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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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Natural 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 (aNKR), adhesion, and inhibitory (iNKR) 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 aNKRs (4). However, following genotoxic stress (5) or virus infection (6), aNKR ligands 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/V)xyYxx(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 aNKR signaling pathways, and 2) induced tyrosine phosphorylation of the adaptor Crk, which relocates 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 Ser394 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 (TfR), low-density lipoprotein receptor, and epidermal growth factor receptor (21–23). AP-2 is a heterotetrameric 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, NKI, Jurkat, HEK293T and LentiX 293T cells (Clontech, Mountain View, CA) were cultured as described (8, 25, 26). Healthy volunteer blood donors were recruited by informed consent 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 Pharbio, San Jose, CA), NKI cells expressing 3DL1-cherry and EYFP-Rab4 or EGFP-Rab7 (1 d after passage and stimulation with IL-2) were cooled on ice for 15 min and then labeled with DX9–brilliant blue 421 (BioLegend, San Diego, CA) for 30 min. Slides were subsequently washed three times in PBS at room temperature and fixed in prewarmed PBS containing 3% paraformaldehyde for 15 min at room temperature. Slides were mounted with 0.16 mm coverslips (Thermo Fisher, Pittsburgh, PA) in ProLong Gold antifade reagent (Life Technologies, Eugene, OR). Images were acquired using a rigorous method developed by Manders et al. (28, 29), which measures the degree of overlap of pixels in two separate fluorescent channels independent of their intensity and relative to the total intensity within each channel. Manders coefficients were quantified for each channel independent of their intensity and relative to the total intensity which measures the degree of overlap of pixels in two separate fluorescent channels. Manders coefficients were quantified for each time point. 3DL1 internalization was analyzed by quantifying the colocalization of DX9 mAb and Rab4 or Rab7. Colocalization was quantified using a rigorous method developed by Manders et al. (28, 29), which measures the degree of overlap of pixels in two separate fluorescent channels independent of their intensity and relative to the total intensity within each channel. Manders coefficients were quantified for each 0.3-μm slice of each z-stack with the Just Another Colocalization Plugin in ImageJ after thresholding (http://imagej.nih.gov/ij/, http://rsbweb.nih.gov/ij/plugins/track/jacop.html). The resulting coefficients were compared for significance with the Wilcoxon rank sum test using R software (R Foundation; http://www.r-project.org). A Manders coefficient of 0 corresponds to nonoverlapping distribution, whereas a value of 1 signifies 100% colocalization. To better visualize the colocalization in Fig. 1D, six of the 0.3-μm slices from the center of representative cells were merged as a maximum projection to generate the images shown.

Cytotoxicity assay

The CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) was performed according to the manufacturer’s instructions. Briefly, 721.221 cells (1 × 10^5; lacking or expressing the 3DL1 ligand [HLA-B8]) were mixed with NKI cells to achieve NK cell/target cell ratios of 12:1 to 10:1. Cells were incubated together in a U-bottom plate at 37°C in a humidified atmosphere of 7% CO2. After 4 h, supernatants were harvested, exposed to substrate, and absorbance (490 nm) was measured on a BioTek EL808 microplate reader (BioTek, Winooski, VT). Background absorbance from a blank media control was subtracted from all values. Target cell maximum was determined after target cell incubation with basis buffer, whereas spontaneous values were computed from NK or target cells alone. Specific lysis was calculated as: 100 × [experimental absorbance − NK cell spontaneous − target cell spontaneous]/[target cell maximum − target cell spontaneous].

