In contrast to anti-tumor activity, YT cell and primary NK cell cytotoxicity for *Cryptococcus neoformans* bypasses LFA-1

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Abstract

NK cell cytotoxicity requires two positive signals for killing of tumors. Activation receptors induce polarization of the microtubule organization center and degranulation, while leukocyte function-associated antigen (LFA)-1 is required for conjugate formation and actin polymerization and under some circumstances may be sufficient for NK cell cytotoxicity. Although the receptor for direct killing of fungi is not known, CD18, the β2 chain of LFA-1, binds components of the capsule and cell wall of the opportunistic pathogen *Cryptococcus neoformans*, namely the polysaccharides glucoronoxylomannan and galactoxylomannan. Herein, we also demonstrate that LFA-1 was concentrated in regions of the NK cell surface interacting with *C. neoformans*. Consequently, there was compelling evidence to hypothesize that NK cells would also use LFA-1 to recognize and kill *C. neoformans*. Using a combination of NK cell lines that did or did not express LFA-1 or by using a CD18-specific functional blocking antibody, we confirm that NK cell anti-tumor activity is critically dependent upon the expression of LFA-1. Duplicating the events of tumor cytotoxicity, NK cells form conjugates with cryptococcal targets, rearrange the cell cytoskeleton to develop an NK immunologic synapse and release perforin-containing granules; however, each of these events occurred independently of LFA-1. Furthermore, NK cell-mediated killing of *C. neoformans* was detectable in both NK cells pre-treated with CD18-blocking antibodies and in NK cells lacking cell surface LFA-1 expression. These results demonstrate that in the absence of LFA-1 expression, NK cells are fully capable of recognizing a target (*C. neoformans*) and retain all of the events required for cytotoxicity.

Introduction

NK cells recognize infected and transformed host cells by a sophisticated and growing number of mechanisms [reviewed in (1)]. These recognition systems involve a fine balance of both stimulatory and inhibitory receptors that recognize ligands encoded by the host genome, alone or in combination with viral gene products. Recently, the ability of NK cells to recognize fungi directly, with an entirely different genetic make up to the host, and induce direct microbial cytotoxicity has promised to bring to light unique insights into NK cell function.

Leukocyte function-associated antigen (LFA)-1 (also known as CD11a/CD18 or αLβ2) is a member of the integrin family, a large family of heterodimeric membrane glycoproteins that are well known for their ability to facilitate cell–cell and cell–extracellular matrix adhesions. LFA-1 expression on lymphocytes allows for improved adhesion to vascular endothelium (2, 3), while LFA-1-mediated adhesion between antigen-presenting cells and T cells greatly facilitates antigen presentation and subsequent T cell activation (4, 5). In addition to its role in adhesion, signaling events elicited by LFA-1 ligation also contribute to T cell activation and effect subsequent T cell differentiation (6–8). Of importance to the results presented herein, LFA-1 also plays an essential role in NK cell-mediated immunity. Compared with cells isolated from wild-type animals, cytokine-activated NK cells isolated...
NK cell anti-fungal activity is LFA-1 independent

NK cell anti-fungal activity is LFA-1 independent in vitro. It has been previously demonstrated that the polysaccharides glucoronoxylomannan (GXM) and galactoxylomannan (GaIXM), components of C. neoformans capsules and cell wall, bind to the β2 chain (CD18) of LFA-1 on neutrophils (31). CD18 is involved in phagocytosis and production of cytokines by macrophages (32), and anti-CD18 mAb treatment of Cryptococcus-infected mice increases the burden of organisms in the brain, demonstrating the importance of CD18 in optimal host defense (33). Thus, given that LFA-1 plays an important role in NK cell cytolytic function (9–11, 34, 35), there was compelling evidence to believe that NK cells would also use LFA-1 to recognize and kill C. neoformans. We have previously shown that certain aspects of the signaling pathways essential for NK cell anti-tumor activity are also critical for NK cell anti-fungal activity (30). However, the results presented herein, namely the demonstration of LFA-1-independent recognition, conjugate formation, cytoskeletal reorganization, perforin release and effective lysis of fungal targets, demonstrate a fundamental difference in the way in which these events are initiated.

Materials and methods

Preparation of C. neoformans

The non-encapsulated C. neoformans strain CAP67 [American Type Culture Collection (ATCC) 52817] and its parental encapsulated strain B3501 (ATCC 34873) were obtained from the ATCC (Manassas, VA, USA), and the organisms were maintained on Sabouraud Dextrose Agar Slants (BD Biosciences, Mississauga, Canada) as previously described (36). Organisms were transferred from slants to Sabouraud dextrose broth (BD Biosciences) placed onto an orbital shaker at 32°C for 48 h and then stored at 4°C to act as stock cultures. Prior to experiments, C. neoformans stocks were subcultured at 32°C for 24 h, following which they were counted using a hemacytometer and suspended in complete medium (RPMI 1640, 10% fetal bovine serum, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids; all from Invitrogen Life Technologies, Burlington, Canada).

