then labeled with DX9–brilliant blue 421 (BioLegend, San Diego, CA) for 30 min. Slides were analyzed on a Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) microscope equipped with a 405 nM laser for nuclear staining and 488/405 and 561/568 nM lasers for fluorescence detection. Images were analyzed with ImageJ software.

**Yeast two-hybrid screen**

A LexA-based yeast two-hybrid screen was performed as described (25). For the screen, the human 3DL1*0010101 cytoplasmic domain (aa 340–423) was generated from pGEX–4T1 plasmid (GE Healthcare, Piscataway, NJ) in BL21 bacteria, purified on glutathione-agarose (Thermo Scientific, Rockford, IL), incubated with HEK293T cell lysates, and washed as previously described (16, 32). Adsorbed proteins were separated by SDS-PAGE, transferred to polyvinyliden difluoride, and immunoblotted with anti-μ2 Ab (Sigma-Aldrich, St. Louis, MO) or anti-α-adaptin mAb (BD Biosciences, Mountain View, CA).

**GST pull-down assay**

GST-3DL1 fusion proteins (wild-type [WT] and Y to A mutant 3DL1 cytoplasmic domains; aa 340–423) were generated from pGEX–4T1 plasmid (GE Healthcare, Piscataway, NJ) in BL21 bacteria, purified on glutathione-agarose (Thermo Scientific, Rockford, IL), incubated with HEK293T cell lysates, and washed as previously described (16, 32). Adsorbed proteins were separated by SDS-PAGE, transfereed to polyvinyliden difluoride, and immunoblotted with anti-μ2 Ab (Sigma-Aldrich, St. Louis, MO) or anti-α-adaptin mAb (BD Biosciences, Mountain View, CA).

**Immunoprecipitation and immunoblotting**

For experiments in Fig. 2, 3DL1* KHYG-1 cells were lysed in mRIPA buffer (1% IGEPAL CA-630, 0.5% sodium deoxycholate, 150 mM NaCl, 10 mM Tris [pH 7.5], 0.1% SDS, 2 mM sodium orthovanadate, and 1 μg/ml each of aprotinin, leupeptin, and soybean trypsin inhibitor [Sigma-Aldrich]), 3DL1 was immunoprecipitated with protein G–agarose (Millipore, Billerica, MA) precoupled with DX9 mAb. Proteins were separated as above, immunoblotted with anti-α-adaptin mAb (BD Transduction Laboratories) and rabbit anti-KIR Ab (16), followed by HRP-conjugated secondary Ab. The resulting blots were visualized with ECL (Millipore) and exposure to autoradiography film (Denville Scientific, Metuchen, NJ). The intensities of μ2, α-adaptin, and KIR bands were quantified using ImageJ software. The ratio of μ2 or α-adaptin/KIR protein levels for WT was arbitrarily set to 100%. In experiments for Fig. 5, KHYG-1 cells (4 d after passage and IL-2 stimulation) were stimulated for 15 min with pervanadate alone, 7.5 μg DX9 alone (a saturating concentration), or both in combination for 10 min on ice prior to lysis, as described (33). These cells were lysed in IP buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 μg/ml protease inhibitors as above, 2 mM sodium orthovanadate, 1 mM NaF, 2 mM EGTA, and 0.5% Triton X-100). 3DL1 was immunoprecipitated from these lysates with 5.133 μM Ab coupled to cyanogen bromide–activated Sepharose beads (GE Healthcare). Proteins were separated as above and immunoblotted with anti-α-adaptin polyclonal Ab (Proteintech, Chicago, IL), 4G10 mAb (Millipore, anti-SHP-1 polyclonal Ab (Santa Cruz Biotechnology, Dallas, TX), and anti-KIR polyclonal Ab (no. ARP53462; Aviva, San Diego, CA) followed by anti-mouse IR680 and anti-rabbit IR800 (LI-COR Biosciences, Lincoln, NE). Proteins were visualized as described (26). Band intensity was quantified with ImageJ.

