

## T cell-to-T cell clustering enhances NF- $\kappa$ B 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- $\kappa$ B, 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- $\kappa$ B activity compared to unclustered cells. Inhibition of PI3K signaling with wortmannin decreased the clustering-mediated NF- $\kappa$ B signal. Over-expression of a dominant negative form of Cbl-b, an endogenous inhibitor of PI3K, in unclustered cells rescued NF- $\kappa$ B 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- $\kappa$ B inactivation, but not TCR/CD28 mediated NF- $\kappa$ B 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.

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T cells require stimulation through two surface receptors in order to become fully activated. The binding of the TCR-CD3- $\zeta$  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- $\kappa$ B, 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- $\kappa$ B. Using antibodies to the TCR and CD28 molecules to activate T cells, we show that the presence of cell clustering increased NF- $\kappa$ B 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- $\kappa$ B 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  $\mu$ g/ml ciprofloxacin as previously described [14]. At the time of transfection,  $5 \times 10^6$  cells and 1–10  $\mu$ g DNA were electroporated at 250 V and 950  $\mu$ F 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  $\mu$ g/ml of anti-CD3 antibody and 2  $\mu$ g/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  $\mu$ g/ml of anti-CD3 antibody and 2  $\mu$ g/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  $\mu$ g/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  $\pm$  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- $\kappa$ B 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- $\kappa$ B activity, Jurkat T cells that were transfected with a luciferase reporter plasmid containing the DNA-binding site for NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B-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- $\kappa$ B 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- $\kappa$ B activity at the level of unstimulated cells. Taken together, these findings suggested again that cells that were allowed to cluster increased NF- $\kappa$ B

