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NK cells are key components of the immune response to virally infected and tumor cells. Recognition of target cells initiates a series of events in NK cells that culminates in target destruction via directed secretion of lytic granules. Ral proteins are members of the Ras superfamily of small GTPases; they regulate vesicular trafficking and polarized granule secretion in several cell types. In this study, we address the role of Ral GTPases in cell-mediated cytotoxicity. Using a human NK cell line and human primary NK cells, we show that both Ral isoforms, RalA and RalB, are activated rapidly after target cell recognition. Furthermore, silencing of RalA and RalB impaired NK cell cytotoxicity. RalA regulated granule polarization toward the immunological synapse and the subsequent process of degranulation, whereas RalB regulated degranulation but not polarization of lytic granules. Analysis of the molecular mechanism indicated that Ral activation in NK cells leads to assembly of the exocytosis, a protein complex involved in polarized secretion. This assembly is required for degranulation, as interference with expression of the exocyst component Sec5 led to reduced degranulation and impaired cytotoxicity in NK cells. Our results thus identify a role for Ral in cell-mediated cytotoxicity, implicating these GTPases in lymphocyte function. The Journal of Immunology, 2011, 187: 2433–2441.

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atural killer cells are essential for the elimination of tumor and virally infected cells. In NK cells, recognition of target cells triggers several signaling cascades that culminate in the formation of the immunological synapse. The synapse allows directional secretion of lytic granules to the target cell membrane. These granules contain perforin, granzyme, and other hydrolytic enzymes able to induce apoptosis in target cells. Cell-mediated cytotoxicity proceeds through several precisely regulated steps; target recognition is followed by extensive reorganization of the actin cytoskeleton and formation of a stable conjugate between the cytotoxic and the target cell. Shortly after conjugate formation, the secretory machinery is polarized. The microtubule-organizing center (MTOC), normally located near the nuclear membrane, moves toward the contact zone between the two cells. The lytic granules then move along the microtubules toward the synapse; when they reach the plasma membrane at the secretion site, the granules dock and fuse to the membrane, releasing their enzymatic content (1). Several molecules such as Rab27, Munc13-4, and syntaxin 11 are implicated in granule docking and fusion (2–4), but the molecular mechanisms that regulate granule secretion are not yet fully understood.

The Ral GTPases belong to the Ras superfamily of small GTPases. Mammals have two Ral genes (RalA and RalB) that encode ubiquitous proteins with 85% sequence identity (5). Ral proteins mediate some Ras functions in the cell, as certain Ral guanine exchange factors that promote Ral activation can interact directly with active Ras. Ral GTPases regulate gene transcription, vesicular trafficking, migration, and cytokinesis and contribute to tumor development. To date, three Ral effectors have been identified: RalBP1 (6), the exocyst complex (7, 8), and the ZonaB transcription factor (9). Both RalA and RalB also bind constitutively to phospholipase D1 and phospholipase C-6 and are needed for their activation (10–12).

The exocyst is a protein complex involved in tethering post-Golgi secretory vesicles to specific plasma membrane domains. It is composed of eight evolutionarily conserved subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (13). The exocyst is involved in establishing epithelial polarity, membrane delivery, polarized growth, and polarized secretion (14). Exocyst complex function is regulated by several small GTPases including Ral and members of the Rab and Rho families. Active RalA and RalB interact directly with the exocyst subunits Exo84 and Sec5 (7, 8, 15), although RalB binds to these proteins less efficiently than RalA (16). Ral GTPases regulate exocyst assembly, and disruption of Ral interaction with the exocyst alters protein targeting to the basolateral membrane in polarized epithelial cells (7). The exocyst also mediates Ral functions such as cell transformation, cytokinesis, and cell migration. As Ral GTPases participate in polarized secretion, we studied Ral function in the regulation of NK cytotoxicity and analyzed their effect on granule polarization and degranulation through exocyst assembly.