**Retroviral and lentiviral constructs and transduction**

To disrupt human μ2 (NM_001025205; American Type Culture Collection, Manassas, VA) binding to tyrosine-based motifs, residues D176 and W420 were mutated to alanine (μ2 dominant negative [DN]). To visualize KIR localization within the endosomal compartment, 3DL1*00101 fusion in-frame with mCherry (Genewiz, South Plainfield, NJ) and EYFP-Rab4 or EGFP-Rab7 (gift from Dr. Mario Zerial, Max Planck Institute, Dresden, Germany) were subcloned into pBMN-NoGFP to generate retroviruses as described (26, 34). For primary NK cell expression, constructs were subcloned into pCDH-EF1-MCS-T2-A-copGFP (System Biosciences, Mountain View, CA) to generate lentivirus by transfecting LentiX 293T cells (35). Primary NK cells were infected in LentiX 293T cells with pCDH, pMD2.G (VSV-G), and psPAX2 (gag/pol) plasmids (from Dr. Sam Kung, University of Manitoba, Winnipeg, MB, Canada). Lentiviral supernatants were harvested 48–72 h after infection, concentrated by ultracentrifugation or polyethylene glycol precipitation. Viral titers were determined in Lentix 293T cells (35). Primary NK cells were infected on 2 consecutive days with lentivirus (multiplicity of infection of 20–40 in 8 μg/ml Polybrene). To alleviate compensatory mechanisms resulting from long-term expression of exogenous proteins, cells were assayed 48–72 h after the second infection.

**FACS-based KIR internalization assay**

NK cell lines and primary NK cells were always assayed 3–4 d after passage into fresh IL-2–containing medium to improve consistency of the results. NK cells were stained with anti-3DL1 (PE-conjugated DX9) or anti-CD71 (TIR; BioLegend) mAbs at 4˚C. A sample was left on ice (0 min) and...
remaining cells were incubated at 37°C for 5–180 min. Cells were moved to ice-cold HBSS plus 1% FBS plus 0.1% NaCl, stained with Alexa Fluor 647–conjugated anti-mouse IgG (Invitrogen, Grand Island, NY) at 4°C, and analyzed by flow cytometry on a BD LSR II (BD Biosciences). Cells aggregates were gated out by forward scatter height versus forward scatter area analysis, and viable NK cells were gated by forward scatter height versus side light scatter and lack of propidium iodide (Invitrogen) staining. Lentiviral-transduced cells were subgated into GFP versus side light scatter and lack of propidium iodide (Invitrogen) stain-

After Ab engagement, 3DL1 moves to early/recycling and late endosomes

Previously, we provided evidence that 3DL1 internalizes and recycles back to the cell surface in NK cells (16). In that work, we showed that turnover of surface 3DL1 on transduced NK-92 cells was not changed during a 4-h assay when 50 μg/ml cycloheximide was added to the cells, indicating that 1) surface expression is quite stable, 2) recycling is occurring, and 3) trafficking to degra-

Results

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turalization of Ab-labeled KIR3DL1 moving from the cell surface at time 0 min to merge with intracellular compartments that include Rab4+ endosomes by 15 min and Rab7+ late endosomes by 30 min.

The μ2 subunit of AP-2 interacts with the cytoplasmic domain of 3DL1

As a means to identify proteins responsible for 3DL1 internali-

zation, we performed a yeast two-hybrid screen using the cyto-

plasmic domain of 3DL1 as bait. In this screen, we identified five clones encoding the μ2 component of the AP-2 clathrin adaptor complex (residues 146–435; data not shown). The 3DL1/μ2 inter-

受 was subsequently confirmed in yeast, along with the interaction of μ2 with the cytoplasmic domain of CD5 (which directly interacts with μ2) (38) but not CD4 (which can only interact indirectly with μ2 through the HIV protein Nef) (39) (Fig. 2A). The μ2 protein interacts with cargo containing tyrosine-based motifs (Y=X-X-φ; X is any amino acid and φ is a hydro-

phobic residue) (19). 3DL1 has two potential μ2 binding sites located within the N- and C-terminal ITIMs, that is, VTY377AQL and IIY407TEL, respectively. We mutated each tyrosine to alanine and assayed their interactions with μ2 in a yeast two-hybrid re-

porter assay. Individual Y377A and Y407A mutants exhibited significantly decreased interaction with μ2, with the Y377A mu-

tant being most affected (Fig. 2B). Disruption of both tyrosines completely abrogated the μ2/3DL1 interaction.

