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

*J Immunol* published online 19 September 2014
http://www.jimmunol.org/content/early/2014/09/19/jimmunol.1303406

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/09/19/jimmunol.1303406.DCSupplemental

Why *The JI*?
- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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.

Received for publication December 20, 2013. Accepted for publication August 21, 2014.

This work was supported by National Institutes of Health Grants CA083859 (to K.S.C.), CA009035 (to A.K.P. and D.A.A.A.), and CA06927 (to the Fox Chase Cancer Center), 2014. 2014. 193: 000–000.

The online version of this article contains supplemental material.

Abbreviations used in this article: AA, Y377F407A; aNKR, activating NKR; 3DL1, killer cell Ig-like receptor 3DL1 (or KIR3DL1); DN, dominant negative; iNKR, inhibitory NKR; KIR, killer cell Ig-like receptor; MFI, mean fluorescence intensity; MHC-I, MHC class I; PV, pervanadate; TIR, transferrin receptor; WT, wild-type.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/S16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1303406
(19, 21). Although the mechanism of KIR endocytosis is unknown, the CD94/NKG2A, iNKR 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) were recruited by appointment at 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 Pharningen, San Jose, CA), NK cells expressing 3DL1-cherry and EYFP-Rab4 or EGF-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. 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 60× oil objective with EZ-C1 3.80 software on an inverted Nikon TE2000 with a C1 confocal scan head (Nikon, Melville, NY). Z-stacks (0.3 μm step size) were collected from at least 25 cells expressing high levels of Rab and 3DL1 for each time point. 3DL1 internalization was analyzed by quantifying the colocalization of DX9 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 ¥ 105) lacking or expressing the 3DL1 ligand [HLA-B51+] were mixed with NK cells to achieve NK cell/target cell ratios of 1:125:1 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. Effector to target ratios were computed for each target cell 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). Bait 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 QuickChange 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 polyvinylidene 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 deoxycholic acid, 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 4 h 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 mAb 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–SHIP-1 polyclonal Ab (Santa Cruz Biotechnology, Dallas, TX), and anti-KIR polyclonal Ab (no. ARPS3462; 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*0010101 fused in frame to mCherry (Geneviz, South Plainfield, NJ) and EYFP-Rab4 or EGF-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-EP1-MCS-T2A-copGFP (System Biosciences, Mountain View, CA) to generate lentivirus by transfecting Lentix 293T cells with pCDH, pMD2.G (VSVG-V), and psPAX2 (gag/pol) plasmids (from Dr. Sam Kung, University of Manitoba, Winnipeg, MB, Canada). Lentiviral supernatants were harvested 48–72 h later, filtered, and concentrated by ultracentrifugation or polyelectlyene 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% 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). Cells aggregated 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⁺ (infected) and GFP⁻ (uninfected) populations. For each sample, the percentage internalization = 100 – [(2˚ Ab mean fluorescence intensity (MFI) at t min/2˚ Ab MFI at 0 min) × 100]. Jurkat T cells (see Fig. 3C) were subjected to an acid wash stripping assay as described (16). Brefeld, following internalization, cells were treated with 200 µg/ml 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 relocalizes 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 punctuate 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. 1F, top). In contrast, significant colocalization of anti-3DL1 mAbs in Rab7⁺ late endosomes did not occur until the 30 min time point (Fig. 1F, 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 screen 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, VTY³⁷⁷AQL 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 coinmunoprecipitated 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 coinmunoprecipitated 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 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 TIR Ab, we compared the changes in MFI 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).
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⁺ 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.
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.
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 interact 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 cytoplasmic 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 phosphorylated under these conditions. Instead, we found that the KIR/AP-2 interaction is most profoundly diminished following engagement 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 independent 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 mechanism has not been defined, if Ab binding is characteristic of ligand engagement, 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 endocytosis of 3DL1 would presumably occur more readily when NK cells are engaged with MHC-I-deficient cells, thereby more efficiently removing the iKIRs from the immune synapse to allow more efficient cytotoxicity. aKIRs (KIR2DS, KIR3DS) lack full efficient removing the iKIRs from the immune synapse to allow more efficient cytotoxicity. aKIRs (KIR2DS, KIR3DS) lack full IIITMs and would therefore not be able to directly recruit AP-2. It is possible that the aKIRs can be endocytosed through another mechanism, however, including potential μ2 binding to the ITAM tyrosines of the associated DAP12 adaptor, similar to a recent report of AP-2-mediated BCR internalization through interaction with an ITAM on CD79b (48).

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

![](http://www.jimmunol.org/)
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 KIR3DL1 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 (46, 47, 53), but to our knowledge, our data are the first to provide evidence 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.

Acknowledgments

We thank Drs. David Wiest and Alana O’Reilly for constructive critique of the manuscript, Drs. Sam Kung and Erica Golemis for reagents and advice, Drs. Alexander MacFarlane IV and Sam Litwin for help with the Wilcoxen rank sum test, and the DNA Sequencing, Flow Cytometry, Bioinformatics and Biostatistics, and Cell Culture Facilities at the Fox Chase Cancer Center for materials and technical support.

Disclosures

The authors have no financial conflicts of interest.

References


The following references are included in the text:


The following references are included in the text:


The following references are included in the text:


The following references are included in the text:


The following references are included in the text:


The following references are included in the text:


Figure S1. Decrease in secondary antibody fluorescence is not due to a loss of primary antibody staining during internalization assay. Compilation of data from 3DL1-WT experiments in Fig. 3A comparing the MFI of PE-conjugated DX9 or TfR (primary Ab; Bottom) and AlexaFluor 647-conjugated anti-mouse IgG (secondary Ab; Top) in NKL cells at 0-30 min of internalization. The mean of 4 independent experiments is represented by a black line with filled in icons. p values were generated from the paired Students t-test, n.s. = not significant, * designates ≤ 0.05 and ** denotes ≤ 0.01.
Figure S2. µ2-DN expression results in a transient elevation in surface levels of TfR and KIR on NKL cells. Mean fluorescence intensity (MFI) measurements of TfR and 3DL1 surface levels on NKL cells were determined by FACS on the indicated days after infection with retrovirus to express µ2-DN (open bars) or in control (uninfected; filled bars). The mean ± S.D. of triplicate samples are shown for each time point, with corresponding p values generated from the paired Students t-test where * designates ≤ 0.05, ** denotes ≤ 0.01, and n.s. = not significant.
Figure S3. Lentivirus infection alone does not significantly affect the rate internalization or surface expression of 3DL1. Compilation of data from experiments in Fig. 4 comparing the percent internalization (Left panel) or MFI of surface expression (Right panel) of 3DL1 in primary NK cells infected with lentivirus generated with empty pCDH vector (GFP⁺ = infected, GFP⁻ = not infected). Differences between the groups were not significant (n.s.) using the Student’s t test.