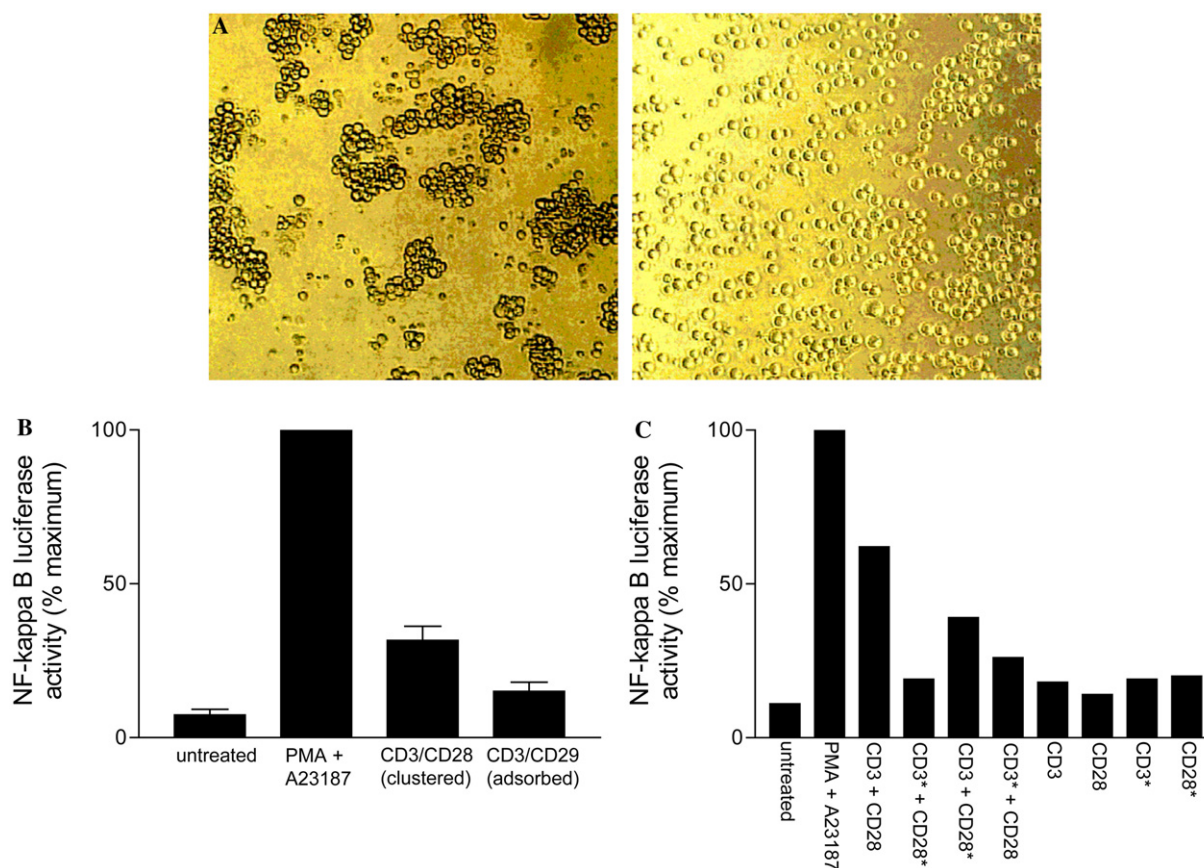


Fig. 1. Clustered T cells have greater NF- $\kappa$ B activity than adsorbed T cells. Jurkat T cells transfected with the NF- $\kappa$ B luciferase reporter were stimulated for 6 h with the indicated treatments. (A) Cells were stimulated with anti-CD3 and anti-CD28 antibodies under conditions which either permitted (left panel) or prevented (right panel) cell clustering. Cells were photographed at 4 h of stimulation. (B) Following stimulation, cells were lysed and luciferase assays were performed. Results are expressed as means  $\pm$  SEM of the percent maximum NF- $\kappa$ B luciferase activity for five independent experiments. (C) Representative of two independent experiments. \*Cells adsorbed to the reaction vessel by this antibody.

activity compared to unclustered cells and that both the CD3 and CD28 signals were required for this effect.

#### *Increase in NF- $\kappa$ B activity is due to cluster formation*

The increase in NF- $\kappa$ B activity in clustered cells over adsorbed cells could be due to either an enhancing signal provided by the clustering of cells or an inhibitory signal initiated by the immobilization of the cells. To explore this question, we examined whether the receptor engaged by immobilization affected stimulation. NF- $\kappa$ B luciferase reporter transfected Jurkat T cells were adsorbed to the side of the reaction vessel by antibodies to the adhesion molecule, LFA-1, or by poly-lysine which cells adhere to non-specifically, and then stimulated with soluble anti-CD3 and anti-CD28 antibodies. NF- $\kappa$ B in cells immobilized by anti-LFA-1 or poly-lysine could not be stimulated, suggesting that the lack of activation did not depend on the immobilization strategy (Fig. 2A). To specifically examine whether the enhanced NF- $\kappa$ B activity was inhibited in these experiments directly by surface immobilization, or because

immobilization blocked cell clustering, cells were stimulated when suspended in media containing methylcellulose. The methylcellulose forms a viscous media that allows cells to remain suspended such that cells neither adsorbed to the reaction vessel nor clustered to each other. Preventing clustering of suspended cells in this manner also abrogated NF- $\kappa$ B activation.

To see if the addition of an excess of adsorbing antibody could overcome the decrease in NF- $\kappa$ B activity seen in adsorbed cells, dose-response studies were performed. Jurkat T cells transfected with the NF- $\kappa$ B reporter were stimulated with anti-CD3 antibodies at a concentration of 10  $\mu$ g/ml and anti-CD28 antibodies at concentrations of 50, 5, and 0.5  $\mu$ g/ml under conditions in which the cells were either allowed to cluster or were adsorbed to the side of the reaction vessel by the anti-CD28 antibodies. While at lower concentrations of the adsorbing antibody the amount of NF- $\kappa$ B activity remains lower in the adsorbed cells, at the concentration of 50  $\mu$ g/ml, the amount of NF- $\kappa$ B activity is similar to that present in the cells that clustered together (Fig. 2B). In contrast, clustered cells were maximally stimulated at all antibody