Cell lines and primary NK cells

The human NK leukemia cell lines, YT (a gift from C. Clayberger, Stanford University), YT-1(+) and YT-1(−), cells were cultured in complete medium. YT-1(−) cells are a mutant sub-clone of YT-1(+) cells that are deficient for CD18 expression due to a mutation in the CD18 gene. Consequently, these cells lack both CD18 and CD11a cell surface expression, although intracellular expression of CD11a is maintained (37). Primary human NK cells were isolated using a MACS NK Cell Isolation Kit II and the autoMACSTM automatic magnetic cell sorter (both from Miltenyi Biotec). The CD16/56 profile of isolated NK cells indicated highly pure (>96%) CD56⁻/CD16⁺ NK cells (data not shown). The following human tumor cell lines were used: K562 cells (hematopoietic malignant cells, a gift from Oliver Bathe, University of Calgary); Raji cells (malignant lymphoblast-like cells, a gift from Julie Deans, University of Calgary) and 721.221 cells from LFA-1-deficient mice demonstrate reduced anti-tumor activity due to impaired conjugate formation with tumor cell targets (9, 10). Furthermore, LFA-1-specific antibodies block human NK cell-mediated lysis of K562 tumor cell targets (11, 12). Several independent studies have demonstrated that LFA-1 signaling is directly coupled to mobilization of perforin-containing lytic granules (13–15). Experiments using insect cells transduced to express the LFA-1 ligand (intra-cellular adhesion molecule-1, CD54) demonstrate that LFA-1 was sufficient for resting NK cells to bind to targets and initiate signaling events leading to perforin polarization to the site of target cell contact (13, 14). Furthermore, in IL-2-activated NK cells, LFA-1 signaling alone was able to induce not only polarization but also degranulation of perforin-containing granules (16). In addition, ligand-induced LFA-1 clustering on CD3⁺CD8⁺CD56⁺ NK cells directly facilitates perforin release, which is inhibited by LFA-1-blocking antibodies (15). Recently, it has been shown that LFA-1 is required for conjugate formation and actin polymerization, while NK cell activation receptors are required for polarization of the microtubule-organizing center and degranulation and that both are required for cytotoxicity (17). Thus, taken together, these studies demonstrate that signaling through LFA-1 can be necessary for NK cell cytolytic activity.

Although the majority of studies highlights a role for LFA-1 during NK cell anti-tumor responses, there is evidence that NK cells may bypass this requirement (14, 18). First, NK cells from leukocyte adhesion deficiency 1 patients that are stimulated with IL-2 and PHA, presumably to markedly increase their cytotoxic potential, have a significant reduction in the requirement for LFA-1 for several functions including anti-tumor cytotoxicity and IFN-γ release (18). Second, insect cells transduced to express multiple ligands for NK cell-activating receptors (e.g. IgG and CD48, ligands for CD16 and 2B4, respectively) are killed by NK cells in the absence of LFA-1 signaling (14). Thus, it is possible that appropriate ligation of multiple alternative NK cell receptors may circumvent the requirement for LFA-1 during NK cell cytolytic activity.

Cryptococcus neoformans is an encapsulated yeast that is the anamorph of the basidiomycete Filobasidiella neoformans. Cryptococcus neoformans causes infection in humans via the pulmonary route. In immunosuppressed individuals, such as patients with HIV/acquired immunodeficiency syndrome, C. neoformans infection results in a devastating pulmonary and meningeal disease (19, 20). In non-immunosuppressed individuals, C. neoformans infection often results in a sub-clinical respiratory infection that is generally resolved by a multi-faceted host immune response [reviewed in (21)]. Seminal studies demonstrated that murine NK cells possess anti-cryptococcal activity in vitro and are required for optimal clearance of cryptococci from tissues in vivo (22–26). In addition, we and others have shown that human NK cells also possess the ability to directly kill C. neoformans (27–30). These studies demonstrated that NK cell-mediated anti-cryptococcal activity did not require pre-activation and employs perforin as the cytotoxic effector molecule. In addition, cell contact is required for the transfer of perforin to the microbial target. However, the receptor–ligand apparatus mediating this anti-cryptococcal activity remains unknown.

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(lymphoblast B-cell line, a gift from R. Chris Bleackley, University of Alberta). All tumor cells were maintained in complete medium.

