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Dynamin 2 Regulates Granule Exocytosis during NK Cell-Mediated Cytotoxicity

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NK cells are innate immune cells that can eliminate their targets through granule release. In this study, we describe a specialized role for the large GTPase Dynamin 2 (Dyn2) in the regulation of these secretory events leading to cell-mediated cytotoxicity. By modulating the expression of Dyn2 using small interfering RNA or by inhibiting its activity using a pharmacological agent, we determined that Dyn2 does not regulate conjugate formation, proximal signaling, or granule polarization. In contrast, during cell-mediated killing, Dyn2 localizes with lytic granules and polarizes to the NK cell–target interface where it regulates the final fusion of lytic granules with the plasma membrane. These findings identify a novel role for Dyn2 in the exocytic events required for effective NK cell-mediated cytotoxicity. The Journal of Immunology, 2008, 181: 6995–7001.

Materials and Methods

Reagents, cells, and Abs

All reagents were obtained from Sigma-Aldrich unless stated otherwise. The P815 murine mastocytoma line was obtained from American Type Culture Collection. The MHC class I-deficient B lymphoblastoid cell line 721.221 was provided by Peter Parham (Stanford University, Palo Alto, CA). Human NK cells were cloned and passaged as described (10). Rabbit polyclonal Abs to PLC-γ2, SLP-76, and Vav1 were generated and characterized as previously described (11–14). Rabbit polyclonal Abs to Dyn2 were generated as described (8). Monoclonal anti-NKG2D (R&D Systems), anti-phosphorylated tyrosine (4G10; Upstate Biotechnology), anti-LFA-1 (MMH23), anti-perforin, anti-CD107a-PE (BD Biosciences), and IgG1 (50327; MP Biomedicals) were purchased as indicated. Goat anti-mouse IgG F(ab)2 was obtained from MP Biomedicals.

Plasmids and recombinant vaccinia

The sequence-encoding Dyn2 used to generate vaccinia constructs were amplified and subcloned into the previously described pSP11.FLAG plasmid, and recombinant vaccinia were generated by homologous recombination as previously described (8, 14). The cDNA sequence of Dyn2 was verified by PCR. Infections with vaccinia were conducted for 5 h in serum-free media at a multiplicity of infection of 20:1.

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Abbreviations used in this paper: MTOC, microtubule organizing center; siRNA, small interfering RNA; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Dyn2, Dynamin 2; CMAC, 7-amino-4-chloromethylcoumarin; TPA, tetradecanoyl phorbol myristate.

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Cytotoxicity assay

The \(^{51}\text{Cr}\)-release assays were performed as described previously (10). In some assays, NK cells were pretreated with the Dyn2 inhibitor dynasore for 30 min at the indicated concentrations.

Conjugate formation

NK cells were labeled for 1 h at 37°C with 100 \( \mu \text{M} \) sulfofluorescein (Molecular Probes), and the K562 target cells were labeled for 1 h at 37°C with 40 \( \mu \text{g/ml} \) hydroethidine (Polysciences). NK cells were pretreated with a final concentration of 10 \( \mu \text{g}/\mu \text{l} \) IgG1 control or anti-LFA-1 Ab for 10 min on ice. The cells were then washed and resuspended at a concentration of 5 \( \times 10^6 \) cells/ml. The effectors and targets (25 \( \mu \text{l} \) each) were mixed together, and allowed to incubate at 37°C for 10 min before adding 1 ml of ice-cold RPMI 1640 medium. Conjugate formation was assessed using a FACScan (BD Biosciences) and is revealed by the simultaneous emission of green and red fluorescence. Results are expressed as the percentage of total NK cells that formed conjugates. In some experiments, NK-target conjugates were labeled with CD107a-PE.

Cell stimulation, immunoprecipitation, and immunoblot analysis

Vaccinia infection of NK cells, cell stimulation, protein immunoprecipitation, and detection of tyrosine phosphorylation were done as described (15).