We next engineered WT and mutant 3DL1 cytoplasmic domains as recombinant GST fusion proteins and probed 293T (Fig. 2C) or KHYG-1 cell lysates (data not shown) for interaction with the AP-

2 complex. Consistent with the yeast two-hybrid results, 3DL1-

WT interacted with both μ2 and α-adaptin of AP-2. In this assay, consistent with the yeast reporter assay, mutation of either Y377 alone or both tyrosines to alanine eliminated interaction with μ2 or α-adaptin, whereas the Y407A mutation only partially disrup-

ted binding (Fig. 2C). Taken together, these in vitro data and the in vivo findings in yeast indicate that the μ2 subunit of AP-2 interacts with the cytoplasmic ITIM tyrosines of 3DL1. Whereas Y377 is crucial for interaction with μ2, Y407 contributes but is less imperative to binding.

We also tested whether AP-2 could be coimmunoprecipitated with full-length 3DL1 from NK cells. 3DL1 was isolated from a sorted subset of either 3DL1- or 3DL1+ KHYG-1 cells and probed for AP-2 by immunoblot. Consistent with the GST pull-
down data, α-adaptin coimmunoprecipitated with 3DL1 (Fig. 2D).

AP-2 promotes 3DL1 internalization through interaction with ITIM tyrosines

In view of our observations by confocal microscopy that DX9 mAb causes endocytosis of 3DL1, we quantified endocytosis by first labeling cell surface 3DL1 with PE-conjugated DX9 at 4°C, incu-
bating the cells for various times at 37°C, and then staining with a fluorescein-tagged secondary Ab to determine the amount of DX9 retained on the cell surface (see Materials and Methods). To determine whether disruption of the 3DL1/μ2 interaction affects internalization of 3DL1, we compared the endocytic rate of WT and Y377/407A (AA) receptor upon Ab binding in NKl cells. The rate of internalization of 3DL1-WT was slow, with <25% endocy-
tosed by 30 min (Fig. 3A), consistent with our microscopy studies (Fig. 1). In contrast, internalization of the 3DL1-AA mu-
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endocytosis and surface level of TfRs were consistent in these same cells expressing 3DL1-WT or 3DL1-AA (Fig. 3A, 3B).

These data demonstrate that the methodology used to generate KIR-expressing NKL cells (e.g., retroviral transduction) did not globally affect receptor endocytosis and that the 3DL1-AA mutation specifically impacted 3DL1. To confirm that the results represented receptor internalization, rather than dissociation of primary DX9 or TfR Ab, we compared the changes in MFIs of differentially fluorophore-conjugated primary and secondary Abs throughout the time course of the assay. Consistent with internalization, we observed a significant decrease in secondary Ab surface staining fluorescence over time, which did not track with a similar decrease in primary Ab fluorescence during the same time course (Supplemental Fig. 1). We also compared the inter-