Yeast two-hybrid screen

A LexA-based yeast two-hybrid screen was performed as described (25). Briefly for the screen was the human 3DL1*0010101 cytoplasmic domain (aa 340–423, http://www.ebi.ac.uk/ipd/kir) fused with the LexA-DNA binding domain, which was expressed from the pEG202 plasmid. Control bait constructs encoded the cytoplasmic domains of CD5 (aa 402–495) or murine CD4 (aa 418–457) (30, 31). The 3DL1 ITIM tyrosines were mutated to alanines (Y377A, Y407A, and Y377/407A) using QuikChange II (Stratagene, Santa Clara, 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, transferred to polyvinylidine 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% IGEPSH, 630, 0.5% sodium deoxycholic acid, 150 mM NaCl, 10 mM Tris [pH 7.5], 0.1% SDS, 2 mM sodium orthovandate, 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 10 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-CHCl [pH 7.5], 150 mM NaCl, 1 μg/ml protease inhibitors as above, 2 mM sodium orthovandate, 1 mM NaF, 2 mM EGTA, and 0.5% Triton X-100). 3DL1 was immunoprecipitated from these lysates with 5.133 mMAb 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. AFR53462; 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*001 fusion in-frame 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-NeoGFP to generate retroviruses as described (26, 34). For primary NK cell expression, constructs were subcloned into pCDH-CEF-MCS-T2A-copGFP (System Biosciences, Mountain View, CA) to generate lentivirus to transfect 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 later, filtered (0.45 μm), and 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 (TR; 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% NaN₃, 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). Cell 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 sub gated into GFP⁺ (uninfected) and GFP⁺ (infected) populations. For each sample, the percentage internalization = 100 – [F₂ [Ab mean fluorescence intensity (MFI) at t min]/Ab MFI at 0 min] × 100]. Jurkat T cells (see Fig. 3C) were subjected to an acid wash stripping assay as described (16). Briefly, following internalization, cells were treated with 200 μM HBSS containing 100 mM glycine and 100 mM NaCl (pH 2.5) at 4°C to remove surface-bound Ab. Cells were washed twice with HBSS plus 1% FBS, and 3DL1 expression was analyzed by FACS.

Results

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 degradation pathways is minimal in the absence of de novo protein synthesis. To better define the subcellular distribution of 3DL1 and determine whether 3DL1 relocates to endosomal compartments following internalization from the cell surface, we expressed a fluorescently tagged version of 3DL1 (3DL1-Cherry) in NKL cells. First, we confirmed that the C-terminal tag did not disrupt inhibitory function (Fig. 1A), which is consistent with a previous report using 3DL1-EGFP (36). Next, we quantified the amount of 3DL1 expressed on both the cell surface and within the endosomal compartment in fixed NKL cells coexpressing 3DL1-Cherry and either EYFP-Rab4, which marks early/recycling endosomes, or EGFP-Rab7, a late endosomal marker. As expected, a significant fraction of 3DL1-Cherry localized to the plasma membrane (median Manders coefficient of 0.3165 and 0.466 in EYFP-Rab4- and EGFP-Rab7-expressing cells, respectively), consistent with its established role in inhibitory signaling (Fig. 1B) (3). We also found a significant amount localized to punctate internal structures, with a small pool coinciding with Rab4⁺ endosomes (median Manders coefficient of 0.0615, Fig. 1C, left) and a more sizeable fraction colocalizing with Rab7⁺ endosomes (median Manders coefficient of 0.222, Fig. 1C, right). Taken together, these data indicate that 3DL1 traffics from the cell surface through the endosomal compartments.