**Antibodies and reagents**

The following reagents were used: anti-CD11a-phycocerythrin-cyanin 5 (PeCy5) (clone HI111) and the FITC-conjugated Perforin Antibody Reagent Set (both BD Biosciences); mouse anti-human CD18-blocking antibody (clone IB4; Cabilochern); normal mouse IgG (Santa Cruz); Alexa Fluor 350 phalloidin (Invitrogen Molecular Probes); PKH26 Red Fluorescent Cell Linker Kit (Sigma) and carboxyfluorescein diacetate succinimidyl ester (CFSE) (Guava Technologies, Hayward, CA, USA).

**Conjugate formation assay**

Cell conjugation assays were performed as previously described (13), with minor modifications. Briefly, tumor cell lines (721.221 and Raji cells) or *C. neoformans* were pre-labeled with the green fluorescent dye CFSE, while NK cell lines were pre-labeled with the yellow fluorescent dye PKH26, following the manufacturer's protocol. Tumor cells (2 × 10^5) or *C. neoformans* (5 × 10^3) were mixed with NK cells (1 × 10^3) in 200 μl final volume of cold complete medium, centrifuged at 300 r.p.m. for 5 min and incubated at 37°C for the indicated time periods. Cells were then fixed in ice-cold PBS containing 0.5% formalin and data acquired immediately using the Guava EasyCyte flow cytometer (Guava Technologies) and analyzed using the FlowJo software package (Tree Star, Ashland, OR, USA). Baseline denotes cells that were fixed after mixing without any incubation at 37°C, which due to the constraints of the assay occurred 5 min after the initial mixing. Cells were identified based on forward scatter and side-scatter profiles and the percentage of NK cells forming conjugates calculated as follows: [(number of NK cells co-labeling with green)/(total number of NK cells)] × 100.

**Immunofluorescence microscopy**

NK cells were mixed with *C. neoformans* strain B3501 at an E:T ratio of 1:5, centrifuged at 300 r.p.m. for 3 min and cultured at 37°C for 24 h. Cells were then fixed in PBS containing 3.7% formalin and cell surface stained with anti-CD11a-PeCy5. For analysis of cell cytoskeleton organization, NK cells were pre-labeled with the yellow fluorescent dye PKH26, following the manufacturer's protocol. Tumor cells (2 × 10^5) or *C. neoformans* (5 × 10^3) were mixed with NK cells (1 × 10^3) in 200 μl final volume of cold complete medium, centrifuged at 300 r.p.m. for 5 min and incubated at 37°C for the indicated time periods. Cells were then fixed in ice-cold PBS containing 0.5% formalin and data acquired immediately using the Guava EasyCyte flow cytometer (Guava Technologies) and analyzed using the FlowJo software package (Tree Star, Ashland, OR, USA). Baseline denotes cells that were fixed after mixing without any incubation at 37°C, which due to the constraints of the assay occurred 5 min after the initial mixing. Cells were identified based on forward scatter and side-scatter profiles and the percentage of NK cells forming conjugates calculated as follows: [(number of NK cells co-labeling with green)/(total number of NK cells)] × 100.

**Flow cytometric cytotoxicity assay**

NK cell anti-tumor activity was measured using the Guava EasyCyte Flow Cytometry Kit (Guava Technologies) following the manufacturer's protocol. Briefly, tumor cell targets were pre-labeled with 2.5 μM CFSE and cultured in triplicate at 37°C for 4 h with NK cells at different E:T ratios, following which 40 μl of 7-AAD [a dye that labels dead cells (38)] was added to each well and the cells incubated for a further 10 min at room temperature in the dark. Samples were acquired on the Guava EasyCyte flow cytometer and analyzed using the Guava Cytotoxicity software. Viable tumor cells were quantified as the percentage of CFSE-positive cells that were negative for 7-AAD labeling. Results are expressed as the mean ± standard error of the mean (SEM).

**Anti-cryptococcal activity of NK cells**

A colony-forming unit (CFU) assay was performed as previously described (29). *Cryptococcus neoformans* (2 × 10^3 per well) were incubated in quadruplicate with or without NK cells at the indicated effector:target (E:T) ratios. In some experiments, NK cells were pre-incubated with CD18-specific blocking antibody (clone IB4) or normal mouse IgG prior to culture with *C. neoformans*. The number of CFU of *C. neoformans* per well was determined at the indicated time points by lysing the effector cells with dH_2_O, followed by diluting and spotting onto Sabouraud Dextrose Agar plates. Results are expressed as the number of *C. neoformans* per milliliters (mean ± SEM).