FIGURE 1. 3DL1 localizes to early and late endosomes after Ab engagement. (A) Nonradioactive colorimetric assay comparing the cytotoxicity of either KIR− (squares; NKL) or 3DL1-Cherry+ NKL cells (circles; KIR+ NKL) against 721.221 target cells lacking (No KIR ligand) or expressing the 3DL1 ligand HLA-B*051 (KIR ligand) at various NK cell/target cell (NK:T) ratios. The means ± SD of data from five independent experiments are shown. For each experiment, the data were first divided by the “NKL, No KIR ligand” sample (arbitrarily set to 100%) to generate the percentage of total cytotoxicity. Statistical analysis comparing matched samples for each NK cell line was calculated using the Student t test. *p ≤ 0.05, **p ≤ 0.01. (B) 3DL1 and Rab4/ Rab7 localization in representative NKL cells 1 d after IL-2 stimulation. Following fixation, cells expressing 3DL1-Cherry and either EYFP-Rab4 or EGFP-Rab7 were labeled with brilliant blue 421–conjugated DX9 (BV421-DX9). Shown are maximum projected images of six 0.3-μm slices through the center of representative individual cells. Cherry, 3DL1-Cherry (red); αKIR, brilliant blue 421–conjugated DX9 mAb bound to cell surface 3DL1 (blue); Rab4, EYFP-Rab4 (green); Rab7, EGFP-Rab7 (green). (C) Compilation of data as in (B). Thresholded Manders coefficients of 3DL1-Cherry colocalized with DX9 mAb (cell surface), Rab4 (Rab4+ endosome), and Rab7 (Rab7+ endosome). The median of each data set is indicated by a horizontal bar. Data are pooled from two independent experiments where each icon represents a separate cell. Black-filled icons designate values derived from the representative NK cells shown in panel (B). (D and E) Intracellular localization of 3DL1 with Rab4 (D) or Rab7 (E) is shown in representative NKL cells after 0–30 min exposure to DX9 mAb. 3DL1-Cherry+ NKL cells were stained with brilliant blue 421–conjugated DX9 mAb on ice to label the receptor at the cell surface and then kept on ice (time 0) or incubated at 37˚C for 15 or 30 min. Shown are maximum-projected images of six 0.3-μm slices through the center of representative individual cells. (F–H) Compilation of data as in panel (C) Thresholded Manders coefficients of DX9 mAb colocalized with Rab4 or Rab7 (F, top and bottom panels, respectively), 3DL1-Cherry colocalized with Rab4 and Rab7 (G, top and bottom panels, respectively), and DX9 mAb colocalized with 3DL1-Cherry in the same cell populations (H). Black-filled icons designate values derived from the representative NK cells shown in panels (D) or (E). Statistical analysis used the Wilcoxon rank sum test. *p ≤ 0.05, **p ≤ 0.01. Data are pooled from two independent experiments where each icon represents a separate cell. All images were taken using a 60× oil objective.
FIGURE 2. The μ2 subunit of AP-2 interacts with the ITIM tyrosine residues of 3DL1. (A) EGY50 yeast cells were cotransformed with prey plasmid encoding μ2 (aa 146–435) and bait plasmids encoding 3DL1, CD5, or CD4 cytoplasmic domains or empty vector (vector). Yeasts were plated on leucine-deficient medium (+ 2% glucose [Glu, left] or 2% galactose + 0.2% raffinose [Gal, right]) to induce protein expression. Results are means ± SD of β-galactosidase assays of EGY50 cells cotransformed with prey plasmid containing μ2 and bait plasmid with cytoplasmic domains of WT or alanine mutant 3DL1 (YA, AY, or AA). Data are individual determinations from four independent experiments, and paired values derived from individual experiments are connected by lines. Statistical analysis used the Student t test. *p ≤ 0.05, **p ≤ 0.01. (B) Quantitative β-galactosidase assay of EGY50 cells cotransformed with prey plasmid containing μ2 and bait plasmid with cytoplasmic domains of WT or alanine mutant 3DL1 (YA, AY, or AA). Data are means ± SD of β-galactosidase production from three experiments. (C) GST fusion proteins exposed to 293T cell extracts were immunoblotted with anti-μ2 or anti-α-adaptin Ab. The degree of μ2 and α-adaptin pulldown was determined by quantifying the ratio of μ2 or α-adaptin/KIR protein levels for Y to A mutants relative to WT (set to 100%). Data are representative of three independent experiments. (D) 3DL1′ (lane 1 only) and 3DL1 + KHYG-1 cells were lysed, immunoprecipitated with anti-3DL1 mAb, and immunoblotted for α-adaptin, SHP-1, and KIR. E, empty lane; L, ladder; WCL, whole-cell lysate. Approximate protein molecular mass is indicated on the left-hand side of each immunoblot. Data are representative of two independent experiments.