Cell microscopy

Immunofluorescence of fixed NK-containing conjugates was performed similarly to what we have described for T cell-B cell conjugates (16). In brief, 200 \( \mu \text{l} \) of NK cells (1 \( \times 10^6 \) cells/ml) were mixed with 200 \( \mu \text{l} \) of 7-amino-4-chloromethylcoumarin (CMAC)-stained 721 target cells (1 \( \times 10^6 \) cells/ml), and pelleted together at 500 rpm in serum-free RPMI 1640 cell culture medium (Invitrogen). These pelleted cells were then incubated at 37°C for 72 h at 37°C with 1 \( \times 10^6 \) cells/ml. The effectors and targets (25 \( \mu \text{l} \) each) were mixed together, and allowed to incubate at 37°C for 10 min before adding 1 ml of ice-cold RPMI 1640 medium. Conjugate formation was assessed using a FACScan (BD Biosciences) and is revealed by the simultaneous emission of green and red fluorescence. Results are expressed as the percentage of total NK cells that formed conjugates. In some experiments, NK-target conjugates were labeled with CD107a-PE.

Stimulation and measurement of secretion

Following infection, electroporation, or treatment with dynasore, NK cells were resuspended in RPMI 1640 plus 10% FBS and stimulated with tetradecanoyl phorbol myristate (TPA) and ionomycin or added to high-binding plates (Costar) that had been precoated with mAb at the indicated concentration. After 4 h of treatment at 37°C, the supernatant was harvested, and assayed for granule enzyme activity. Dyn2 localization with lytic granules at the NK cytolytic synapse. Clonally derived, non-transformed, human NK cells were conjugated with CMAC-stained 721 target B cells (blue) and, at the indicated times following conjugate formation, the cells were fixed and stained with anti-Dyn2 (green) and anti-perforin (red) to label lytic granules.

Results

Dyn2 localizes with lytic granules at the NK cytolytic synapse

Clonally derived, non-transformed, IL-2-activated human NK cells were used for this and all subsequent assays. We have previously

Dyn2 suppression

The following Dyn2-specific small interfering RNA (siRNA) construct was obtained from Dharmacon: 5'-NNGCACGCACUGAACAAGA-3'. On day 4–5 after passage, actively dividing NK cells were electroporated as previously described (16) with 300 pmol of negative control siRNA (AF488, Qiagen) or Dyn2 siRNA and then were resuspended at a density of 7 \( \times 10^6 \) cells/ml in RPMI 1640 medium (Invitrogen) plus 10% human serum, 1% l-glutamine (Mediatech), 1% sodium pyruvate (Invitrogen), and 10 U/ml of IL 2 (Chiron). Cells were analyzed 48 h after nucleofection.

Statistical analyses

Statistical analyses were done using either Microsoft Excel or R, and paired Student’s \( t \)-tests were used to calculate \( p \) values.
shown that Dyn2 accumulates at the T cell–APC synapse (8) and thus wanted to examine the spatial and temporal localization of Dyn2 during the development of cell-mediated cytotoxicity. To examine this, NK cells were conjugated to 721 target B cells and at different times following conjugation, cells were fixed and stained for endogenous Dyn2 and perforin to label the lytic granules. Dyn2 showed a diffuse staining pattern in both NK cells and 721 target cells. Initially upon target cell recognition, Dyn2 did not localize with perforin-containing granules in NK cells (Fig. 1, top panel). However, at later time points, Dyn2 and the lytic granules colocalized, and subsequently polarized toward the cytolytic synapse (Fig. 1, lower three panels). Although we cannot rule out the possibility that Dyn2 from the 721 cells contributes to the Dyn2 enrichment at the cytolytic synapse, it is clear that Dyn2 localizes and translocates with the lytic granules upon NK cell activation. These data suggest the possibility that Dyn2 may participate in the generation of cell-mediated killing by NK cells.

Dyn2 modulates cell-mediated cytotoxicity

We next examined the role of Dyn2 in NK-mediated cytotoxicity. We infected cells with wild type vaccinia (WR) or FLAG-Dyn2-encoding vaccinia, and examined cytotoxicity using a redirected killing assay against P815 tumor targets that bind anti-FcR mAb or anti-NKG2D mAb. Enhanced expression of Dyn2 consistently increased redirected cytotoxicity through both the FcR and NKG2D receptors (Fig. 2, top panels), as well as enhanced natural cytotoxicity of the targets tested (Fig. 2, lower panels). These results suggest that Dyn2 positively regulates cell-mediated killing by NK cells.