**Statistical analysis**

Statistical analysis was performed using analysis of variance with the Bonferroni multiple comparisons post hoc test, using the GraphPad Prism version 3 software package (GraphPad Software, San Diego, CA, USA).
Results

NK cells interact with C. neoformans in a LFA-1-independent manner

Given that both GXM and GalXM bind to the β2 chain (CD18) of LFA-1 on neutrophils (31) and LFA-1 plays a critical role in NK cell cytolytic function (9–11, 34, 35), we hypothesized that LFA-1 would be the receptor for C. neoformans or at least contribute to the anti-cryptococcal activity of NK cells. Indeed, immunofluorescence microscopy revealed that LFA-1 expression was distributed evenly around the surface of the NK cell line, YT cells (Fig. 1A and B), but was polarized to regions of close contact with C. neoformans (Fig. 1C and D), suggesting a role for LFA-1 in recognition of cryptococcal organisms.

To investigate the functional role of LFA-1 in the anti-cryptococcal response, we utilized YT-1(−/C0) cells which due to a mutation in the CD18 gene lack cell surface expression of the β2 integrins (37). Flow cytometric analysis confirmed the lack of cell surface CD18 and CD11a expression on YT-1(−/C0) cells and the presence of both on the wild-type YT-1(+)+ cells (data not shown). Thus, the YT-1(−) and YT-1(+) cells will be referred to as LFA-1(−) and LFA-1(+) NK cells, respectively, from here on. As NK cells isolated from LFA-1-deficient mice demonstrate impaired conjugate formation with tumor cell targets (9, 10), we initially compared the impact of LFA-1 deficiency on NK cell binding interactions with either tumor cell targets or C. neoformans. Conjugate formation between LFA-1(+) NK cells and 721.221 tumor cells was evident at 30 min post-mixing and reached maximum levels by 60 min (Fig. 2A). In contrast, LFA-1(−) NK cells demonstrated a reduced capacity to form conjugates with 721.221 tumor cells, even after 120 min post-mixing. Similar results were obtained using Raji tumor cells as targets (data not shown).

In contrast to tumor cell targets, both LFA-1(+) and LFA-1(−) NK cells exhibited similar baseline levels of conjugate formation with the encapsulated C. neoformans strain B3501, which increased to the same extent over the culture period (Fig. 2B). Similarly, both LFA-1(+) and LFA-1(−) NK cells demonstrated conjugate formation with C. neoformans strain CAP67 (Fig. 2C). The experiments were repeated twice with similar results.

Fig. 1. Polarization of LFA-1 to regions of interaction with Cryptococcus neoformans. YT cells were cultured in the absence (A and B) or presence (C and D) of C. neoformans strain B3501. Left hand panels show differential interference contrast (DIC) images while right hand panels show LFA-1 expression. Panel (C) shows two C. neoformans organisms (asterisked) in close association with a YT cell. All images were recorded at ×60 objective magnification; bar represents 15 μm.

Fig. 2. Loss of LFA-1 abrogates NK cell conjugate formation with tumor cell targets but not Cryptococcus neoformans. PKH26-labeled NK cells were cultured with CFSE-labeled tumor cells or C. neoformans for up to 120 min at 37°C and the percentage of NK cells forming conjugates with target cells quantified as described in the Materials and methods. LFA-1(−) NK cells demonstrate reduced conjugate formation with (A) 721.221 tumor cells over time, while both LFA-1(+) NK cells and LFA-1(−) NK cells demonstrate conjugate formation with (B) encapsulated C. neoformans strain B3501 and (C) non-encapsulated C. neoformans strain CAP67. The experiments were repeated twice with similar results.
cells demonstrated conjugate formation with the non-encapsulated *C. neoformans* strain CAP67 (Fig. 2C). Thus, in contrast to tumor cell targets, NK cells formed conjugates with *C. neoformans* in the absence of LFA-1.

The interaction of LFA-1 with ligands on tumor targets not only acts as an adhesion event to stabilize conjugate formation but also leads to signaling events that mediate the cellular cytoskeleton reorganization that is required for the formation of the central NK immunologic synapse (cNKIS) and the polarization of cytolytic granules to the NK cell-tumor cell interface [reviewed in (39)]. Therefore, we next investigated whether these LFA-1-mediated events were affected during the killing of *C. neoformans* by LFA-1-deficient NK cells. To investigate the impact of LFA-1 deficiency on the cellular cytoskeleton reorganization that occurs in NK cells upon contacting a target, NK cells were cultured with either CFSE-labeled tumor cells or CFSE-labeled *C. neoformans* and subsequently stained with phalloidin to detect F-actin. Polarization of F-actin to the site of contact with tumor cells was observed in LFA-1(+) NK cells (Fig. 3A, arrowed); however, this was not observed in LFA-1(−) NK cells (Fig. 3B) even though the cells were in contact with one another. In contrast, F-actin polarization to the site of contact with *C. neoformans* was observed in NK cells irrespective of LFA-1 expression (Fig. 3C and D, arrowed). These studies demonstrate that F-actin polarization to the site of contact with *C. neoformans* occurred independently of LFA-1 expression.