To visualize internalization specifically, we labeled 3DL1 expressed on the cell surface with the DX9 mAb at 4°C and quantified the colocalization of the anti-3DL1 mAb with EYFP-Rab4 (Fig. 1D) and EGFP-Rab7 (Fig. 1E) at 0–30 min of internalization at 37°C (37). We found a significant increase of anti-3DL1 mAbs colocalizing in Rab4⁺ endosomes at 15 min, which stabilized to a similar degree at 30 min (Fig. 1E, top). In contrast, significant colocalization of anti-3DL1 mAbs in Rab7⁺ late endosomes did not occur until the 30 min time point (Fig. 1E, bottom). Similarly, we also observed a significant increase in 3DL1-Cherry colocalized with Rab4⁺ endosomes at 15 min and with Rab7⁺ endosomes at 30 min (Fig. 1G, top and bottom, respectively). We also observed a significant increase in anti-KIR mAbs colocalizing with 3DL1-Cherry at 15 and 30 min (Fig. 1H), presumably due to an accumulation of the Ab-bound receptor with denser pools in endosomal compartments after internalization. Taken together, these data are consistent with a slow rate of internalization 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 internalization, we performed a yeast two-hybrid screening using the cytoplasmic 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 interaction 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 hydrophobic residue) (19). 3DL1 has two potential μ2 binding sites located within the N- and C-terminal ITIMs, that is, VTY⁴³⁷AQY and ILY⁴⁰⁷TEL, respectively. We mutated each tyrosine to alanine and assayed their interactions with μ2 in a yeast two-hybrid reporter assay. Individual Y377A and Y407A mutants exhibited significantly decreased interaction with μ2, with the Y377A mutant 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 disrupted 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, incubating the cells for various times at 37°C, and then staining with a fluorophore-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% endocytosed by 30 min (Fig. 3A), consistent with our microscopy studies (Fig. 1). In contrast, internalization of the 3DL1-AA mutant was significantly delayed in NKL cells as compared with 3DL1-WT (Fig. 3A). Also, surface expression of 3DL1-AA was consistently higher than 3DL1-WT (Fig. 3B), indicating that disruption of association with the AP-2 clathrin adaptor results in accumulation of 3DL1 on the NK cell surface. In contrast, the rate
of endocytosis and surface levels 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).
normalization of both 3DL1-WT and 3DL1-AA in Jurkat T cells using an acid-stripping protocol. Again, 3DL1-AA was internalized at a slower rate and expressed at higher surface levels, as compared with 3DL1-WT (Fig. 3C). Unfortunately, primary NK cells and NK cell lines were found to be extremely sensitive to acid wash, which restricted the use of this assay to Jurkat cells.

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.

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

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
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/)

Expression of DN μ2 (μ2-DN) delays internalization and increases surface expression of 3DL1 in primary NK cells. (A) TIR internalization (top panel) and surface expression (bottom panel) were determined as in Fig. 3 in control (−), μ2-WT–expressing, or μ2-DN–expressing KHYG-1 cells. Results are from individual determinations at 10 min (top panel) or 0 min of internalization (bottom panel) from four independent experiments, with values derived from individual experiments connected by lines. (B) Infected primary NK cells are marked by GFP expression following lentiviral transduction, and μ2-DN–expressing cells exhibit reduced surface expression of 3DL1. 3DL1+ primary human NK cells were infected with lentivirus containing μ2-WT or μ2-DN. The percentage of GFP+ and GFP− cells for each condition is indicated. Bottom panels: 3DL1 surface expression in the GFP+ populations at time 0 and following 150 min at 37˚C with MFI of 3DL1 is shown. (C) Data from three experiments performed as in (B) comparing 3DL1+ primary cells infected with lentivirus containing μ2-WT or μ2-DN. Paired values derived from individual experiments are connected by lines, and different donors are represented as distinct icons. (D) 3DL1 internalization (left panel) and surface expression (right panel) are shown in primary NK cells infected with lentivirus (Lenti) generated with empty vector (−) or μ2-DN (DN) lentivirus. Shown are 12 experiments with NK cells from five healthy donors (separate icon/donor), and paired values derived from individual experiments are connected by lines. Statistical analysis used the Student t test. *p ≤ 0.05, **p ≤ 0.01.
performed as in (A) the mean value shown as a horizontal bar. The immunoblot shown in (A) band in each lane. Each icon represents an independent experiment with relative band intensity was calculated as a ratio to the intensity of the KIR
square icons in (A) was used to generate the band intensity data designated by the gray-filled mAb (PV+DX9). IPs were immunoblotted for phosphotyrosine (pY), the primary function of TfRs is to internalize iron.

