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Perforin-Dependent Cryptococcal Microbicidal Activity in NK Cells Requires PI3K-Dependent ERK1/2 Signaling

Jeremy C. D. Wiseman,* Ling Ling Ma,† Kaleb J. Marr,* Gareth J. Jones,‡ and Christopher H. Mody2†‡

Previously, NK cells have been reported to kill the opportunistic fungal pathogen Cryptococcus neoformans through a perforin-dependent mechanism; however, the receptor and signaling involved are unknown. In this report we sought to identify the signaling pathways activated and required for direct perforin-mediated killing of microbes. In this study, using the NK-like cell line YT and primary peripheral blood NK cells, it is demonstrated that YT cells kill C. neoformans and that the killing is accompanied by the activation of PI3K. We demonstrate that inhibition of either the catalytic subunit (using a pharmacological inhibitor) or the α-regulatory subunit (using small interfering RNA knockdown) of PI3K significantly inhibited the killing of C. neoformans. Downstream of PI3K, ERK1/2 was activated in a PI3K-dependent fashion and was required for cryptococcal killing. Furthermore, we demonstrate that perforin release from YT cells can be detected by 4 h after contact of the YT cells with C. neoformans and that the release of perforin is blocked by pharmacological inhibition of either PI3K or ERK1/2. Defective degranulation is rooted in the inability to polarize perforin-containing granules toward the target. Finally, we demonstrate that PI3K-ERK1/2-dependent signaling is activated and required for the killing of C. neoformans by primary NK cells. Taken together, these data identify a conserved PI3K-ERK1/2 pathway that is used by NK cells during the direct killing of C. neoformans and demonstrate that the pathway is essential in the formation and activation of the microbicidal mechanism. The Journal of Immunology, 2007, 178: 6456–6464.
signaling cascade converging on the activation of PI3K is involved. Sequential activation of PI3K, Ras-related C3 botulinum toxin substrate-1 (Rac-1), \(^{3}\) p21/Cdc42/Rac1-activated kinase (PAK), MEK1/2, and ERK1/2 has been documented to control the redirection of perforin-containing granules. However, not all signaling by NK cells is mediated by PI3K. Previous studies have indicated that signaling can occur in the absence of PI3K catalytic activity; Vav-1 and phospholipase C\(\gamma\), important for the transduction of Rac-1-dependent signaling and Ca\(^{2+}\) mobilization, respectively, in response to NKGD ligation can occur in the presence of the specific PI3K inhibitor wortmannin but requires tyrosine phosphorylation of the YINM motif on the cytoplasmic tail of DAP10 (30). Thus, the focus of these studies was to determine whether the fundamentally different microbialic activity is PI3K dependent or independent and whether the PI3K-ERK1/2 pathway participates in signaling the direct NK cell-mediated killing of \(C.\) neoformans.

**Materials and Methods**

**Preparation of NK cells and \(C.\) neoformans**

The YT thymic lymphoma cell line was a gift from Dr. C. Clayberger (Stanford University, Palo Alto CA). YT cells were grown in complete RPMI medium consisting of RPMI 1640, 10% heat inactivated FBS, 0.1 mM nonessential amino acids, 100\(\mu\)M penicillin 100 \(\mu\)g/ml streptomycin, 1 mM sodium pyruvate, and 2 mM \(\gamma\)-glutamine (all from Invitrogen Life Technologies).

Primary NK cells were isolated using a MACS NK cell isolation kit II and the autoMACS automatic magnetic cell sorter, both from Miltenyi Biotec. \(C.\) neoformans strain CAP67 was obtained from the American Type Culture Collection (ATCC; catalog no. 52817). The fungus was grown on Sabouraud's dextrose agar (Difco) and maintained on slants, passaged each month as described (31). \(C.\) neoformans was grown to log phase in Sabouraud's dextrose broth (Difco) at 32°C with gentle rotation for 24 h before using it in experiments. For all experiments with primary NK cells the encapsulated \(C.\) neoformans strain B3501 was obtained from ATCC (catalog no. 34873) and maintained in the same fashion as \(C.\) neoformans CAP67.