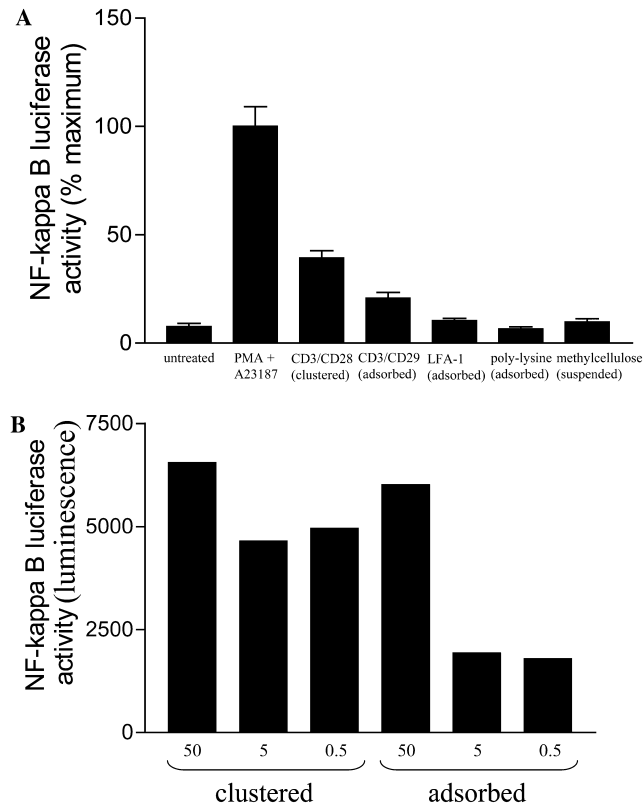


Fig. 2. Cluster formation increases NF- $\kappa$ B activity. Jurkat T cells transfected with the NF- $\kappa$ B luciferase reporter were stimulated for 6 h with anti-CD3 and anti-CD28 antibodies under conditions which either permitted cell clustering, or prevented clustering by adsorbing cells to the reaction vessel wall either with the indicated coatings or with methylcellulose-containing media. Following stimulation, cells were lysed and luciferase assays were performed. (A) Results are expressed as means  $\pm$  SEM of the percent maximum NF- $\kappa$ B luciferase activity for three independent experiments. (B) Representative of two independent experiments.

concentrations. Together, these results suggest that cell clustering is not essential to a T cell response and can be replaced by excessive receptor stimulation, but also that clustering significantly enhances the ability of T cells to activate in response to low level stimuli.

#### Dominant negative Cbl-b rescues NF- $\kappa$ B activity in unclustered cells

While CD28 is the most well known co-stimulatory molecule in T cells, other co-stimulatory molecules have been identified [17,18]. Many of these molecules and their ligands are present on the surface of Jurkat T cells, raising the possibility that cell clustering could stimulate additional signals via a ligand on one cell binding to its receptor on a neighboring cell. We tested the possibility that the decrease in NF- $\kappa$ B activity seen in the unclustered cells was from the absence of one of these signals. Combinations of known stimulants of co-stimulatory molecules were added to the cells adsorbed by anti-

CD3 and anti-CD28 antibodies to attempt to rescue activation. Among the receptors stimulated were LFA-1, ICAM-1, and CD4, however, their activation did not increase NF- $\kappa$ B activity in the unclustered cells (data not shown).

Since the decrease in NF- $\kappa$ B activity in the unclustered cells did not appear to be due to the lack of a major co-stimulatory signal from a membrane bound receptor, we then investigated how the inhibition of biochemical signaling pathways would affect the clustering-induced NF- $\kappa$ B activity. PI3K is a key early regulator involved in T cell activation [8,9]. In order to test the role of PI3K in clustering-mediated NF- $\kappa$ B activity, Jurkat T cells transfected with the NF- $\kappa$ B luciferase reporter were incubated with wortmannin, an inhibitor of PI3K activity, or with DMSO as a vehicle control (Fig. 3A). Wortmannin treatment appeared to decrease