Materials and Methods

Cells and reagents

The human NK-like YTS tumor cell line and the HLA class I-negative 721.221 B lymphoblastoid cell line were cultured in RPMI 1640 medium with 10% FBS. The Drosophila cell line SC2 B7.1/ICAM-1 was
maintained in Schneider medium containing 2 mM l-glutamine and 10% FBS. Expression of transfected ligands in SC2 cells was induced by 24-h incubation with 1 mM CuSO₄.

Human NK cells were isolated from healthy donor PBMC using Dynabeads Untouched Human NK Cells Kit (Invitrogen). Pure NK cells (>95% CD56⁺CD3⁻) were cultured in RPMI 1640 with 10% human serum, 100 U/ml purified human IL-2, and autologous mitomycin-treated (1 μg/ml, 90 min, 37˚C) PBL feeder cells (1:1).

The following Abs were used: anti-RalA (BD Biosciences), anti-RalB (Upstate), anti-perforin (Kamiya Biomedical), anti-α-tubulin (DM1A; Calbiochem), anti-Sec5 (Proteintech), anti-Sec8 (BD Biosciences), anti-Sec10 (Abcam), and anti-CD107a (BD Pharmingen).

Short hairpin RNA

Short hairpin RNA (shRNA) lentiviral plasmids (pLKO.1-puro; Sigma-Aldrich) were stably infected in YTS cells to downregulate RalA and RalB expression; we used the sequences RalA1 (5’-CCGGCGAGCTAATGTTCAGAAGTGTTCCTCCAAAACTCATCGTTTTT-3’), RalA2 (5’-CCGGCAAGGTGTTCCTCCAAAACTCATCGTTTTT-3’), RalB1 (5’-CCGGCACTCGAGAATTAGGTCAAAGAACACCTTGTTTTTG-3’), and RalB2 (5’-CCGGCCTTTACAGCAACTGCCGAATCTCGAGATTTCGGCAGTTGCTGTAAAGGTTTTTG-3’). As a negative control, we used a short-hairpin sequence containing 5 bp mismatches to any known human gene (SHC002; Sigma-Aldrich). To silence Sec5, a synthetic small interfering RNA (siRNA) was used (Dharmacon): 5’-GGUCGGAAAGACAAGGCAGAUCdTdT-3’.

Cell activation

Before cell activation, human NK cells and YTS cells were starved (2 h) in RPMI 1640 containing 0.1% low-endotoxin BSA (Sigma-Aldrich). When 721.221 cells were used as targets for pulldown assays, these cells were starved as above, fixed with 4% paraformaldehyde to avoid activation of endogenous Ral, and washed thoroughly with RPMI 1640. YTS and human NK cells were activated by coincubation with SC2 B7.1/ICAM-1 or 721.221 target cells at a 1:1 ratio for various times at 37˚C. After activation, cells were washed with cold PBS and lysed as described later.

Ral pulldown experiments and immunoprecipitation

Cells were lysed (30 min) in PBS with 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml each aprtinin and leupeptin. Lysates containing 500 μg protein were incubated with GST-RalBP1 Ral binding domain (RBD)-conjugated glutathione–Sepharose beads (17) (1 h). Beads were washed once with lysis buffer and twice with 25 mM Tris (pH 7.2), boiled, and samples analyzed by Western blot using anti-RalA or anti-RalB Abs.

For immunoprecipitation, cells were lysed with 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.1 mM DTT, 1% Triton X-100, and 10% glycerol (30 min, 4˚C). The resulting extract (500 μg) was incubated with the indicated Abs (2 μg) and precipitated with protein A-coupled Sepharose beads. Beads