To further substantiate a role for Dyn2 in cell-mediated cytotoxicity, we sought to initially inhibit Dyn2 function using a recently identified pharmacological inhibitor of Dyn1, Dyn2, and the mitochondrial dynamin Drp1 (17). This agent, dynasore, inhibits the GTPase activity of dynamins, an event that is known to be required for the fusion and pinching of vesicles (17). NK clones were pretreated with varying concentrations of dynasore and then their lytic activity was assessed toward 721 and in a redirected killing assay using anti-FcR or anti-NKG2D. Dynasore treatment of NK cells inhibits the development of natural cytotoxicity, as well as FcR- and NKG2D-dependent cytolytic activity in a dose-dependent manner (Fig. 3A).

To determine whether NK cells require Dyn2 for cellular cytotoxicity, we...
used siRNA toward Dyn2 to specifically deplete Dyn2 protein levels in NK clones (Fig. 3B, inset). As shown in Fig. 3B, depletion of Dyn2 resulted in decreased natural cytotoxicity toward 721, as well as FcR-dependent cytolytic activity. Taken together, these data indicate that Dyn2 is involved in the development of cell-mediated killing by NK clones.

Dyn2 does not modulate conjugate formation

Initiation of cytotoxicity occurs after binding of NK cells to a susceptible target through a variety of adhesion receptors, including LFA-1, and establishment of a stable effector cell-target cell conjugate. No differences in cell surface expression of FcR or LFA-1 were observed when Dyn2 expression was enhanced or suppressed (data not shown). To determine whether Dyn2 plays a role in target-effector binding, we assessed the ability of NK cells to bind to the NK-sensitive chronic myelogenous leukemia cell line K562. Conjugate formation was assessed by two-color flow cytometry of NK cells intracellularly stained with sulfofluorescin and K562 target cells stained with hydroethidine; conjugates were determined by simultaneous emission of green fluorescence (NK cells) and red fluorescence (K562). No affect on conjugate formation was observed when the expression of Dyn2 was enhanced, although conjugate formation could be inhibited by the addition of blocking anti-LFA-1 Abs (Fig. 4A). Moreover, siRNA depletion of Dyn2 did not affect the number of NK–721 conjugates that formed over the indicated time course (Fig. 4B). Thus, Dyn2 does not affect cytotoxicity by altering the ability of NK cells to form conjugates with target cells.

Proximal signaling is unchanged by Dyn2

To determine whether Dyn2 affects the generation of signaling by activating receptors on NK cells, the proximal tyrosine phosphorylation of PLC-γ2, Vav1, and SLP-76 was examined following the activation of FcR (Fig. 5A). NK clones infected with WR or Dyn2 recombinant vaccinia viruses were stimulated for the indicated times with anti-FcR and then lysed. PLC-γ2, Vav, and SLP-76 were then immunoprecipitated from the lysates followed by SDS-PAGE and immunoblot for p-tyr or the indicated proteins. B, NK clones electroporated with negative control siRNA or Dyn2 suppressing siRNA were stimulated for the indicated times with anti-FcR and then lysed. PLC-γ2, Vav, and SLP-76 were then immunoprecipitated and analyzed as in A. The experiments shown are representative of 3 independent experiments. Lysates were equalized for protein expression and blotted for Dyn2 to verify suppression of the protein (inset, B). C, NK clones were electroporated with control or Dyn2 siRNA. Forty-eight hours post electroporation, calcium mobilization in response to anti-FcR/goat anti-mouse was measured using Indo-1 staining and flow cytometry. Data shown are representative of two independent experiments.

Dyn2 does not affect granule polarization.

A, Primary human NK cells were transfected with control siRNA or siRNA directed against Dyn2. After 72 h, these cells were fixed and stained with anti-Dyn2 (green) to analyze suppression and anti-perforin (red) to label lytic granules. B, Primary human NK cells were transfected as in A and then conjugated to CMAC-stained 721 target B cells (blue) and incubated for 30 min at 37°C. These NK-721 conjugates were then fixed and stained with anti-Dyn2 (green) and anti-perforin (red). C, Conjugates from B were scored for perforin-containing granule polarization to the NK-721 cytolytic synapse. Data shown are representative or two experiments performed in triplicate.
ligation of FcγRIIIA (FcR). Cells were infected with control WR vaccinia or recombinant virus encoding FLAG-Dyn2 and stimulated with anti-FcR followed by goat anti-mouse cross-linking Ab. PLC-2, Vav1, and SLP-76 were then immunoprecipitated and no change in tyrosine phosphorylation compared with control was observed (Fig. 5A). To further confirm these results, cells were transfected with control siRNA or siRNA against Dyn2 and analyzed for PLC-γ2, Vav1, and SLP-76 phosphorylation in the same manner. No change in proximal signaling from control cells was observed when Dyn2 was suppressed (Fig. 5B). Similarly, no differences in calcium signaling were observed upon stimulation of the FcR, with either overexpression or suppression of Dyn2 (Fig. 5C and data not shown). These results suggest that Dyn2 is not affecting the most proximal signals initiating NK cell activation, indicating that its role in cytotoxicity is further downstream.