**Antibodies**

All anti-v-akt murine thymoma viral oncogene homologue (c-Akt) and MAPK Abs were obtained from Cell Signaling Technology. The anti-p85\(\alpha\) Abs was part of the PI3K p85\(\alpha\) siRNA/siAb assay kit from Upstate Signaling Solutions and the p85\(\gamma\)-actin mAb was obtained from Santa Cruz Biotechnology. FITC mouse anti-perforin, mouse anti-perforin, and tetramethyl rhodamine isothiocyanate (TRITC)-goat anti-mouse Abs and isotypes were obtained from BD Biosciences. An isotype-matched Ab was used with mouse anti-perforin (BD Bioscences) primary Ab and TRITC-goat anti-rabbit secondary Abs according to the manufacturer's instructions. Densitometry was performed using LI-COR Odyssey instrument software.

**Gene silencing with siRNA**

Silencing of PI3K p85\(\alpha\) subunit was performed using the PI3K p85\(\alpha\) siRNA/siAb assay kit (Upstate). To label siRNA for transfection efficiency, the Silencer siRNA labeling kit was used according to the manufacturer’s instructions (Ambion). YT cells were transfected by electroporation. Briefly, YT cells were washed in serum-free medium and resuspended in serum-free medium at (5 \(\times\) 10\(^5\)/ml). These cells (2 \(\times\) 10\(^6\)) were added to quartz electroporation cuvettes along with 2.5 \(\mu\)g of FAM-labeled, SMARTpool p85\(\alpha\)-specific siRNA (consisting of four separate 21-nt siRNAs; Upstate), 2.5 \(\mu\)g of nonspecific siRNA (21 nt), or medium alone. A capacitance of 950 and voltage of 275 V was used to electroporate the cells.

**NK cell degranulation flow cytometry assay**

YT cells were incubated alone or at an E:T ratio of 1:100 with \(C.\) neoformans. YT cells were fixed and made permeable using the Cytofix/Cytoperm kit (BD Biosciences). Cells were stained using the FITC anti-perforin kit (BD Biosciences) and analyzed using a FACSscan flow cytometer (BD Biosciences). At least 5000 events were recorded during each analysis.

**Immunofluorescence microscopy**

For immunofluorescence microscopy, YT cells were mixed together at an E:T ratio of 1:10 with \(C.\) neoformans, centrifuged at 27 \(\times\) g for 5 min, resuspended, transferred to a polylisine-coated, chambered coverslass (Nunc), and incubated for 1 h at 37°C and 5% \(C.\) neoformans. Cells were fixed and permeabilized with Cytofix/Cytperm (BD Biosciences) and stained with mouse anti-perforin (BD Biosciences) primary Ab and TRITC-goat anti-mouse secondary Ab (BD Biosciences). An isotype-matched Ab was used with secondary TRITC-goat anti-mouse to control for nonspecific labeling. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). For \(C.\) neoformans labeling, 10 \(\times\) 10\(^5\) \(C.\) neoformans were incubated in 1 ml PBS with 1 mM CFSE (Sigma-Aldrich) for 15 min (37°C and 5% \(C.\) neoformans) and the cells were then washed three times in PBS and transferred to complete RPMI medium and incubated 30 min at 4°C, before incubation with YT cells.

Immunofluorescence microscopy was performed on the Olympus IX70 microscope equipped with digital deconvolution (DeltaVision; Applied Precision). Cells were imaged in transmitted light to identify conjugates. Conjugate formation was based on three criteria: 1) YT cells must have been in apparent contact with \(C.\) neoformans; 2) demonstrated invagination of cytoplasm; and 3) an elongated morphology. Fluorescence images were then recorded at \(\times 60\) objective magnification using DAPI/PE-Cy5 and FITC filter sets. The z-sections of fluorescent images were recorded at successive 0.2-\(\mu\)m intervals through the entire thickness of the conjugated YT cells. Image analysis (Softworks software) was used to perform a full iterative or nearest neighbor deconvolution, and images shown in this paper represent a single z-section corresponding to an in-focus NK-fungal synapse.

**Statistics**

Data expressed are the mean \(\pm\) the SEM. An ANOVA was performed to establish equal variance and Student’s t-test with Bonferroni correction was applied to determine statistical significance.