We have previously demonstrated that NK cell-mediated anti-cryptococcal activity is critically dependent on the effector molecule perforin (29), wherein upon binding to *C. neoformans*, NK cells polarize perforin-containing granules to the site of conjugate formation and degranulate (30). Thus, we investigated cryptococcal-induced perforin degranulation by quantitating the intracellular perforin level in NK cells stimulated with *C. neoformans* (Fig. 4). Freshly isolated primary human NK cells constitutively express intracellular perforin (Fig. 4A, solid black line). Following stimulation with *C. neoformans* strain B3501, intracellular perforin expression is reduced (Fig. 4A, solid gray lines), demonstrating cryptococcal-induced perforin degranulation. In addition, stimulation with *C. neoformans* induced cell surface expression of CD107a on primary NK cells (data not shown), providing further evidence for cryptococcal-induced degranulation. Given that ligand-induced LFA-1 clustering and formation of the cNKIS directly facilitates perforin release (15), we next investigated whether cryptococcal-induced perforin degranulation occurred independently of LFA-1 signaling by repeating the experiments using the LFA-1(+) and LFA-1(−) NK cell lines. Similar to primary NK cells, the wild-type LFA-1(+) NK cells constitutively express perforin, which is lost following culture with excess numbers of *C. neoformans* (Fig. 4B). Furthermore, cryptococcal-induced perforin loss was also observed in the mutant LFA-1(−) NK cells (Fig. 4C), demonstrating that cryptococcal-induced perforin degranulation can be triggered through an LFA-1-independent mechanism.

**LFA-1 expression is not required for NK cell anti-cryptococcal activity**

We next investigated whether LFA-1 expression was essential for NK cell-mediated killing of either tumor cells or cryptococcal targets. LFA-1(+) NK cells demonstrated robust killing of both Raji tumor cell targets (Fig. 5A) and 721.221 tumor cell targets (data not shown), while LFA-1(−) NK cells exhibited significantly reduced anti-tumor activity at all E:T ratios studied. In contrast, a significant reduction in the growth of the encapsulated *C. neoformans* strain B3501 was observed when the organisms were cultured in the presence of LFA-1(−) NK cells at an E:T ratio of 100:1 (Fig. 5B). Furthermore, this reduction in growth was comparable to that observed when *C. neoformans* was cultured in the presence of the wild-type parental LFA-1(+) NK cells. Although LFA-1 was not required for YT cell anti-cryptococcal activity at an E:T ratio of 100:1, we also investigated whether LFA-1 contributed to the response at lower E:T ratios. Both wild-type LFA-1(+) NK cells and the mutant LFA-1(−) NK cells demonstrated significant anti-cryptococcal activity at reduced E:T ratios of 50:1 and 10:1 (Fig. 5B), although this activity was less pronounced at the lower E:T ratio of 10:1 in both cell types. Of importance, there was no significant difference between the anti-cryptococcal activities of these two cell lines at any of the E:T ratios investigated. Similar results were also obtained using the non-encapsulated *C. neoformans* strain CAP67 (Fig. 5C).

The NK cell line, YT cells, expresses abundant cell surface CD18 and CD11a but exhibit limited expression of the other β2 integrin α-chains CD11b and CD11c (data not shown). Thus, although YT cells lack CR3/Mac-1 (CD11b/CD18) and CR4/p150/95 (CD11c/CD18), they do express cell surface LFA-1 (CD11a/CD18). Pre-treatment of YT cells with a

