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Research paper

Quantitative measurement of F-actin accumulation at the NK cell immunological synapse

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ABSTRACT

NK cells are lymphocytes of the innate immune system that can kill target cells after activation signal-induced directional secretion of lytic granule contents. This process depends upon F-actin polymerization at the NK cell immunological synapse (NKIS), which is the dynamic organization of molecules at the interface between the NK cell and target cell. Although F-actin accumulation at the NKIS is easily visualized, the ability to quantify F-actin at the NKIS is required to understand how F-actin reorganization and accumulation enable NK cell function. Here, we demonstrate several novel algorithms for measuring the content of F-actin accumulated at the NKIS with special emphasis upon actin contributed by the NK cell. These algorithms do not rely upon overexpressing fluorescent proteins or preincubating cells with vital fluorescent dyes. Using models of the activating and inhibitory NKIS as well as NK cells expressing fluorescent protein — cell surface receptor fusion proteins, these algorithms were tested and were used to quantitatively demonstrate that F-actin accumulates at the activating, but not at the inhibitory NKIS. With these approaches, we have also established mathematical formulas that should prove valuable in the comprehensive quantitative evaluation of the NKIS and be more broadly applicable in the measurement of the accumulation of any fluorophore at an intercellular junction.

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1. Introduction

1.1. The NK cell immunological synapse

The immunological synapse can be defined as the interface between an immune cell and the cell with which it interacts and has the potential to facilitate signal transduction events to enable immunological functions (Davis and Dustin, 2004). A specialized immunological synapse formed between an NK cell and its target is referred to as the NK cell immunological synapse (NKIS) (reviewed in Orange (2008)). Here, NK cells can receive activating and inhibitory signals to ultimately form either an activating or inhibitory NKIS. The commitment to one or the other depends upon the relative strength and integration of these signals (Almeida and Davis, 2006). The inhibitory NKIS is transient and results in rapid detachment from the target cell (Burshtyn et al., 2000). The activating NKIS induces NK cell function that can include the directed secretion of lytic granule contents to mediate cytotoxicity of the target cell (Liu et al., 2009). This latter type of NKIS is also referred to specifically as a lytic NKIS. NK cells form the lytic NKIS through a sequential progression of steps that involve coordinated function of cell surface receptors, signaling molecules, cytoskeletal proteins, and cellular organelles (reviewed in Orange (2008)). One of the earliest steps in the maturation of the lytic NKIS is the accumulation of filamentous (F)-actin at the synapse (Orange et al., 2003; Wulfing et al., 2003), which facilitates the formation of a supramolecular activation cluster (SMAC). An

Abbreviations: F-actin, filamentous actin; KIR, killer cell immunoglobulin-like receptor; mAbs, monoclonal antibodies; MTOC, microtubule organizing center; NK, natural killer; NKIS, NK cell immunological synapse; SMAC, supramolecular activation cluster.

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F-actin rich peripheral SMAC (pSMAC) is required for the polarization of the microtubule organizing center (MTOC) along with lytic granules to the NKIS to allow for directed secretion of the lytic granule contents and cytotoxicity (Orange et al., 2003). The MTOC and microtubules are then required for the delivery of the lytic granules to the NKIS. Specifically, the blockade of microtubule function prevents lytic granule polarization to the NKIS without affecting F-actin accumulation at the pSMAC (Orange et al., 2003; Banerjee et al., 2007). Thus, F-actin accumulation is an early and critical event in maturation and function of the lytic NKIS. This is specifically exploited by the inhibitory NKIS, which appears to utilize inhibitory signaling to directly prevent maturation of a cytolytic NKIS by interfering with F-actin accumulation (Masilamani et al., 2006).