\(^{3}\) Abbreviations used in this paper: Rac-1, Ras-related C3 botulinum toxin substrate-1; Akt, v-akt murine thymoma viral oncogene homolog; DAPL, 4',6'-diamidino-2-phenylindole; PAK, p21/Cdc42/Rac1-activated kinase; TRITC, tetramethyl rhodamine isothiocyanate; siRNA, small interfering RNA.
Results

NK cell killing of *C. neoformans* is accompanied by activation of PI3K

To study the signaling of NK cell-mediated anticyptococcal activity, the NK-like cell line YT was used. To determine the effect of YT cells on *C. neoformans*, the number of viable organisms, as described in previous investigations, was determined (32). Either *C. neoformans* alone or YT cells mixed with *C. neoformans* were incubated for 24 h at various E:T ratios (Fig. 1A). These experiments indicated that at the lowest E:T ratio tested (100:1) YT cells mediated a significant (*p < 0.0125*) inhibition of cryptococcal growth and at higher ratios (≥500:1) the CFU were lower than the starting inoculum, demonstrating cryptococcal killing (Fig. 1A).

To determine whether PI3K signaling pathways were activated during YT cell killing of *C. neoformans*, YT cells were stimulated with *C. neoformans* and lysed and immunoblotting was performed for phosphorylated Akt/ PKB, which is immediately downstream and dependent upon the activation of PI3K. Akt/PKB phosphorylation was detected within 2 min and returned to unstimulated levels after 30 min of stimulation with *C. neoformans* (Fig. 1B). These results indicate that YT cell killing of *C. neoformans* is accompanied by the activation of PI3K.

PI3K is required for anticyptococcal activity

Although it was determined that the stimulation of YT cells with *C. neoformans* induced activation, we sought to determine whether PI3K activity was required for the anticyptococcal activity. Experiments were performed to determine whether a pharmacologic inhibitor of PI3K, LY294002, affected the activity. LY294002 works by reversibly inhibiting the ability of the p110 subunit of PI3K to catalyze the transfer of phosphate from ATP to its substrate (33). YT cells were pretreated with DMSO (as a control) or LY294002 and were compared with untreated YT cells for their ability to kill *C. neoformans*. In these experiments the viability of YT cells in the presence of the LY294002 was >90%. A significant (*p < 0.007*) abrogation of anticyptococcal activity was observed in the presence of the inhibitor LY294002 but not in the presence of vehicle alone (Fig. 2A). Indeed, there was no significant difference between the CFU of *C. neoformans* alone without YT cells and the CFU of *C. neoformans* incubated with LY294002-treated YT cells, indicating that the killing mediated by YT cells was abrogated in the absence of PI3K signaling.

The role of the regulatory subunit of PI3K, p85α, which is specifically required for the catalytic activity of PI3K, was examined. For this purpose, siRNA was used for targeted knockdown of p85. To determine the transfection efficiency following electroporation, the siRNA was labeled with FAM. Transfection efficiency was determined by flow cytometric analysis of the transfected cells at 24 h after transfection. Thick line, Mock transfected YT cells; thin line, FAM-labeled anti-p85 siRNA. C. Lysates from YT cells transfected with siRNA to the p85 subunit of PI3K were separated by SDS-PAGE and immunoblotting was performed for p85α protein expression and β-actin (loading control) at 72 h after transfection. Lanes from left to right: Mock transfected, nonspecific siRNA (NS), and p85α-specific siRNA. D. The anticyptococcal activity of YT cells transfected with anti-p85α-siRNA was compared with YT cells that has been mock transfected or transfected with nonspecific siRNA at 72 h after transfection. *, *p < 0.0125. Data are representative of two experiments.

FIGURE 1. YT cells kill *C. neoformans* and activate PI3K following contact with *C. neoformans*. A, YT cells were incubated at the indicated E:T ratios with *C. neoformans* at 37°C and 5% CO2 for 24 h. The CFU of *C. neoformans* alone minus the starting inoculum (*C. neoformans*) ± SEM was compared with growth in the presence of YT cells minus the starting inoculum at various E:T ratios (from 100:1 to 1000:1). *, *p < 0.0125 as compared the growth of *C. neoformans* alone. The data are representative of three experiments. B, YT cells were left unstimulated (Un) or stimulated with *C. neoformans* at an E:T ratio of 1:100 for the indicated time period (2–30 min). Phospho-Akt (Ser473) was detected by immunoblot, stripped, and reprobed for pan-Akt. The data are representative of two experiments.