FIGURE 1. RalA and RalB are activated after induction of a cytotoxic response. A, YTS NK cells were coincubated with SC2 target cells expressing B7.1/ICAM-1 at a 1:1 ratio for the indicated times. RalA-GTP and RalB-GTP levels were analyzed by pulldown assay followed by Western blot using anti-RalA or anti-RalB Abs. Graphs show mean ± SD of Ral signals in arbitrary units normalized for Ral levels at t = 0 (n = 4). *p < 0.05 (two-tailed Student t test). B, RalA-GTP and RalB-GTP levels were determined as above using the 721.221 cell line as target. (n = 3). *p < 0.05 (two-tailed Student t test). C, Primary IL-2–stimulated human NK cells were coincubated with 721.221 target cells for the indicated times. Levels of active RalA and RalB were determined as above. Data were quantitated as in A (n = 4). *p < 0.05 (two-tailed Student t test). AU, arbitrary units.
were washed once with lysis buffer and three times with 50 mM Tris-HCl pH 7.5, boiled, and analyzed by Western blot.

**Immunofluorescence**

Cells were plated on poly-l-lysine–pretreated coverslips and incubated for 10 min at 37°C, then fixed with ice-cold 100% methanol (3 min) and washed in PBS. Cells were blocked with PBS plus 0.1% Triton X-100, 10% heat-inactivated goat serum, and 0.5% BSA (30 min), then stained with primary Ab and incubated with fluorochrome-coupled secondary Abs. After washing in PBS, coverslips were mounted with PBS plus 50% glycero1 and analyzed with an Olympus FluoView confocal microscope. To quantify the percentage of NK-target cell conjugates that show Ral translocation, we determined the extent of Ral and perforin colocalization by computation of the Pearson correlation coefficient (r) in productive contacts (Olympus FluoView software). Conjugates showing r ≥ 0.5 were considered positive for translocation.

**Conjugation, degranulation, and cytotoxicity assays**

YTS cells were stained with PKH67 Green Fluorescent Cell Linker and 721.221 cells with PKH26 Red Fluorescent Cell Linker (both from Sigma-Aldrich). YTS and 721.221 cells were coincubated at a 1:2 ratio for different times, fixed in 4% paraformaldehyde, and analyzed by FACS. Events positive for red and green fluorescence were considered conjugates, and the percentage of conjugation was calculated as (conjugated YTS cells/total YTS cells) × 100.

For degranulation assays, YTS cells were stained with PKH26 and incubated with 721.221 cells at a 1:2 ratio with 4 nM monensin and FITC-labeled anti-human CD107a or an isotype control Ab (4 h, 37°C), after which samples were analyzed by flow cytometry.

To assay cytotoxicity, target 721.221 cells (5 × 10⁴) and various concentrations of effector YTS cells were cultured in RPMI 1640 with 10% Alamar blue (overnight, 37°C; Ref. 18). Samples were analyzed using a CytoFluor 2350 (Millipore) with an excitation wavelength of 530 nm and reading at 590 nm. The specific percentage of cytotoxicity was calculated as: ([AF target cells – (AF experimental – AF effector cells)]/AF target cells) × 100, where AF represents (sample fluorescence − fluorescence of the culture medium). Complementary cytotoxicity assays were performed using the JAM test (19). Briefly, 721.221 cells (10⁶ per experimental condition) were labeled with [³H]thymidine (5 mCi/ml; 16 h, 37°C) and incubated with various concentrations of effector YTS cells (4 h, 37°C). Cells were harvested with a FilterMate harvester (PerkinElmer), and the [³H]thymidine signal was measured in a 1450 Microbeta counter (PerkinElmer). Cytotoxicity was calculated as [1 – (experimental signal/target cell signal)] × 100.

**Polarization assays**

YTS cells were incubated with 721.221 target cells at a 1:4 ratio (15 min, 37°C), and cells were prepared on coverslips as above. Contacts were analyzed by confocal microscopy.

**Statistical analysis**

Two-tailed Student t test was calculated using GraphPad Prism 5.0 (GraphPad Software). Unless otherwise indicated, data represent the mean ± SD, and p < 0.05 was considered significant.