![Fig. 3. Polarization of F-actin to the site of contact with Cryptococcus neoformans occurs independent of LFA-1 expression. LFA-1(+) and LFA-1(−) NK cells were cultured in the presence of CFSE-labeled Raji tumor cell targets (A and B) or *C. neoformans* strain CAP67 (C and D) as described in Materials and methods. Arrows indicate F-actin polarization to site of target cell contact. T denotes tumor cell, C denotes *C. neoformans* and NK denotes NK cells.](image-url)
CD18-specific functional blocking antibody, but not with control antibody, significantly inhibited their anti-tumor activity to Raji target cells (Fig. 6A). In contrast, blocking CD18 failed to inhibit the anti-cryptococcal activity of YT cells to the encapsulated C. neoformans strain B3501 (Fig. 6B) even at the reduced E:T ratio of 10:1. Similarly, blocking CD18 also failed to inhibit the anti-cryptococcal activity to the non-encapsulated C. neoformans strain CAP67 (Fig. 6C). Having observed that antibody blockade of CD18 failed to impair YT cell anti-cryptococcal activity, we felt that it was important to extend these observations to primary human NK cells. Isolated primary human NK cells effectively killed Raji tumor cell targets, which was significantly impaired by pre-treatment with the CD18-specific functional blocking antibody (Fig. 7A). In accordance with previous studies (28, 30), freshly isolated primary human NK cells also exhibited anti-cryptococcal activity as evidenced by a reduction in the CFU for C. neoformans strain B3501 (Fig. 7B). However, in contrast to anti-tumor activity, blocking CD18 had no effect on the anti-cryptococcal activity of primary human NK cells. Taken together, anti-cryptococcal activity is detectable in both CD18-blocked NK cells and in NK cells that lack cell surface CD11a and CD18, suggesting that the anti-cryptococcal response is not dependent on LFA-1 expression.

Discussion

The studies presented herein demonstrate that, in contrast to their anti-tumor activity, NK cells are able to bypass LFA-1 ligation and signaling for (i) formation of stable conjugates with C. neoformans, (ii) reorganization of the cellular cytoskeleton and formation of a cNKIS, (iii) cryptococcal-induced perforin degranulation and (iv) subsequent killing of both encapsulated and non-encapsulated strains of C. neoformans.

LFA-1 plays a central and essential role in mediating adhesive interactions between numerous cells of the immune system. T cells utilize LFA-1 to form the peripheral supramolecular activation cluster, a component of the specialized immunological synapse that forms between T cells and antigen-presenting cells during T cell activation (40) and between CD8+ CTL and susceptible target cells during cytolysis (41, 42). LFA-1 not only acts to stabilize adhesion between these cells but also initiates signaling events, including activation of phosphoinositide 3-kinase (PI3K) (43) and extracellular signal-regulated kinase (Erk)1/2 (15), that are required for optimal T cell activation (7) and the recruitment of cytolytic granules to the site of target contact (44). Members of the β2 integrin family also play essential roles during NK cell cytolytic activity. LFA-1 and Mac-1 are both components of the peripheral region of the cNKIS that forms at the contact site between NK cells and susceptible targets (45), where F-actin polymerization triggered by LFA-1 signaling serves to strengthen conjugation between NK cells and C. neoformans.

Fig. 4. Cryptococcus neoformans-induced NK cell perforin degranulation occurs independently of LFA-1 expression. (A) Primary human NK cells, (B) LFA-1(+) NK cells and (C) LFA-1(-) NK cells were cultured with or without C. neoformans (E:T ratio of 1:10) in complete medium at 37°C for 48 h, following which cells were harvested, made permeable and immunolabeled with either anti-perforin or isotype control antibodies. Perforin expression in unstimulated (solid black lines) and C. neoformans-stimulated (solid gray lines) NK cells was analyzed as described in Materials and methods. Dotted lines represent isotype control antibodies. The experiments were repeated twice with similar results.
their targets (17). Furthermore, ligand-induced LFA-1 clustering has also been demonstrated to induce signaling events that directly facilitate granule polarization and perforin release (13–16).

Thus, given the central role of LFA-1 in mediating numerous cell–cell interactions occurring within the immune system, we investigated the role of LFA-1 in NK cell recognition and killing of the pathogenic yeast *C. neoformans*. Indeed, previous studies identified GXM and GalXM, components of the *C. neoformans* cell wall and capsule, as ligands for β2 integrins (31). Furthermore, signaling of NK cell anti-cryptococcal activity involves PI3K and Erk1/2 (30), signaling molecules that...
were surprised to find that neither conjugate formation upon the cell surface expression of LFA-1. However, we confirmed that NK cell anti-tumor activity was critically dependent on LFA-1 ligation for NK cell-mediated anti-tumor and anti-fungal activity. In agreement with previous studies, we confirmed a fundamental difference in the dependence on LFA-1 for primary human NK cells and report herein for the first time that NK cells deficient for LFA-1 are impaired in their ability to form conjugates with tumor targets (Fig. 2B and C), and in the rare conjugates that are formed between these cells, there is no accumulation of F-actin at the site of contact (Fig. 3B), confirming the role of LFA-1 in mediating this cytoskeleton reorganization during NK cell anti-tumor responses. In contrast, F-actin accumulation at contact sites with C. neoformans occurred independently of LFA-1 expression (Fig. 3C and D). Thus, while formation of an NKIS (as demonstrated by F-actin polarization to the site of target contact) is critically dependent upon LFA-1 when NK cells encounter tumor cells, our data demonstrate that the NK cell receptors responsible for recognition of cryptococcal targets initiate this process independently of LFA-1.