FIGURE 3. Mutation of the ITIM tyrosines slows 3DL1 internalization in NK cells. (A) Internalization assay and (B) surface expression of 3DL1 (left panels) and TIR (CD71, right panels) in NKL cells expressing WT (●) or AA mutant (○) 3DL1. Cells were labeled with PE-conjugated anti-3DL1 or anti-CD71 mAbs at 4˚C and incubated for 0–30 min at 37˚C. The remaining surface-bound mAb was labeled with Alexa Fluor 647–conjugated anti-mouse IgG at 4˚C. The percentage internalized was quantified by FACS. Surface expression (DX9 mAb MFI) was measured at time 0 min. Results in (A) and (B) are from the same four experiments, and paired values derived from individual experiments are connected by lines. (C) KIR internalization was assessed using an acid-stripping assay in Jurkat T cells (see Materials and Methods). The fraction of 3DL1 internalized was quantified during a 2-h time course (left panel), and surface expression was assessed at time 0 min (right panel) in Jurkat T cells that were retrovirally transduced to express 3DL1-WT or 3DL1-AA. Results are individual determinations from four independent experiments, and paired values derived from individual experiments are connected by lines. (D) Internalization assay (performed as in (A), left panel) and surface expression at time 0 min (right panel) of 3DL1-WT or 3DL1-AA expressed in primary NK cells by lentiviral transduction. 3DL1−NK cells sorted from three healthy donors were transduced to express 3DL1-WT or 3DL1-AA. Presented data are from seven experiments using cells derived from six independent transductions. Paired values derived from individual experiments are connected by lines, and values from individual donors are represented by distinct icons. Statistical analysis used the Student t test. *p ≤ 0.05, **p ≤ 0.01. (E) Representative histogram of 3DL1-WT and 3DL1-AA surface expression from an experiment shown in (D).

Expression of DN AP-2 reduces 3DL1 internalization
We next tested the impact of expressing a DN form of the μ2 subunit of AP-2 on 3DL1 surface expression and internalization in primary NK cells. A D176A/W421 mutant of μ2 (designated μ2-DN) disrupts the interaction of μ2 to Y-X-X-Φ--bearing cargo (similar to TRs and KIRs) without affecting either the formation of human primary NK cells. 3DL1-AA internalization was also significantly delayed in primary NK cells, and surface expression was significantly elevated compared with 3DL1-WT (Fig. 3D, 3E). Collectively, we conclude that the endocytosis of 3DL1 depends, at least partially, on the cytoplasmic ITIMs, because tyrosine mutation significantly slowed internalization and increased surface expression in cell lines and primary NK cells.

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Next, we compared surface expression and internalization rates in human primary NK cells. We used lentiviral transduction and sorting to express 3DL1-WT or 3DL1-AA in CD3+CD56+3DL1+ human primary NK cells. 3DL1-AA internalization was also significantly delayed in primary NK cells, and surface expression was significantly elevated compared with 3DL1-WT (Fig. 3D, 3E). Collectively, we conclude that the endocytosis of 3DL1 depends, at least partially, on the cytoplasmic ITIMs, because tyrosine mutation significantly slowed internalization and increased surface expression in cell lines and primary NK cells.
of the AP-2 complex or the internalization of dileucine motif-based cargo (21, 40, 41). Because primary NK cells express very low levels of TIRs (our unpublished observations), we first showed that μ2-DN expression effectively delayed internalization and increased surface expression of TIRs in KHYG-1 cells (Fig. 4A). We also measured surface levels of 3DL1 in NKL cells following expression of μ2-DN. Importantly, μ2-DN expression caused a significantly greater increase in the surface levels of TIRs than in KIR surface levels on NKL cells. Furthermore, the impact was transient, as the elevation of cell surface expression for both receptors was lost after 1 wk of culture (Supplemental Fig. 2). From these results, we conclude that KIR surface expression levels are more tightly regulated than TIRs, and compensatory mechanisms rapidly diminish the efficacy of μ2-DN in NK cell lines. To avoid these compensatory mechanisms, we next analyzed the impact of short-term μ2-DN expression on 3DL1 internalization in primary NK cells. To this end, μ2-WT or μ2-DN were next expressed by lentiviral transduction in 3DL1+ human primary NK cells, and the transduced populations were identified by coordinate GFP expression (Fig. 4B). Expression of μ2-DN significantly delayed endocytosis (Figs. 4C, 4D) and increased surface expression of 3DL1 (Fig. 4D) as compared with expression of μ2-WT (Fig. 4C) or control transduction with empty vector (Fig. 4D). In contrast, lentivirus infection and resulting GFP expression alone did not impact 3DL1 internalization or surface expression (Supplemental Fig. 3). These data confirm that the AP-2 clathrin adaptor can significantly contribute to the endocytosis of 3DL1 and thereby influence the levels of receptor surface expression on NK cells.