**Results**

**RalA and RalB are activated in cytotoxic cells**

Initiation of NK cell cytotoxic function requires signaling through several intracellular pathways, including activation of the Ras oncogene (20, 21). We used YTS cells, a thymic lymphoma-derived NK cell line, to determine whether Ral GTPases mediate some Ras functions in natural cytotoxicity. YTS-mediated cytolysis is dependent on CD28 and LFA-1 ligation (22, 23); we therefore stimulated YTS with SC2 Drosophila cells expressing B7.1 and ICAM-1 (24) (Supplemental Fig. 1). Levels of GTP-loaded RalA and RalB were subsequently determined by pulldown assays using GST/RalBP1 RBD as bait (17). RalA and RalB showed rapid, transient activation with similar kinetics. Both isoforms exhibited biphasic activation, with a first peak of Ral activation at 3–5 min post-YTS stimulation; active Ral levels then decreased almost to basal levels, and a second activation peak was observed at 15 min post-stimulation (Fig. 1A). Ral activation profiles were similar when the MHC class I-deficient B cell line 721.221 was used as target (Fig. 1B).

To corroborate the biological relevance of the observations in the YTS cell line, we examined Ral-GTP levels in primary human NK cells stimulated with 721.221 target cells. RalA-GTP levels showed a biphasic increase shortly after stimulation, similar to that of YTS cells; in contrast, RalB showed a single activation peak at 0.5–1 min (Fig. 1C). These observations confirmed results in the YTS cell line, suggesting that Ral activation is important during cell-mediated cytotoxicity in NK cells.

![FIGURE 2. RalA and RalB localize to lytic granules. A, YTS cells were incubated alone or with 721.221 target cells at a 1:4 ratio (20 min), fixed, stained with anti-RalA (left panel) or anti-RalB (right panel) (both green) and anti-perforin (red), and analyzed by confocal microscopy. B and C, Freshly isolated human NK cells (B) and IL-2-activated NK cells (C) were processed as above. Asterisk denotes 721.221 target cells. Original magnification ×60, zoom 6×.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.2435359)
RalA and RalB localize to the lytic granules

To get insight into Ral function during cytotoxicity, we determined Ral subcellular localization in YTS cells by confocal microscopy. Ral GTPases are geranyl-geranylated and associate to the plasma membrane and vesicular structures (25). In several cell types with secretory function such as platelets, endothelial cells, and neurons, Ral localizes to secreted granules (26–28). We costained YTS cells with anti-RalA or anti-RalB and anti-perforin Ab as a marker of lytic granules. Both Ral isoforms colocalized with perforin in resting YTS cells (Fig. 2A, upper panels). Stimulation of YTS cells with the MHC class I-deficient B cell line 721.221 led to Ral translocation to the effector–target interphase, where it continued to colocalize with perforin-containing granules (Fig. 2A, lower panels). Ral translocation was observed in the majority of conjugates (98%) in which perforin polarized to the cell–cell contact zone.

We also analyzed Ral localization in human NK cells freshly isolated from peripheral blood (Fig. 2B) and in IL-2–activated NK cells (Fig. 2C). In both cases, RalA and RalB colocalized with lytic granules, confirming observations in the YTS cell line. The percentage of conjugates showing Ral translocation to the immunological synapse was slightly lower than in YTS cells (RalA, 76% translocation; RalB, 87%); these results could be explained by the heterogeneity of cell activation in primary NK cell culture.

In conjunction with the Ral activation observed after cytotoxicity induction, these data suggest a role for Ral in cell-mediated cytotoxicity.

Interference with Ral impairs YTS cytotoxic response

To examine the role of Ral GTPases in NK cell function, we generated stable YTS cells lines expressing lentiviral vector-driven shRNA for RalA and RalB. Two independent stable cell lines were generated using two distinct shRNA sequences for RalA and two for RalB. These cell lines were used in Alamar blue cytotoxicity assays with 721.221 as target cells. Despite partial RalA knockdown, the shRNA reduced YTS cell killing of target cells (Fig. 3A); interference with RalB also impaired cytotoxicity, although to a lesser extent than interference with RalA (Fig. 3B). Both RalA and RalB shRNA were specific and reduced expression of the corresponding isoform by ∼80% (Fig. 3E). A complementary assessment of the cytotoxic activity of RalA shRNA- and RalB shRNA-expressing YTS cells using the JAM test (see Materials and Methods) also showed reduced cytotoxicity in both RalA shRNA and RalB shRNA cells compared with that in controls (Fig. 3C, 3D). The differences between controls and cells with reduced Ral levels were higher in the JAM test than in the Alamar blue assay.