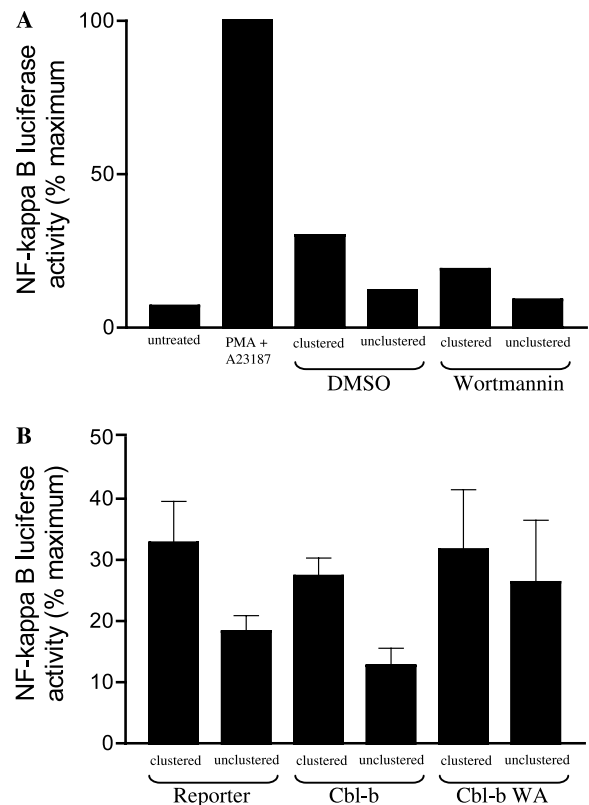


Fig. 3. The presence of dominant negative Cbl-b rescues NF- $\kappa$ B activity in unclustered cells. (A) Jurkat T cells transfected with the NF- $\kappa$ B luciferase reporter and incubated in either 100 nM wortmannin or DMSO (vehicle control) were stimulated for 6 h with anti-CD3 and anti-CD28 antibodies under conditions which the cells clustered together or were adsorbed. Following stimulation, cells were lysed and luciferase assays were performed. Representative of three independent experiments. (B) Jurkat T cells transfected with the NF- $\kappa$ B luciferase reporter alone or with Cbl-b or Cbl-b WA were stimulated for 6 h with anti-CD3 and anti-CD28 antibodies under conditions which the cells clustered together or were adsorbed. Following stimulation, cells were lysed and luciferase assays were performed. Results are expressed as means  $\pm$  SEM of the percent maximum NF- $\kappa$ B luciferase activity for four independent experiments.

the clustering-mediated NF- $\kappa$ B activity while having little effect in unclustered cells. These findings suggested that PI3K may be involved in the clustering-mediated NF- $\kappa$ B activity.

Cbl-b, a RING-type E3 ubiquitin ligase member of the Cbl family of proteins, is an endogenous negative regulator of T cell activation [11,19], and appears to do so by ubiquitination and inactivation of PI3K [20,21], and by inhibiting lipid raft formation and receptor oligomerization [22]. To test the role of Cbl-b in the clustering-mediated NF- $\kappa$ B activity, Jurkat T cells were transfected with the NF- $\kappa$ B luciferase reporter alone or co-transfected with DNA encoding full-length or a dominant negative Cbl-b (Cbl-b WA) [21], and stimulated with anti-CD3 and anti-CD28 antibodies under conditions where the cells were allowed to cluster or not. After 6 h, the cells were removed, lysed, and NF- $\kappa$ B activity was determined (Fig. 3B). While over-expression of wild type Cbl-b did not alter NF- $\kappa$ B activity, over-expression of Cbl-b WA resulted in an increase in NF- $\kappa$ B activity in the unclustered cells to the level seen in clustered cells. Thus, while PI3K inhibition with wortmannin blocked clustering-mediated activation, mutated Cbl-b appears to rescue the clustering effect in unclustered cells, suggesting that clustering activates NF- $\kappa$ B through Cbl-b inactivation.

#### *Rho signaling is required for cluster-mediated NF- $\kappa$ B activation*

Both cell-to-cell adhesion and PI3K signaling appear to modulate cytoskeletal organizations important to T cell activation, possibly through the Rho-family GTPase, RhoA [10,12]. To test the role of Rho in clustering-mediated NF- $\kappa$ B signaling, Jurkat T cells were

transfected with the NF- $\kappa$ B luciferase reporter alone or with DNA encoding constitutively active RhoA (RhoV14) or dominant negative RhoA (RhoN19), and stimulated with anti-CD3 and anti-CD28 antibodies under conditions where the cells were allowed to cluster or not. After 6 h, the cells were removed, lysed, and NF- $\kappa$ B activity was determined (Fig. 4). Expression of RhoV14 did not alter NF- $\kappa$ B activity in either clustered or unclustered cells over controls, but RhoN19 selectively decreased cluster-mediated NF- $\kappa$ B activity. Taken together, these results suggest that RhoA is necessary but not sufficient for the increase in NF- $\kappa$ B activity seen in clustered cells.