The KIR/AP-2 interaction is regulated by Ab binding to 3DL1

The ability of KIRs to inhibit NK cell cytotoxicity is dependent on tyrosine phosphorylation of the ITIM tyrosines. Because we have found that AP-2 associates with KIRs through these same tyrosines, we next tested whether the KIR/AP-2 interaction is regulated by the phosphorylation state of these tyrosines. We hypothesized that because μ2 binds unphosphorylated tyrosines (19), the KIR/AP-2 interaction would be enhanced when the KIR ITIMs are not phosphorylated, but decreased when the ITIM tyrosines are phosphorylated. To test this hypothesis, we immunoprecipitated 3DL1 from unstimulated cells or cells stimulated with 1) pervanadate (PV) alone to induce robust and stable tyrosine phosphorylation, 2) DX9 mAb alone, which mimics MHC-I engagement and should transiently increase ITIM phosphorylation (36, 42), or 3) both together. In accordance with previous publications (8, 43), PV-treated cells exhibited a high degree of KIR tyrosine phosphorylation and SHP-1 association. Furthermore, we found that the association of the α-adaptin subunit of AP-2 was significantly diminished following receptor engagement with DX9 in the presence or absence of PV, whereas PV alone reduced α-adaptin association only modestly, which did not reach statistical significance (Fig. 5). Collectively, these data show that the KIR/AP-2 association is most pronounced in unmanipulated cells, and although tyrosine phosphorylation can reduce the association, Ab engagement seems to further promote AP-2 displacement. In fact, DX9 mAb engagement alone significantly displaced α-adaptin binding but did not induce tyrosine phosphorylation above baseline in this assay. We were unable to reproducibly observe differences in 3DL1 expression levels on NK cells that had been conjugated with target cells bearing or lacking HLA-B*51 ligand (data not shown). This could be due to inefficiency of ligand engagement under these conditions, however, resulting in only a minor fraction of the total surface 3DL1 being affected, thereby limiting detection of changes in surface levels on a per cell basis. This is consistent with the work of Treanor et al. (44) that showed that only a small fraction of KIRs in an immune synapse are phosphorylated in microclusters. In contrast, DX9 Ab has the potential to bind all of the 3DL1 on the cell surface, and if DX9 binding is consistent with ligand engagement, our data suggest that KIRs may be more susceptible to AP-2–dependent internalization when not engaged with ligand, whereas engagement with ligand would displace AP-2 to stabilize the receptor on the surface, where it can mediate prolonged inhibitory signaling to maintain NK cell tolerance.

Discussion

Our results show that 3DL1 can be slowly internalized, first to early/recycling endosomes and subsequently to late endosomes (Fig. 1). Moreover, at least part of this endocytic process is me-

![FIGURE 4](http://www.jimmunol.org/)
The KIR/AP-2 interaction is regulated by KIR engagement and ITIM phosphorylation. (A) 3DL1 was immunoprecipitated (IP) from unstimulated (Unstim) 3DL1+ KHYG-1 cells or the same cells after treatment for 10 min on ice with PV, DX9 mAb (DX9), or PV and DX9 mAb (PV+DX9). IPs were immunoblotted for phosphoryrosine (PY), α-adaptin, SHP-1, and KIR. (B) Compilation of data from five experiments performed as in (A). Band intensities were quantified by ImageJ, and the relative band intensity was calculated as a ratio to the intensity of the KIR band in each lane. Each icon represents an independent experiment with the mean value shown as a horizontal bar. The immunoblot shown in (A) was used to generate the band intensity data designated by the gray-filled square icons in (B). Statistical analysis used the Student t test. \( p \leq 0.05, \quad \ast \ast p \leq 0.01. \)