To confirm that the impaired cytotoxic response was not due to a reduction in the amount of lytic proteins contained in the lytic granules in silenced YTS cells, we measured perforin levels: RalA shRNA- and RalB shRNA-expressing YTS cells and controls showed similar perforin levels (Fig. 3F). Reduction of RalB and, more clearly, of RalA thus resulted in reduced cell-mediated cytotoxicity.

Ral GTPases regulate granule polarization and secretion

Efficient cell-mediated killing requires the completion of three steps: formation of a stable conjugate with target cells, polarization of the secretory machinery, and finally, granule secretion. To determine which of these processes is regulated by activation of Ral GTPases, we first analyzed the effect of Ral silencing on YTS conjugate formation with 721.221 target cells by two-color FACS analysis. Interference with neither RalA nor RalB affected YTS cell conjugation with target cells (Fig. 4A).

Ral is linked to various polarized secretion events that require vesicle targeting to specific membrane domains; these include basolateral secretion in epithelia (16), synaptic vesicle release in neurons (29), and secretion of Weibel–Palade bodies in endothelial cells (30). Ral might thus be involved in lytic granule polarization during cell-mediated cytotoxicity. To analyze the effects of Ral silencing on granule polarization, we induced conjugate formation between YTS cells stably expressing RalA shRNA and RalB shRNA and target 721.221 cells, then stained for perforin and tubulin as markers of lytic granules and the MTOC, respectively (Fig. 4B). Incubation with target cells led to MTOC reorientation and perforin polarization to the contact zone in control YTS cells; however, in cells expressing RalA shRNA, we observed aberrant localization of both MTOC and lytic granules.
FIGURE 4. Effect of Ral GTPases in conjugate formation, polarization, and degranulation. A. YTS cells stably expressing control or RalA shRNA (left panel) or control or RalB shRNA (right panel) were labeled with PKH67 and coincubated with PKH26-labeled 721.221 target cells at a 1:2 E:T ratio. The percentage of conjugation was calculated as (conjugated YTS cells/total YTS cells) × 100. The mean ± SD of three independent experiments is shown. Differences between groups were not significant. n.s., not significant.

B, YTS cells stably expressing control, RalA, or RalB shRNA were incubated alone or with 721.221 target cells, fixed, stained with anti-α-tubulin and anti-perforin Abs, and analyzed by confocal microscopy. Asterisk denotes 721.221 target cells.

C and D, Quantification of MTOC and lytic granule polarization frequencies in control- versus RalA-silenced (C) or control- versus RalB-silenced (D) YTS cells. Data show mean ± SD of three independent experiments; at least 50 conjugates were scored in each experiment. *p < 0.01 (two-tailed Student’s t-test).