FIGURE 2. PI3K is required for the killing of *C. neoformans* (*C. neo*). A, YT cells were pretreated with 50 μM LY294002 (LY) for 2 h and the anticyptococcal activity was compared with untreated and vehicle control (DMSO)-treated cells. *, *p < 0.007. B, A pool of four distinct 21-bp siRNAs to the p85α subunit of PI3K was labeled with FAM. Transfection efficiency was determined by flow cytometric analysis of the transfected cells at 24 h after transfection. Thick line, Mock transfected YT cells; thin line, FAM-labeled anti-p85 siRNA. C, Lysates from YT cells transfected with siRNA to the p85 subunit of PI3K were separated by SDS-PAGE and immunoblotting was performed for p85α protein expression and β-actin (loading control) at 72 h after transfection. Lanes from left to right: Mock transfected, nonspecific siRNA (NS), and p85α-specific siRNA. D, The anticyptococcal activity of YT cells transfected with anti-p85α-siRNA was compared with YT cells that has been mock transfected or transfected with nonspecific siRNA at 72 h after transfection. *, *p < 0.0125. Data are representative of two experiments.
72 h after transfection demonstrated a marked reduction in the expression of p85 in cells transfected with specific siRNA compared with those transfected with either the nonspecific siRNA or medium alone (Fig. 2C). Although a small reduction in p85 expression was observed in nonspecific siRNA with respect to mock-transfected cells despite consistent protein expression as assessed by β-actin (Fig. 2C), this did not affect the ability of YT cells to kill C. neoformans. YT cells transfected with siRNA to p85, nonspecific control siRNA, or medium alone were analyzed for anticycrococcal activity (Fig. 2D). There was a significant (p < 0.0125) reduction in the cryptococcal killing by those cells transfected with p85α-specific siRNA, but not in the control cells (Fig. 2D). Transfection with p85α-specific siRNA had no adverse effect on YT cell viability compared with control or mock-transfected cells, where viability under all conditions was >90%. Thus, inhibition of the catalytic activity of PI3K or silencing of the p85 regulatory subunit indicates that PI3K activity is required for the effective killing of C. neoformans by NK cells.

**ERK1/2 are activated and required for killing of C. neoformans by YT cells**

MAPK signaling has been reported to participate in signaling during NK-mediated tumor cell lysis (28). To determine whether MAPK, ERK1, and ERK2 were activated in YT cells during direct anticycrococcal activity, YT cells were stimulated with C. neoformans over a time course and immunoblotting for dual tyrosine/threonine phosphorylation of p42/44 MAPK (ERK 1/2) was performed (Fig. 3A and B). ERK1/2 became transiently phosphorylated in YT cells following contact with C. neoformans (Fig. 3A and B). Relative phosphorylation, as determined by densitometry of phosphorylated and total ERK1/2, exhibited an activation course similar to that observed for Akt with the maximum phosphorylation at 5 min, returning to baseline within 30 min (Figs. 1B and 3B).

Although like Akt MAP kinase signaling was activated in YT cells following contact with C. neoformans, it was not known whether ERK1/2 activation was required for cryptococcal killing. To address this question, YT cells were pretreated with U0126, a specific inhibitor of MEK1 and MEK2, which are the immediately upstream tyrosine/threonine kinases to ERK1 and ERK2. The ability of treated cells to kill C. neoformans was compared with that of untreated or control (DMSO)-treated YT cells (Fig. 3C). YT cells treated with the MEK1/2 inhibitor U0126 were significantly (p < 0.007) impaired in their ability to kill C. neoformans (Fig. 3C). The viability of YT cells in the presence of U0126 was >90%.