NK cells from attacking normal MHC-I+ cells in the body, whereas
cordance with the critical role that these receptors play in tolerizing
expression is more tightly controlled than TfRs, presumably through
endocytosis of both receptors, we conclude that 3DL1 surface ex-
3DL1 (Fig. 4A, Supplemental Fig. 2). Based on these observa-
endocytosis and the roles of sequence elements outside of the
emphasize that endocytosis of TfRs, which is considered to pri-
Sequence motifs in KIRs that exist outside of the cytoplasmic
diately by interaction between 1) the μ2 subunit of the AP-2 clathrin adaptor complex and 2) the ITIM motifs in the KIR cyto-
plasmic domain (Figs. 2–4). Our findings are consistent with a previous report by Chwae et al. (45) that found AP-2 interaction with a chimeric 3DL1 receptor construct in Jurkat T cells; how-
ever, an ITIM-mediated basis for the interaction was not defined. The same group also provided evidence that the N-terminal ITIM
and several additional sequence elements in the 3DL1 cytoplasmic domain are involved in endocytosis of this chimeric receptor in response to treating the Jurkat cells with protein kinase C agonists (17). We cannot rule out alternative mechanisms that can also mediate KIR endocytosis, but our experiments studied the full-length receptor to characterize the role of AP-2 binding to ITIM tyrosines in primary NK cells. Furthermore, it is important to emphasize that endocytosis of TfRs, which is considered to pri-
arily involve AP-2/clathrin (21), was diminished to a similar degree as 3DL1 by expression of μ2-DN in our experiments (Fig. 4). Alternatively, μ2-DN expression resulted in significantly greater elevation of TIR surface expression as compared with 3DL1 (Fig. 4A, Supplemental Fig. 2). Based on these observa-
tions, although AP-2/clathrin can mediate similar degrees of endocytosis of both receptors, we conclude that 3DL1 surface ex-
pression is more tightly controlled than TfRs, presumably through
more efficient recycling of KIRs back to the cell surface. The more efficient retention of iKIR expression on the cell surface is in ac-
cordance with the critical role that these receptors play in tolerizing
NK cells from attacking normal MHC-I+ cells in the body, whereas
the primary function of TIRs is to internalize iron.

Following engagement with MHC-I at the immune synapse, KIRs are phosphorylated on ITIM tyrosines in aggregated micro-
clusters (44), leading to the recruitment of SHP-1/SHP-2 and inhibitory signaling (3, 7, 8). Because μ2 associates with unphosphorylated tyrosine-based motifs, we expected μ2 to in-
teract with 3DL1 and induce endocytosis only when not engaged with ligand. A similar mechanism has been described for CTLA-4, on which phosphorylation disrupts recruitment of μ2 to a cyto-
plasmic tyrosine to regulate endocytosis (46, 47). Although we
were surprised that PV-induced tyrosine phosphorylation of 3DL1 did not significantly displace α-adaptin binding, it is possible that the pool of 3DL1 associated with AP-2 was not efficiently phos-
phorylated under these conditions. Instead, we found that the KIR/
AP-2 interaction is most profoundly diminished following en-
gagement with DX9 Ab in the presence or absence of PV (Fig. 5). The lack of significant detectable tyrosine phosphorylation of
3DL1 by treatment with DX9 alone suggests that Ab-mediated displacement of AP-2 may result through a mechanism indepen-
dent of ITIM tyrosine phosphorylation. It is possible that Ab
binding induces additional changes in the receptor cytoplasmic
domain (in addition to just tyrosine phosphorylation) to more
effectively dissociate the clathrin adaptor. Although this mecha-
nism has not been defined, if Ab binding is characteristic of ligand
gengagement, our results suggest that engaged KIRs are maintained at the target cell interface to mediate prolonged inhibitory signaling and sustained self-tolerance toward normal MHC-I-bearing cells. Furthermore, although DX9 engagement for 10 min decreased the interaction of KIRs with AP-2 (Fig. 5), the Ab-engaged receptor was ultimately slowly internalized by an ITIM/AP-2–
dependent process during a longer time course, as shown in our internalization assays (Figs. 3, 4) and microscopy studies (Fig. 1). In contrast, our data further imply that AP-2–mediated endocy-
tosis of 3DL1 would presumably occur more readily when NK

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 TL9 at that location (12).
Surprisingly, relocalization in that context was reportedly inde-
pendent of the cytoplasmic domain, because truncation distal to
the transmembrane domain had no impact upon KIR3DL2 inter-
nalization. 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, how-
ever, 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

![FIGURE 5. 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 phosphotyrosine (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 ≤ 0.05, **p ≤ 0.01.](http://www.jimmunol.org/ Downloaded from)
identified the interaction of the AP-2/clathrin complex with the cytoplasmic ITIMs of 3DL1 as one mechanism shutting 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-1/2-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 (46, 47, 53), but to our knowledge, our data are the first to demonstrate that AP-2 can internalize an inhibitory receptor through an ITIM binding site. This mechanism may more generally target additional ITIM-bearing receptors for endocytosis.

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Disclosures

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