E, YTS cells stably expressing control, RalA (left panel), or RalB (right panel) shRNA were labeled with PKH26 and incubated with 721.221 target cells at a 1:2 ratio. The assay was performed in the presence of Ab to the degranulation marker CD107a; cells were then harvested and analyzed by flow cytometry. Graphs show the percentage of CD107a+ (degranulating) cells within the PKH26-labeled population (mean ± SD, n = 3). Statistical differences were determined using a two-tailed Student’s t-test: *p < 0.05.
FIGURE 5. Ral GTPases regulate exocyst assembly during the cytotoxic response. A, YTS cells were stimulated with SC2 target cells expressing B7/ICAM-1 for the indicated times. Lysates were immunoprecipitated with anti-RalA or anti-RalB Abs and analyzed by Western blot with anti-Sec8 Ab. Two internal controls were used, a sample containing cell lysate and protein A but no Ab (“NoAb”) and a sample containing Ab and protein A but no cell lysate (“NoLys”). As a protein loading control, Sec8 levels were tested in whole-cell lysates including the NoAb sample (middle panel). Graph shows mean ± SD of Sec8 signals in arbitrary units normalized for Sec8 levels at t = 0 (n = 4). *p < 0.05 (two-tailed Student t test). B, YTS cells were stimulated as in A and lysates incubated with GST-RalBP1 RBD-conjugated beads to pull down active Ral GTPases. Levels of GTP-bound RalA, RalB, and associated Sec5 or Sec8 were determined by Western blot; as controls, whole-cell lysates were tested with anti-RalA, anti-RalB, anti-Sec5, and anti-Sec8 Abs (middle panel). Graphs show mean ± SD of RalA-GTP, RalB-GTP, Sec5, and Sec8 signals in arbitrary units normalized for the levels at t = 0 (n = 4). *p < 0.05 (two-tailed Student t test). C, Confocal microscopy of YTS cells alone or incubated with 721.221 target cells, then stained with anti-perforin (red) and anti-Sec5.
D contrast, RalB silencing did not affect polarization (Fig. 4C); in C cells in which the secretory machinery was polarized (Fig. 4C), RalA knockdown led to a significant decrease in the percentage of exocytosis. The relative orientation and perforin polarization to the contact zone showed that RalA knockdown led to a significant decrease in the percentage of CD107a levels in control cells, as predicted, but did not notably increase degranulation of YTS cells in the absence of RalA or RalB (Fig. 4D).

The results show that both RalA and RalB participate in cell-mediated cytotoxicity; RalA regulates granule polarization and secretion, whereas RalB mediates only the latter step.

Interaction with target cells induces exocyst assembly

Ral GTPases interact with several effector proteins, including the exocyst components Sec5 and Exo84 (7, 8). Assembly of the exocyst complex is necessary for a large number of cell functions involving targeted membrane delivery, and Ral activation is essential for the assembly and localization of the octameric exocyst complex (7, 8). To determine whether the cytotoxic response in YTS cells requires exocyst assembly, we immunoprecipitated RalA and RalB and tested for associated Sec8. After CD28/LFA-1 stimulation, both RalA and RalB coimmunoprecipitated with Sec8 with kinetics resembling those of Ral activation (Fig. 5A). Because Ral GTPases do not bind directly to the Sec8 exocyst subunit, these results indicate that triggering of the cytotoxic response induces exocyst assembly. To determine whether Ral/Sec8 interaction was dependent on Ral activation, we performed a pulldown assay and analyzed the exocyst proteins associated with active Ral. Sec5, one of the exocyst components that binds directly to Ral GTPases, interacted with active RalA and RalB; Sec8 levels also paralleled those of active RalA and RalB, suggesting that exocyst assembly depends on Ral activation (Fig. 5B).

The unassembled exocyst exists as hemisubunits distributed in distinct cell locations; these hemisubunits interact to form the functional octameric complex (8). We used immunofluorescence and confocal imaging to examine the subcellular localization of different exocyst subunits in YTS cells. Staining for Sec5 showed a punctate pattern compatible with vesicular localization. These vesicles differ from lytic granules, however, as Sec5 did not colocalize with perforin (Fig. 5C). After YTS cells were incubated with 721.221 target cells, Sec5-containing vesicles colocalized partially with lytic granules (Fig. 5C).

Analysis of the cellular location of other exocyst proteins before stimulation of YTS cells showed that a fraction of Sec8 colocalizes with perforin, indicating that some exocyst components are found in lytic granules in unstimulated cells (Fig. 5D). In contrast, Sec10 localized more diffusely in cytoplasm and did not colocalize with perforin or Sec8 (Fig. 5D). Incubation of YTS cells with target cells confirmed fusion of perforin-positive lytic granules with vesicles containing exocyst components. Indeed, YTS conjugate formation with 721.221 cells led to Sec8 and perforin translocation to the contact zone, where they colocalized with a large fraction of Sec10 (Fig. 5D); this result is in agreement with the biochemical data for exocyst assembly (Fig. 5A). These findings show exocyst assembly at the contact zone during cytotoxicity, suggesting a role for this complex in lytic granule polarization and secretion.