**MAP kinase signaling is downstream of PI3K during cryptococcal killing**

The specific activation and requirement for both PI3K and ERK1/2 has been confirmed; however, because ERK1/2 may become activated through a number of different signaling pathways (34–40), it was not known whether PI3K and ERK1/2 were being activated independently to contribute to the killing or were functioning as part of a sequential signaling cascade. To address this question, immunoblot analysis for the activation of both PI3K and ERK1/2 was performed in the presence of specific inhibitors to each enzyme (Fig. 4). YT cells were left untreated, treated with vehicle control (DMSO), or pretreated for 2 h with either LY294002 or U0126. Cells were then stimulated with C. neoformans for 5 min or left unstimulated and lysed after 5 min. Lysates were analyzed by immunoblotting for Akt phosphorylation (Fig. 4A and B). A relatively constant low level of Akt phosphorylation was observed in the absence of stimulation (Fig. 4A, odd-numbered lanes, and B, open bars). In the presence of C. neoformans; however, relative phosphorylation was increased and the observed increase was sensitive to PI3K inhibition (LY294002) (Fig. 4A, lane 6) but not to the inhibition of MEK1/2 (U0126) (Fig. 4A, lane 8), indicating that the activation of PI3K was prior to ERK1/2 activation. Conversely, ERK1/2 activation (Fig. 4C, C and D) was sensitive to both PI3K inhibition (Fig. 4C; lane 6) and, as expected, MEK1/2 inhibition (Fig. 4C, lane 8), indicating that ERK1/2 is dependent on and downstream of PI3K.

**MAPK signaling couples PI3K activation to perforin release during YT cell killing of C. neoformans**

The killing of C. neoformans by NK cells is dependent upon the cytolytic effector molecule perforin (18). To determine whether MAP kinase signaling downstream of PI3K activation was controlling the release of perforin during YT cell killing of C. neoformans, a flow cytometric technique was used to analyze the release of perforin in response to the stimulation of YT cells with C. neoformans. YT cells were pretreated with medium, control (DMSO), and U0126 or LY294002 to inhibit PI3K or ERK1/2,
respectively. Cells were stimulated with C. neoformans for 4 h. Following incubation, cells were stained with an isotype-matched control FITC-conjugated mAb or a FITC-conjugated anti-perforin mAb and analyzed for perforin content by flow cytometry (Fig. 5). Unstimulated cells demonstrated high levels of perforin expression that remained over the 4-h incubation period (Fig. 5; unstimulated column). YT cells stimulated with C. neoformans, that were pretreated with medium, or the vehicle control demonstrated a loss of fluorescence (Fig. 5, right column (Stimulated), top two rows (YT Alone and DMSO)). YT cells pretreated with inhibitors to PI3K or MEK1/2, however, did not demonstrate a loss of fluorescence (Fig. 5, right column (Stimulated), bottom two rows (LY294002 and U0126)). Interestingly the geometric mean fluorescent intensity for YT cells pretreated with LY294002 or U0126 and then stimulated with C. neoformans over 4 h was increased, indicating that they did not release perforin in response to stimulation with C. neoformans when PI3K or ERK1/2 were inhibited.

FIGURE 4. ERK1/2 is activated downstream of PI3K during cytotoxicity against C. neoformans. A and B, PI3K activity is insensitive to ERK1/2 inhibition. YT cells were left unstimulated (white bars) or stimulated (black bars) with C. neoformans for 5 min in the presence of medium (Cells Alone), vehicle control (DMSO), 50 μM LY294002, or 50 μM U0126. Lysates were resolved by SDS-PAGE transferred to nitrocellulose and an immunoblotting was performed to detect phospho-Akt (p-Akt) and total-Akt (pan-Akt) as a control; the immunoblotting (A) and the corresponding densitometry (B) are expressed as relative phosphorylation (phospho/total) in arbitrary units. C and D, Cell lysates were prepared as described above and anti-phospho-ERK1/2 (p-ERK1/2) for ERK1/2 activation and total-ERK (pan-ERK1/2) for loading control were analyzed by immunoblotting (C) and the corresponding densitometry (D). The data from each panel are representative of two experiments.

