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T cell-to-T cell clustering enhances NF-κB activity by a PI3K signal mediated by Cbl-b and Rho

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Abstract

Full activation of T cells requires the binding of antigen to the T cell receptor and stimulation of the CD28 molecule, a process which typically occurs when T cells bind to an antigen presenting cell. The transcription factor, NF-κB, is an integration point for these two signals and its activation is critical for T cell function. Using antibodies to the TCR and CD28 molecules to activate Jurkat T cells, we show that cells that were permitted to aggregate into multi-cellular clusters increased NF-κB activity compared to unclustered cells. Inhibition of PI3K signaling with wortmannin decreased the clustering-mediated NF-κB signal. Over-expression of a dominant negative form of Cbl-b, an endogenous inhibitor of PI3K, in unclustered cells rescued NF-κB activation to the same levels caused by cell clustering. Inhibiting signaling through Rho with dominant negative RhoA abrogated both clustering-mediated and dominant negative Cbl-b-mediated NF-κB inactivation, but not TCR/CD28 mediated NF-κB activation. Taken together, these results suggest that in addition to pathways stimulated by classical T cell–APC interactions, another signal arising from T cell clustering can enhance activation.

Keywords: CD28; Clustering; Cell–cell adhesion; Cytoskeleton; TCR; RhoA; PI3K

T cells require stimulation through two surface receptors in order to become fully activated. The binding of the TCR-CD3-ζ chain complex (TCR) to antigen is the primary stimulus, with an additional co-stimulus occurring through the binding of the CD28 receptor to one of its ligands, CD80 (B7-1) and CD86 (B7-2) [1]. While certain intracellular signaling molecules require only TCR binding for activation, the transcription of IL-2 leading to full activation and subsequent clonal expansion of a T cell requires both TCR and CD28-mediated signals [2]. Specific molecules have been identified as integration points for the two signals. Among these is NF-κB, a transcription factor that is essential for IL-2 gene transcription [3–5].

Following the binding to antigen, an immunological synapse forms at the surface of the T cell with a central aggregate of TCRs surrounded by a ring of adhesion molecules [6,7]. This leads to a series of biochemical signals that can alter T cell morphology and function. Among the early biochemical changes that occur following antigen binding is the activation of the phosphoinositide 3-kinases (PI3K) which phosphorylate inositol phospholipids, leading to lipid products that control a wide array of signal transduction molecules [8,9]. Several negative regulators of PI3K, including PTEN and cbl-b, have recently been identified as important modulators in abrogating T cell activation [10,11]. In addition

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to biochemical changes, structural rearrangements in the actin cytoskeleton appear to be critical to T cell activation [6,7,26]. These rearrangements are mediated by the Rho family of small GTPases, including Rho, Rac, and Cdc42 [12].

The presentation of antigen by an antigen presenting cell (APC) to a solitary T cell can occur in high density inflammatory settings, which may contain the presence of numerous accessory T cells. While much is understood about the role of the APC in the initiation of T cell activation, little is known about how the presence of additional T cells and their interactions affects the response to antigen. A recent study by Metcalf et al. [13] showed that cellular crowding of murine spleen cells leads to an increase in colony stimulating factor and IL-3. Our work examines how the presence of T cell crowding affects the downstream activation of NF-κB. Using antibodies to the TCR and CD28 molecules to activate T cells, we show that the presence of cell clustering increased NF-κB activity compared to unclustered cells. Furthermore, our studies suggest that this clustering modulates T cell activation specifically through the PI3K and Rho signaling pathways. Our results suggest that in addition to a membrane receptor binding step, T cell-to-T cell clustering is an important component of full T cell activation.

Materials and methods

Materials. NF-κB luciferase reporter and anti-CD3 antibody were gifts from Ron Wange (National Institute on Aging; Baltimore, MD). Cbl-b expression vectors were gifts from Stan Lipkowitz (National Cancer Institute; Bethesda, MD). Anti-CD28 and anti-LFA-1 antibodies were purchased from Pharmingen (San Diego, CA). Poly-lysine coated coverslips were purchased from BD Biosciences (Bedford, MA). PMA, A23187, and wortmannin were purchased from Sigma (St. Louis, MO). The Rho constructs were gifts from Peter Burbelo (Georgetown University Medical Center; Washington, DC). Polystyrene petri plates were purchased from Falcon.