Sec5 knockdown reduces cytotoxicity and degranulation in YTS cells

To test whether impaired cytotoxicity in Ral-deficient cells was due to defective exocyst assembly, we silenced Sec5 in YTS cells using siRNA (Fig. 6A) and tested their ability to kill 721.221 target cells in an Alamar blue assay. Interference with Sec5 impaired YTS cytolytic capacity, similar to results obtained after RalA silencing (Fig. 6B). Analysis of the mechanism involved indicated that Sec5 silencing did not affect YTS cell capacity to form stable conjugates with target cells (Fig. 6C). Differing from RalA interference, MTOC reorganization and lytic granule polarization to the immunological synapse were also unaffected in Sec5-silenced cells (Fig. 6D). Nonetheless, interference with

![Image](http://www.jimmunol.org/download/6249/a6f.jpg)
Sec5 reduced CD107a translocation to the YTS cell plasma membrane after activation with target cells (Fig. 6E), supporting a role for exocyst assembly during granule secretion.

Discussion

In this study, we show that Ral GTPases regulate cell-mediated cytotoxicity in NK cells. Both RaLA and RaLB are activated rapidly in YTS cells and primary NK cells after interaction with target cells, and interference with either RaLA isoform impaired YTS cell killing ability. Analysis of the essential stages involved in cytotoxicity indicated that RaLA regulates polarization of the lytic machinery to the immunological synapse as well as the degranulation step; in contrast, RaLB regulates only the latter step in cytotoxicity. Study of the molecular mechanism underlying RaLA modulation of cytotoxicity showed that RaLA and RaLB regulated exocyst assembly and that this complex is needed for YTS degranulation and efficient killing of target cells.

Ral GTPases are associated with the plasma membrane and with vesicular structures within the cell. In epithelial cells, RaLA and RaLB are found in the endosomal compartment (16, 32); we observed no RaLA or RaLB colocalization with endosomal markers in YTS cells (data not shown), suggesting that these GTPases have a different location in NK cells. In some secretory cells, Ral localizes to secreted vesicles (26–28). In NK cells, we found that both RaLA and RaLB associated with lytic granules, which constitute a specialized type of lysosome called secretory lysosomes (33); Ral GTPases associate with other secretory lysosomes as the dense granules in platelets (28). This lysosome association is restricted to secretory lysosomes, as we did not detect colocalization of Ral GTPases and the lysosomal marker LAMP2 in 293T cells (data not shown).

NK cells can use several mechanisms to lyse target cells, including release of cytotoxic proteins (perforin, granzyme) contained in lytic granules, and death receptor-mediated apoptosis of target cells via FasL upregulation and secretion of cytokines such as TNF-α (34). Silencing of RaLA and RaLB resulted in deficient lytic granule release and impaired YTS cell cytotoxicity. These defects in cytotoxic capacity were readily observed in short-term assays; they were also visible, although less evident in the longer term (compare JAM test to Alamar blue assay, Fig. 3A–D). These results suggest that either the death receptor pathway compensates for some degranulation takes place with slower kinetics in Rab-deficient cells. RaLA and RaLB share 85% amino acid sequence identity; despite this similarity, RaLA and RaLB are reported to serve no RalA or RalB colocalization with endosomal markers in Ral-silenced cells. RalA and RalB shRNA.

In addition to regulation of granule secretion, RaLA also controls polarization of the secretory machinery in a Sec5-independent manner (Figs. 4C, 6C). Polarization is dependent on microtubules and the actin cytoskeleton (43), and Ral GTPases modulate cytoskeleton dynamics through interaction with RabBP1 and filamin (44, 45). Further study is required to determine whether these effectors are downstream of Ral in cell-mediated cytotoxicity.

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Disclosures

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