Dyn2 does not affect granule polarization

The secretion of lytic granules by cytotoxic cells is preceded by a reorganization of the actin cytoskeleton coupled with reorientation of the MTOC and the lytic granules to a position adjacent to the target cell. Recent work by the Griffiths group (19) demonstrates that in CTLs, the centrosome is used to deliver the granules to the cell-cell interface. Because Dyn2 appears to accumulate with granules and polarize toward the cytolytic synapse during the generation of cell-mediated killing (Fig. 1), we decided to investigate whether depletion of Dyn2 would affect granule polarization. We formed conjugates between CMAC-loaded 721 cells (blue) and NK clones that had been transfected with control siRNA or siRNA toward Dyn2. We subsequently fixed and stained the formed conjugates for perforin and Dyn2. Individual NK cells were also stained and Dyn2-suppressed cells clearly displayed diminished Dyn2 levels compared with control cells, yet contained preformed lytic granules (Fig. 6A). Surprisingly, in the NK cell–721 conjugates, perforin-containing granules localized toward the 721 cells regardless of whether Dyn2 was present (Fig. 6, B and C). These data indicate that Dyn2 suppression does not affect the ability of lytic granules to polarize to the cytolytic synapse, and thus Dyn2 might be participating in a later step such as granule release.

Dyn2 regulates granule secretion

NK cells mediate cytotoxicity by the release of granule contents, including perforin and granzymes, which induce apoptosis of the target cell. To look at the regulation of secretion, we stimulated cells with anti-FcR, which results in proximal tyrosine phosphorylation, inducing degranulation. To determine whether Dyn2 overexpression affected NK cell secretion, we expressed wild-type Dyn2 in NK cells and stimulated using plate-bound Ab against the FcR, and the amount of granzyme A released was assessed by a BLT esterase assay. Expression of Dyn2 enhanced secretion induced by anti-FcR stimulation over that of the WR infected cells.
(Fig. 7A). To determine the effect of Dyn2 suppression on granule release, NK cells were transfected with Dyn2 specific siRNA, and were stimulated and assayed as in Fig. 7A. Suppression of Dyn2 reduced the amount of granzyme A released following anti-FcR ligation (Fig. 7B) although total cellular granzyme levels remained similar in control and Dyn2 suppressed cells (data not shown). Additionally, treatment of NK cells with concentrations of dynasore that inhibited cellular cytotoxicity also abrogated FcR-mediated exocytosis (Fig. 7C). These results demonstrate that the effect of Dyn2 suppression on degranulation correlates with the decrease seen in cell-mediated cytotoxicity. Consistent with this idea, surface expression of the lytic granule membrane protein CD107a in NK cell–target cell conjugates was dramatically reduced in NK cells suppressed for Dyn2 (Fig. 7D).

Exocytosis of lytic granules in NK cells and CTLs requires a transient increase in intracellular Ca$_{2+}$ concentration. This increase in Ca$_{2+}$ is required for the activation of the fusion machinery and docking and fusion of the granule with the membrane (2). Using TPA and ionomycin, we artificially stimulated secretion while bypassing the proximal signaling events. Enhanced expression of Dyn2 had only a modest effect on a strong stimulation of exocytosis by TPA and ionomycin (Fig. 7A). In addition, when using a weak PMA and ionomycin stimulus, Dyn2-suppressed cells (Fig. 7B), as well as dynasore-treated cells demonstrate a decrease in exocytosis (Fig. 7C). Taken together, these data indicate that modulation of cytotoxicity by Dyn2 is downstream of the Ca$_{2+}$ signal.