For the reasons discussed above, we have focused on the role of LFA-1 in mediating NK cell anti-fungal activity. However, components of C. neoformans are also known to interact with other cell surface receptors expressed on immune cells. Although GXM also binds to CD14, toll-like receptor (TLR)-2 and TLR4 (50), we do not believe that these molecules mediate NK cell anti-cryptococcal activity as (i) CD14 is not expressed on NK cells and (ii) TLR2- and 4-specific blocking antibodies had no affect on the ability of NK cells to kill C. neoformans (Supplementary Figure 1, available at International Immunology Online). Lastly, many independent studies have demonstrated that the immune system can respond to fungal pathogens through interaction of dectin-1 with fungal β-glucans (53–56). However, as dectin-1-deficient mice remain resistant to C. neoformans infection (57) and anti-cryptococcal activity persisted in NK cells pre-treated with soluble mannan failed to inhibit NK cell anti-cryptococcal responses (Supplementary Figure 2, available at International Immunology Online). We and others have demonstrated that dendritic cells recognize C. neoformans via interactions between lectin receptors and mannosylated proteins found in the cryptococcal cell wall (51, 52). However, blocking lectin receptors by pre-treating NK cells with soluble mannan failed to inhibit NK cell anti-cryptococcal activity (Supplementary Figure 3, available at International Immunology Online).

The C. neoformans-specific receptors mediating NK cell anti-fungal activity remain elusive and are the subject of intensive investigation in our laboratory. We have recently shown that PI3K-dependent activation of Erk1/2 is essential for polarization of perforin-containing granules, degranulation and subsequent killing of C. neoformans (30), demonstrating that certain aspects of the signaling pathways essential for NK cell anti-tumor activity are also critical for cytolytic activity is the reorganization of the cellular cytoskeleton (as evidenced by accumulation of F-actin) to form an immune synapse at the site of target cell contact (45, 48, 49). Of the numerous co-stimulatory and activating receptors expressed on NK cells, LFA-1 appears to play a dominant role in this process (17). Herein, we demonstrate that NK cells deficient for LFA-1 are impaired in their ability to form conjugates with tumor targets (Fig. 2B and C), and in the rare conjugates that are formed between these cells, there is no accumulation of F-actin at the site of contact (Fig. 3B), confirming the role of LFA-1 in mediating this cytoskeleton reorganization during NK cell anti-tumor responses. In contrast, F-actin accumulation at contact sites with C. neoformans occurred independently of LFA-1 expression (Fig. 3C and D). Thus, while formation of an NKIS (as demonstrated by F-actin polarization to the site of target contact) is critically dependent upon LFA-1 when NK cells encounter tumor cells, our data demonstrate that the NK cell receptors responsible for recognition of cryptococcal targets initiate this process independently of LFA-1.

Fig. 7. Antibody blocking of CD18 impairs primary human NK cell anti-tumor but not anti-cryptococcal activity. (A) Primary human NK cells were pre-treated with the CD18-specific blocking antibody clone IB4 or left untreated as control and cultured with CFSE-labeled Raji tumor cell targets for 4–6 h at the indicated E:T ratios. Viable tumor cells were identified as described in Materials and methods. *P < 0.05, **P < 0.01. (B) Primary human NK cells were pre-treated with CD18-specific blocking antibody clone IB4 or left untreated as control prior to culture with Cryptococcus neoformans strain B3501 at an E:T ratio of 1000:1 and fungal cell growth assessed at 0 (Input) and 48 h. NS denotes non-significant differences in CFU.

A central component to both CTL and NK cell-mediated cytolytic activity is the reorganization of the cellular cytoskeleton with cryptococcal targets, F-actin polarization and perforin degranulation nor subsequent anti-cryptococcal activity appeared to be affected by the absence of LFA-1 expression.