Sequence motifs in KIRs that exist outside of the cytoplasmic domain have also been implicated in contributing to endocytosis. Upon binding to CpG oligodeoxynucleotides, KIR3DL2 reportedly relocalizes from the cell surface to early endosomes, thereby transporting the CpG to interact with TLR9 at that location (12). Surprisingly, relocalization in that context was reportedly independent of the cytoplasmic domain, because truncation distal to the transmembrane domain had no impact upon KIR3DL2 internalization. In that report, 3DL1 internalization was also observed upon binding with CpG DNA, although to a lesser extent than KIR3DL2. Furthermore, KIR2DL4, a unique activating receptor that contains a single ITIM (49), can also internalize to early endosomes, where it can mediate intracellular signaling or be degraded following ubiquitylation (13, 25). Published data, however, suggest that internalization of KIR2DL4 is independent of the transmembrane and cytoplasmic domains, because a chimeric receptor consisting of the extracellular domain of KIR2DL4 and transmembrane/cytoplasmic domains of the plasma membrane-localized gp49B receptor was also targeted to endosomes (50). These studies further reinforce the functional relevance of KIR endocytosis and the roles of sequence elements outside of the cytoplasmic domain in mediating internalization. Our work has
identified the interaction of the AP-2/clathrin complex with the cytoplasmic ITIMs of 3DL1 as one mechanism shunting iKIRs from the cell surface. Although this mechanism is expected to also be operational for other iKIRs, further analysis is warranted to specifically examine the contributions of ITIM/AP-2 interactions on the endocytosis of KIR3DL2 and KIR2DL4.

Given that KIR-dependent inhibition of NK cell activation is rapid (tyrosine phosphorylation and SHP-1/SHP-2 recruitment occur within minutes of ligand engagement) (51), whereas the rate of 3DL1 internalization in human primary NK cells is slow (only 19.7 ± 7.25% internalized by 30 min; Figs. 3D, 4C, 4D), it is unlikely that AP-2-mediated endocytosis of 3DL1 contributes directly to inhibitory function. It is possible, however, that this AP-2-mediated mechanism may also be involved in the KIR-dependent physical transfer of HLA molecules from target cells into NK cells that has previously been reported (52). Alternatively, our data and the consistent expression levels of 3DL1 on the surface of NK cells suggest that KIRs normally undergo constitutive internalization and recycling to maintain inhibitory capacity and tolerance. Although we cannot rule out involvement of other endocytic mechanisms regulating KIR surface expression, our findings define a new functional role for the ITIMs on 3DL1.

CTLA4 has been shown to recruit AP-2 through a non-ITIM tyrosine phosphorylation and SHP-1/SHP-2 recruitment mechanism (64), which is consistent with our data. Alternatively, an AP-2–mediated mechanism may also be involved in the KIR-dependent physical transfer of HLA molecules from target cells into NK cells that has previously been reported (52). Alternatively, the rate of 3DL1 internalization in human primary NK cells is slow (only 19.7 ± 7.25% internalized by 30 min; Figs. 3D, 4C, 4D), it is unlikely that AP-2-mediated endocytosis of 3DL1 contributes directly to inhibitory function. It is possible, however, that this AP-2-mediated mechanism may also be involved in the KIR-dependent physical transfer of HLA molecules from target cells into NK cells that has previously been reported (52). Alternatively, our data and the consistent expression levels of 3DL1 on the surface of NK cells suggest that KIRs normally undergo constitutive internalization and recycling to maintain inhibitory capacity and tolerance. Although we cannot rule out involvement of other endocytic mechanisms regulating KIR surface expression, our findings define a new functional role for the ITIMs on 3DL1.

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Disclosures

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References