**Discussion**

We have shown that Dyn2 is an important modulator of NK cell-mediated cytotoxicity. Using both enhanced expression and suppression of the protein, we demonstrate that Dyn2 affects cell-mediated killing. Recent work in CTLs demonstrates polarization of the MTOC delivers the lytic granules to the cell interface (19). However, polarization of granules is not sufficient for granule release. In fact, some receptors on NK cells have the ability to polarize lytic granules to the site of effecter target contact without being able to stimulate granule exocytosis (20). Other insights into the distinction between granule polarization and exocytosis have also been gained from the study of human and mouse genetic diseases. In addition to the disease states that have been shown to result in normal granules, which are unable to polarize correctly such as Hermansky-Pudlak syndrome, disease states have also been identified in which lytic granules polarize correctly but are unable to be released. In familial hemophagocytic lymphohistiocytosis type 2 defects in Rab27a (4) and Munc13–4–5 (result) in cytotoxic cells with granules that polarize correctly but are unable to fuse with the membrane and deliver their content. Additionally, mutations in the SNARE syntactin 11 is associated with familial hemophagocytic lymphohistiocytosis type 4 and has recently been shown to be involved in NK cell granule exocytosis (21, 22). A role for Dyn2 in secretion following the polarization of the MTOC indicates its modulation of cytotoxicity is similarly in the final steps: granule fusion with the membrane and exocytosis.

An exocytic role for Dyn2 is surprising given its extensive characterization as a GTPase dependent regulator of membrane fission and endocytosis. However, several observations suggest that dynamins may have a broader role in membrane dynamics and can participate in coordinating fission with fusion (23, 24). Dynamins have been shown to play a GTPase-dependent role in coordinating membrane recapture during focal exocytosis of macrophages (3) and in secretory granule exocytosis in chromaffin cells (25) and neuroendocrine cells (24). More recently, Dyn2 has been shown to be involved in the secretion of insulin from preformed granules in response to glucose and other stimuli (26). Dynamins have also been found in complexes with docking SNAREs, such as syntaxin (25), and SNARE-interacting proteins, such as EHSH1 (27) and synaptophysin (28), yet it is unclear if dynamin regulates exocytosis via any of these interactions. Most intriguingly, a yeast homologue of dynamin, Vps1p, binds to Vam3p, a t-SNARE, regulating its ability to mediate fusion (29).

Dyn1 has been found to localize to sites of insulin release in pancreatic β-cells, where it is thought to participate in the reuptake of vesicles by cavicapture (30). Intriguingly, this process occurs independently of the recruitment of proteins important for “classical” endocytosis such as clathrin, amphiphysin, and epsin (30). Similarly, we find that in isolated NK cell clones, perforin-containing granules and Dyn2 do not colocalize; however, upon interaction with a target cell, Dyn2 colocalizes with the polarizing lytic granules and traffics with these granules to the cytolytic synapse. It is of interest that a similar redistribution of Munc13–4–4 with lytic granules has been found in CTLs (5). Similar to what we have found for Dyn2, Munc13–4–4 is not required for vesicle polarization or docking at the cytolytic synapse, but is required for the final step in membrane fusion (5). In contrast, depletion of Dyn2 does not completely block granule secretion or killing, but overall, there is decreased secretion of lytic granule constituents, which might result in a decrease in cell-mediated killing. It remains possible that, in NK cells, there are limited lytic granule fusion sites within the formed cytolytic synapse, and that dynamin-dependent granule fusion of the empty lytic granules, either via a cavicapture or kiss-and-run model, is required for the docking of new lytic granules at the fusion site. In the absence of Dyn2, initial granule release would occur, but subsequent bursts of granule release would be limited due to inefficient removal of empty granules from the cytolytic synapse.

A role for Dyn2 in the modulation of cell-mediated cytotoxicity and granule fusion/fission provides a new understanding of the means by which Dyn2 operates and the processes it regulates. Mutations in Dyn2 have recently been implicated in Charcot-Marie-Tooth disease, a form of peripheral neuropathy. Several mutations in Dyn2 were found in patients with Charcot-Marie-Tooth, localizing to the pleckstrin homology domain and decreasing membrane association of Dyn2 (31). Mutations in Dyn2 were also recently discovered in patients with centronuclear myopathy (32). A better understanding of the signaling pathways and domains of Dyn2 that regulate its localization with lytic granules, as well as the pathways that are regulated by Dyn2 has potential to illuminate how mutations in this protein cause disease.

**Disclosures**

The authors have no financial conflict of interest.

**References**