Despite antibody blockade of CD18 failed to inhibit anti-cryptococcal activity of human peripheral blood lymphocytes (46), we have recently demonstrated that this activity is mediated primarily through CD4+ T cells (47). Thus, we have specifically focused on the anti-cryptococcal activity of NK cells using NK cell lines and freshly isolated primary human NK cells and report herein for the first time that NK cell anti-tumor activity was critically dependent upon the cell surface expression of LFA-1. However, we were surprised to find that neither conjugate formation

are activated downstream of LFA-1 ligation (15, 43). Although antibody blockade of LFA-1 failed to inhibit anti-cryptococcal activity of human peripheral blood lymphocytes (46), we have recently demonstrated that this activity is mediated primarily through CD4+ T cells (47). Thus, we have specifically focused on the anti-cryptococcal activity of NK cells using NK cell lines and freshly isolated primary human NK cells and report herein for the first time a fundamental difference in the dependence on LFA-1 ligation for NK cell-mediated anti-tumor and anti-fungal activity. In agreement with previous studies, we confirmed that NK cell anti-tumor activity was critically dependent upon the cell surface expression of LFA-1. However, we were surprised to find that neither conjugate formation with cryptococcal targets, F-actin polarization and perforin degranulation nor subsequent anti-cryptococcal activity appeared to be affected by the absence of LFA-1 expression.

A central component to both CTL and NK cell-mediated cytolytic activity is the reorganization of the cellular cytoskeleton (as evidenced by accumulation of F-actin) to form an immune synapse at the site of target cell contact (45, 48, 49). Of the numerous co-stimulatory and activating receptors expressed on NK cells, LFA-1 appears to play a dominant role in this process (17). Herein, we demonstrate that NK cells deficient for LFA-1 are impaired in their ability to form conjugates with tumor targets (Fig. 2B and C), and in the rare conjugates that are formed between these cells, there is no accumulation of F-actin at the site of contact (Fig. 3B), confirming the role of LFA-1 in mediating this cytoskeleton reorganization during NK cell anti-tumor responses. In contrast, F-actin accumulation at contact sites with C. neoformans occurred independently of LFA-1 expression (Fig. 3C and D). Thus, while formation of an NKIS (as demonstrated by F-actin polarization to the site of target contact) is critically dependent upon LFA-1 when NK cells encounter tumor cells, our data demonstrate that the NK cell receptors responsible for recognition of cryptococcal targets initiate this process independently of LFA-1.

For the reasons discussed above, we have focused on the role of LFA-1 in mediating NK cell anti-fungal activity. However, components of C. neoformans are also known to interact with other cell surface receptors expressed on immune cells. Although GXM also binds to CD14, toll-like receptor (TLR)-2 and TLR4 (50), we do not believe that these molecules mediate NK cell anti-cryptococcal activity as (i) CD14 is not expressed on NK cells and (ii) TLR2- and 4-specific blocking antibodies had no affect on the ability of NK cells to kill C. neoformans (Supplementary Figure 1, available at International Immunology Online). Lastly, many independent studies have demonstrated that the immune system can respond to fungal pathogens through interaction of dectin-1 with fungal β-glucans (53–56). However, as dectin-1-deficient mice remain resistant to C. neoformans infection (57) and anti-cryptococcal activity persisted in NK cells pre-treated with soluble mannan failed to inhibit NK cell anti-cryptococcal responses (Supplementary Figure 2, available at International Immunology Online). We and others have demonstrated that dendritic cells recognize C. neoformans via interactions between lectin receptors and mannosylated proteins found in the cryptococcal cell wall (51, 52). However, blocking lectin receptors by pre-treating NK cells with soluble mannan failed to inhibit NK cell anti-cryptococcal responses (Supplementary Figure 3, available at International Immunology Online). Lastly, many independent studies have demonstrated that the immune system can respond to fungal pathogens through interaction of dectin-1 with fungal β-glucans (53–56). However, as dectin-1-deficient mice remain resistant to C. neoformans infection (57) and anti-cryptococcal activity persisted in NK cells pre-treated with soluble β-glucans (Supplementary Figure 3, available at International Immunology Online), we do not believe that recognition of C. neoformans β-glucans underlies NK cell anti-cryptococcal activity.
NK cell anti-fungal activity. The demonstration of LFA-1-independent recognition, conjugate formation, cytoskeletal reorganization, perforin release and effective lysis of fungal targets represents an extremely important and fundamental difference in the way in which these events are initiated.

**Supplementary data**

Supplementary figures are available at International Immunology Online.

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**Abbreviations**

ATCC American Type Culture Collection  
CFSE carboxyfluorescein diacetate succinimidyl ester  
CFU colony forming unit  
cNKIS central NK immunologic synapse  
Erk extracellular signal-regulated kinase  
GXM glucoronoxylomannan  
GXM galactoxylomannan  
LFA leucocyte function-associated antigen  
PeCy5 phycoerythrin-cyanin 5  
PI3K phosphoinositide 3-kinase  
SEM standard error of the mean  
TLR toll-like receptor

**References**

NK cell anti-fungal activity is LFA-1 independent