FIGURE 5. PI3K and ERK1/2 are required for perforin degranulation in response to stimulation with C. neoformans. YT cells with no pretreatment (YT Alone), vehicle control (DMSO), or treated with either 50 μM LY294002 or 50 μM U0126 were left unstimulated or stimulated with C. neoformans, at an E:T ratio of (1:100) for 0 or 4 h. Cells were fixed, made permeable, and stained with isotype (dotted line) or FITC-conjugated anti-perforin mAb at 0 h (thin line) and 4 h (thick line). Data are representative of two experiments.

FIGURE 6. PI3K and ERK1/2 are required for effective polarization of perforin-containing lytic granules toward the NK-cryptococcal synapse. C. neoformans cells were labeled with CFSE and incubated together with YT cells pretreated with vehicle control (DMSO) or either 50 μM LY294002 or 50 μM U0126. At 1 h the conjugates were fixed and stained on chambered coverglass with a mouse anti-perforin primary Ab, a TRITC-labeled goat-anti-mouse secondary Ab, and DAPI. Bar, 10 μm. Slides were then analyzed by immunofluorescence microscopy. Data are representative of two experiments.
From left to right the lanes indicate lysates of \textit{C. neoformans} alone (C), NK cells stimulated for 5 min with \textit{C. neoformans} culture supernatant (S), NK cells left unstimulated (U), or NK cells stimulated with \textit{C. neoformans} over the indicated time course (2, 5, 10, 20, and 30 min). D, Inhibition of the killing of \textit{C. neoformans} using the pharmacologic inhibitors of PI3K and ERK1/2, LY294002 and U0126, respectively.

The inability to polarize granules in the absence of functional PI3K-ERK1/2 signaling prevents the release of perforin

Having identified the inability of YT cells to release perforin in response to stimulation with \textit{C. neoformans} under conditions of PI3K or ERK1/2 inhibition, experiments were performed to determine which part of the lytic machinery was defective. Although both PI3K and ERK1/2 are required for the release of perforin, it was possible that PI3K was responsible for controlling the polarization of the granules toward the NK-cell fungal contact point whereas ERK1/2 is important for calcium signaling and granule release (41). Alternatively, sequential activation of PI3K and ERK1/2 may be required for the polarization of perforin-containing granules, because a PI3K-ERK1/2 cascade is used by NK cells killing tumor targets, which controlled the polarization of lytic granules (28). Consequently, we wondered whether the same pathway was active and was controlling the polarization of the granules during direct microbial killing by YT cells. To address this question, YT cells were incubated with CFSE-labeled \textit{C. neoformans} for 1 h. Following conjugate formation (see Materials and Methods), YT cell-\textit{C. neoformans} conjugates were fixed, made permeable, and stained for perforin (Fig. 6). YT cells pretreated with a vehicle control (DMSO) were able to polarize perforin effectively toward the YT-\textit{C. neoformans} synapse (Fig. 6, DMSO), YT cells pretreated with either the PI3K inhibitor (Fig. 6, LY294002) or the MEK1/2 inhibitor (Fig. 6, U0126) formed conjugates but were unable to polarize perforin toward the synapse, suggesting that the inability to release perforin was rooted in the defective polarization of granules during the lytic event.

Primary NK cells signal via the PI3K-ERK1/2-dependent pathway during cryptococcal killing

Having observed that YT cells signal through a PI3K-ERK1/2 pathway controlling the polarization and release of perforin during cryptococcal killing, we felt that it was important to extend these observations to primary NK cells. Experiments were performed using primary NK cells and the encapsulated \textit{C. neoformans} var. neoformans strain B3501. The CD16/CD56 profile of isolated NK cells by magnetic cell sorting indicates highly pure (>96%) CD56\(^{+}\) NK cells (Fig. 7A). Furthermore, isolated primary NK cells effectively killed \textit{C. neoformans} in a dose-dependent manner at both 24 and 48 h (Fig. 7B). It was observed that, in a similar fashion to YT cells, primary NK cells responded to intact \textit{C. neoformans}, demonstrating phosphorylation of Akt that peaked at 5 min and returned to basal levels by 30 min (Fig. 7C, lanes 3–8). Phosphorylation of Akt was not observed when stimulated with the \textit{C. neoformans} culture supernatant for 5 min (Fig. 7C, lane 2), nor did Akt Abs cross-react with the lysate of \textit{C. neoformans} in the absence of NK cells (Fig. 7C, lane 1). Furthermore, the anticytocidal activity of primary NK cells was abrogated in the presence of the PI3K inhibitor LY294002 and the MEK1/2 inhibitor U0126 (Fig. 7D). Thus, the mechanism of activation of primary NK cells using an encapsulated isolate of \textit{C. neoformans} was the same as that for YT cells.