Cell culture, transfections, and stimulations. Jurkat T cells were maintained in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS, 2 mM L-glutamine, and 10 μg/ml ciprofloxacin as previously described [14]. At the time of transfection, 5×10^6 cells and 1–10 μg DNA were electroporated at 250 V and 950 μF using a Gene Pulsar II electroporator (Bio-Rad, Hercules, CA) at room temperature. Equal amounts of DNA were used for samples within a single experiment. Activation of T cells was initiated 16–24 h after transfection by exposing cells to 10 μg/ml of anti-CD3 antibody and 2 μg/ml of anti-CD28 antibody in complete media for the specified times. To prevent clustering, cells were activated in dishes pre-coated overnight with 10 μg/ml of anti-CD3 antibody and 2 μg/ml of anti-CD28 antibody for the specified times. Each experiment included a group of untreated cells and a group of cells stimulated with 50 ng/ml of PMA and 500 ng/ml of A23187 as a positive control. Alternately, cells were immersed in media containing 7% methylcellulose (4000 cp, Sigma). Where noted, some experiments included 5 μg/ml of anti-LFA-1 antibody.

Luciferase assay. Luciferase assays were performed as previously described [15]. Briefly, cells were lysed and normalized to equal amounts of total protein using the Bradford assay (Bio-Rad, Hercules, CA). Analysis of lysates for luminescence was done using a luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Luminescence was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Results are expressed as means ± the standard error of the mean unless otherwise noted.

Photography. Digital photographs were taken using an Olympus OM camera with the Meridian Insights System (Meridian Instrument Company, Kent, WA).

Results

Clustered T cells have greater NF-κB activity than surface-adsorbed unclustered T cells

Once activated, T cells form direct contacts with other T cells to form large T cell aggregates or clusters. In order to determine if the formation of T cells into clusters results in a change in NF-κB activity, Jurkat T cells that were transfected with a luciferase reporter plasmid containing the DNA-binding site for NF-κB were stimulated with anti-CD3 and anti-CD28 antibodies under conditions in which the cells were allowed to form clusters (Fig. 1A, left panel) or where cluster formation was prevented by cell capture onto the surface of the reaction vessel with adsorbed anti-CD3 and anti-CD28 antibodies (Fig. 1A, right panel). For comparison, equivalent amounts of NF-κB reporter transfected Jurkat T cells were left unstimulated or stimulated with a combination of phorbol ester (PMA) and calcium ionophore (A23187) to induce maximum NF-κB activity. After 6 h, the cells were removed from the reaction vessels by gentle pipetting, lysed, and luciferase activity was measured. The stimulation of NF-κB activity in T cells that were able to form clusters was substantially higher than in the cells that were prevented from forming clusters (Fig. 1B).

The full activation of NF-κB requires both a primary signal generated through the binding of the TCR and an accessory signal generated by CD28 binding [3,16]. To examine whether one of these signals was specifically required for the cluster-mediated activation, NF-κB-luciferase transfected Jurkat T cells were stimulated with or without anti-CD3 or anti-CD28 antibody while adsorbed to the culture plate by anti-CD28 antibody or anti-CD3 antibody, respectively. Following 6 h of treatment, the cells were lysed and luciferase assays were performed. The cells that were stimulated with anti-CD3 antibody while immobilized by anti-CD28 antibody, or with anti-CD28 antibody while adsorbed by anti-CD3 antibody, had NF-κB activity greater than that of cells adsorbed by both antibodies, but less than that of cells allowed to form clusters (Fig. 1C). As expected, the cells exposed to either anti-CD3 or anti-CD28 antibodies alone had NF-κB activity at the level of unstimulated cells. Taken together, these findings suggested again that cells that were allowed to cluster increased NF-κB