1.2. Quantification of molecular accumulation at the NKIS

Numerous studies of the NKIS have evaluated the accumulation of molecules at the synapse using confocal fluorescent microscopy of fixed conjugates between NK cells and their targets (Borszcz et al., 2003; Orange et al., 2003; Wulfing et al., 2003; Almeida and Davis, 2006; Masilamani et al., 2006; Banerjee et al., 2007). This has allowed consideration of molecular relocalization to and role in coordinating cell function of the NKIS. In some cases, the molecules under consideration are not expressed in the target cell, such as killer cell immunoglobulin-like receptor (KIR) family members, and thus any accumulation of KIR at the NKIS is contributed by the NK cell. This is unfortunately not the case for many signaling and cytoskeletal molecules, such as F-actin, as they are found both in the NK as well as the target cell. Given the critical nature of F-actin in the formation of the NKIS (Orange et al., 2003; Wulfing et al., 2003) this presents an obstacle to truly comprehending the intricacies of synapse maturation. An additional experimental challenge results from different types of target cells having different amounts of F-actin content in their cortex. For example, two commonly used NK cell target cell lines, the 721.221 Epstein Barr Virus-transformed B cell line and K562 erythroleukemia cell line contain reproducibly different quantities of cortical F-actin (unpublished observations). Although alternative approaches, such as using NK cells expressing vectors encoding GFP-actin, loading cells prior to conjugation with vital fluorescent dyes, or evaluation via transmission electron microscopy can facilitate detecting the content of F-actin that is contributed specifically by an NK cell at the NKIS, they present challenges. Electron micrographs are difficult to quantitate and evaluate through 3 dimensions. Attempts to express fluorescent molecule fusion proteins can be inefficient and overexpression of the fusion proteins has the potential to induce unintended cellular effects. Similarly, incubating cells with vital dyes prior to conjugation has the potential to alter their biological activity. Thus, confocal fluorescence microscopy of conjugates between NK cells and target cells that have been stained with fluorescent antibodies or probes after fixation persists as an essential tool for evaluating F-actin accumulation at and the biology of the NKIS.

Another challenge in the use of fluorescent microscopy to evaluate the NKIS relates to a limited ability to identify smaller or incremental changes in molecular accumulations. For example, if a particular experimental intervention did not prevent the accumulation of F-actin at the NKIS but instead reduced the amount of F-actin accumulated by one-half, could it be detected? Although different studies have utilized various approaches to define molecular accumulation at the NKIS (Borszcz et al., 2003; Orange et al., 2003; Wulfing et al., 2003; Almeida and Davis, 2006; Masilamani et al., 2006; Banerjee et al., 2007; Liu et al., 2009), improved quantitative approaches to discern incremental changes could allow for better understanding of the influences upon temporospatial synapse formation and maturation.

In an effort to be able to apply increased sensitivity in studies of the NKIS, we have developed and evaluated several algorithms for measuring molecular accumulation at the synapse. Although these approaches should be generally applicable to the accumulation of any fluorescent signal at an immunological synapse, we applied and validated our algorithms by measuring the F-actin content in the activating and inhibitory NKIS in 2- or 3-dimensional images. While we utilized GFP-receptor fusion protein-expressing NK cells to validate our approach, we also attempted to estimate the specific contribution of F-actin to the NKIS by the NK cell in the conjugate without an additional dependence upon fluorescent dyes, fluorescent fusion proteins, or cell manipulations. Using these algorithms in NK cells that were not manipulated prior to conjugation, we quantitatively demonstrated that the F-actin content at the lytic NKIS was significantly greater than that found in the cortex of unconjugated NK cells or target cells. We also quantitatively demonstrated that F-actin accumulation at the lytic NKIS was more abundant than that found at the inhibitory NKIS.

2. Materials and methods

2.1. NK cell preparation, NK cell and target cell lines

Leukocyte-enriched blood was obtained from volunteer donors and was used to prepare *ex vivo* NK cells by negative selection using the human NK cell isolation kit II (Miltenyi Biotec) as described (Banerjee et al., 2007). NK cell preparations contained >96% $CD56^+/CD3^-$ cells with less than 1% $CD3^+$ cells as determined by FACS using fluorophore-conjugated mAbs (BD Biosciences). All human samples were obtained with approval of the Institutional Review Board of the Children's Hospital of Philadelphia. The immortalized NK cell line YTS-KIR2DL1-GFP was the gift of Dr. D. Burshtyn, (Borszcz et al., 2003), and YTS CD2-GFP cells were previously generated and described elsewhere (Orange et al., 2003). HLA Cw3-, or Cw4-expressing 721.221 B lymphoblastoid cells (Fassett et al., 2001) and K562 erythroleukemia target cells lines with or without transduced CD86 (KT86, and K562, respectively) were used as target cells (Banerjee et al., 2007).

2.2. Confocal microscopy

Conjugates between effector and target cells at a 2:1 ratio were formed in 200 µL of suspension for 15 min and adhered onto poly-L-lysine-coated glass slides (Polyprep, Sigma-Aldrich) for 10 min, all at 37 °C, as described elsewhere (Banerjee et al., 2007). Fixing, permeabilization, and immunostaining for F-actin and perforin were also performed as previously described (Banerjee et al., 2007). After staining, slides were covered with

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