Discussion

In these investigations we have identified the following crucial aspects of signaling activated in NK cells during microbicidal activity: 1) PI3K is activated and essential to YT cell cytotoxic function against the fungal pathogen \textit{C. neoformans} and both the p110...
enzymatic and p85α regulatory subunits are essential to this function; 2) MAPK signaling, more specifically ERK1/2 signaling, is activated and required for YT cell-mediated microbicidal activity; 3) MAPK activation occurs downstream of PI3K and therefore couples PI3K activity to control the lytic event; 4) the PI3K-ERK1/2 pathway is required for the polarization of perforin-containing granules and the release of perforin following conjugate formation; and 5) PI3K signaling and ERK1/2 signaling are required for the effective killing of *C. neoformans* by primary NK cells.

For NK cell-mediated tumor cytotoxicity it is becoming well documented that PI3K signaling is an important component of the lytic machinery, participating in both Fc receptor-mediated, natural, and NKG2D/DAP10-mediated cytotoxicity (26–29, 42, 43). Furthermore PI3K p85 can be recruited by potentially important costimulatory and accessory molecules involved in NK cell-mediated cytotoxicity (44–47). However, the receptor system involved in microbial killing is entirely unknown and important stimulatory signaling events such as the phosphorylation of Rac-1, the guanine nucleotide exchange factor Vav-1, and phospholipase Cγ can occur in the absence of PI3K (33). We therefore performed experiments to determine the activation state of PI3K following stimulation with *C. neoformans* and the requirement for PI3K catalytic activity for the effective killing of *C. neoformans* by YT cells. As indicated by Akt phosphorylation, PI3K was specifically activated in response to stimulation with *C. neoformans*, likely representing a crucial event for the development of cytotoxicity. As pharmacologic inhibition with LY294002 abrogated observed cytotoxicity, it was clear that the effective killing of *C. neoformans* is dependent upon the catalytic activity of PI3K. Furthermore, previous studies have not distinguished which isoform of the regulatory subunits are essential to this function (27, 28, 48). As indicated by siRNA-mediated gene silencing of the p85α regulatory subunit of PI3K, we have demonstrated that in YT cells the presence of the p85α regulatory subunit is required for PI3K activity during cytolytic function (27, 28, 48). As indicated by siRNA-mediated gene silencing of the p85α regulatory subunit of PI3K, we have demonstrated that in YT cells the presence of the p85α regulatory subunit is required for the killing of *C. neoformans*, likely due to the function of p85 in the recruitment of the catalytic p110 subunit and catalytic activity of PI3K. Although, we have not ruled out novel p110-independent functions for the p85α regulatory subunit, these data confirm that PI3K participates in signaling upon the recognition of *C. neoformans* and that the presence of p85α is essential for perforin-mediated killing.

In tumor cell killing, PI3K initiates the activation of a specific signaling cascade, Rac-PAK-MEK-ERK1/2, which has been demonstrated by Jiang and colleagues to be important in directing the polarization of perforin-containing granules (28). ERK1/2 activation has been demonstrated in a number of models to be important for NK cell-mediated cytotoxicity initiated through a variety of receptor systems (34, 35, 41, 49). Thus, we analyzed the role of MAPK signaling in our model. We first demonstrate that ERK1 and ERK2 are specifically and transiently activated in YT cells following contact with *C. neoformans*, as indicated by immunoblotting for dual tyrosine threonine phosphorylation (36), and are required for killing, as inhibition of the upstream tyrosine/threonine kinases for ERK1/2 (MEK1/2) with U0126 abrogated killing. ERK1/2 activation, however, is observed in a number of diverse signaling pathways in NK cells (37–40), leading us to question whether ERK1/2 and PI3K were activated independently or whether the PI3K-Rac-1-PAK-MEK1/2-ERK1/2 signaling cascade is conserved in the YT cell killing of *C. neoformans*.

It was therefore necessary to determine whether ERK1/2 activation was downstream of PI3K and part of a specific PI3K-ERK1/2 cascade, suggesting that a conserved PI3K-ERK1/2 pathway controls both tumor killing and direct anticytotoxic activity. Our results indicate that a PI3K-ERK1/2 pathway is being activated by the stimulation of YT cells with *C. neoformans*, because immunoblotting for Akt phosphorylation and ERK1/2 phosphorylation in the presence of specific inhibitors of PI3K and MEK1/2 shows that the activation of PI3K is insensitive to ERK1/2 inhibition, but ERK1/2 requires PI3K activity.

Although models of tumor cell killing indicate that ERK1 and ERK2 are important for granule mobilization (28, 34, 35), the precise mechanism by which ERK1/2 contributes to polarization is controversial. In an early report examining spontaneous and Ab-dependent tumor cytotoxicity, ERK2 activation regulated cytotoxicity through Ca2+-dependent signaling but not through control of the cytoskeleton or regulation of conjugate formation (41). We believe the PI3K-ERK1/2 pathway to be controlling the polarization and release of perforin and confirmed this by using a flow cytometry-based degranulation assay to examine the depletion of perforin in response to stimulation with *C. neoformans*. Inhibition with either the specific PI3K inhibitor LY294002 or the MEK1/2 inhibitor U0126 prevented the release of perforin from YT cells in response to stimulation with *C. neoformans*. Furthermore, inhibition of PI3K or ERK1/2 prevented the polarization toward the NK-fungal contact point, demonstrating the requirement of this pathway for polarization of the required effector molecule.

Although the receptor on NK cells that binds *Cryptococcus* and triggers killing is unknown, a number of receptors have been identified that are used for the recognition of *C. neoformans*. These include LFA-1 (CD11a/CD18) as well as the C3 complement receptors, CD35 (CR1), CD11b/CD18 (CR3), and CD11c/CD18 (CR4). Additionally, *Cryptococcus* or its components bind to the mannose receptor, DC-SIGN, FcγR, L-selectin, CD14, TLR-2, and TLR-4 (reviewed in Ref. 50). Of these, LFA-1 (CD11a/CD18) is an important activation receptor for YT cells, which do not kill via FcγR (51). Moreover there is evidence that PI3K and Erk1/2 are involved in LFA-1 signaling (52, 53). CD18 on human neutrophils binds cryptococcal glucuronoxylomannan and galactoxylomannan (54), and CD18 is involved in phagocytosis and the production of cytokines by macrophages in response to *C. neoformans* (55). Finally, anti-CD18 mAb treatment of *Cryptococcus*-infected mice increased the brain loads, demonstrating the importance of CD18 in optimal host defense (56). Consequently, it may be of interest in future studies to examine the receptor that is used in the recognition of *C. neoformans* by NK cells.

Importantly, the signaling observed during the YT cell killing of *C. neoformans* is also observed during the killing of *C. neoformans* by primary NK cells. Akt phosphorylation was observed over a similar time course when stimulating primary NK cells with the *C. neoformans*, and the pharmacologic inhibition of both PI3K and ERK1/2 abrogated the killing of the fungus.

We have observed that anti-microbial killing shares a conserved PI3K ERK1/2-dependent signaling pathway used in the lysis of tumor targets. This is of particular interest because NK cells interact with *C. neoformans* though a novel and unconventional mechanism by which various microvilli penetrate the cryptococcal capsule and contact the cell wall; however, there is no broad region of contact that is observed during NK-tumor conjugation (57). Furthermore, there is no evidence for the formation of a traditional cytolytic NK cell immune synapse, an important aspect of tumor killing, which provides supramolecular organization and costimulatory signals (52, 58). Thus, we have a novel situation by which conserved signaling pathways can be activated in NK cells during
the recognition and killing of microbial targets, providing evidence for the existence of a novel NK-microbial cytolytic synapse.

In conclusion, we have established a foothold on the signaling used by NK cells during direct microbial killing and have identified a novel situation by which conserved cytolytic machinery is activated in NK cells by microbial targets.

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Disclosures

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