#### *Dominant negative Cbl-b-induced NF- $\kappa$ B activation requires Rho signaling*

The clustering-mediated NF- $\kappa$ B signaling appears to be rescued by downregulation of Cbl-b and requires Rho signaling. To determine whether the Rho requirement for clustering-mediated NF- $\kappa$ B activation occurs upstream or downstream of the Cbl-b control point, Jurkat T cells were transfected with the NF- $\kappa$ B luciferase reporter alone or with DNA encoding Cbl-b WA, or both Cbl-b WA and RhoN19. Following transfection, T cells were stimulated with anti-CD3 and anti-CD28 antibodies under conditions where the cells were allowed to cluster or not. After 6 h, the cells were removed, lysed, and NF- $\kappa$ B activity was determined. As expected, the presence of Cbl-b WA increased NF- $\kappa$ B activity in the unclustered cells compared to cells that were transfected with only the reporter (Fig. 5). Importantly, expression of RhoN19 abrogated the Cbl-b WA

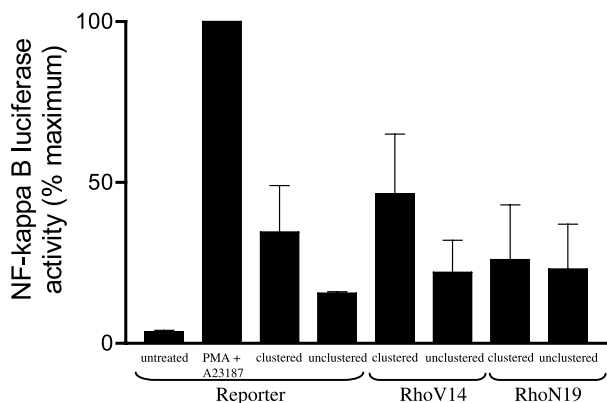


Fig. 4. Rho is required for clustering effect. Jurkat T cells transfected with the NF- $\kappa$ B luciferase reporter alone or with RhoV14 or RhoN19 were stimulated for 6 h with anti-CD3 and anti-CD28 antibodies under conditions which the cells clustered together or were adsorbed. Following stimulation, cells were lysed and luciferase assays were performed. Results are expressed as means  $\pm$  SEM of the percent maximum NF- $\kappa$ B luciferase activity for two independent experiments.

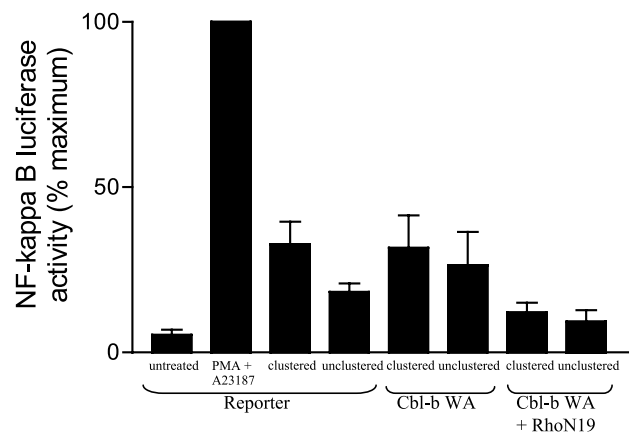


Fig. 5. Rho is downstream of Cbl-b in regulating NF- $\kappa$ B activity. Jurkat T cells transfected with the NF- $\kappa$ B luciferase reporter alone or with either Cbl-b WA, or both Cbl-b WA and RhoN19 were stimulated for 6 h with anti-CD3 and anti-CD28 antibodies under conditions which the cells clustered together or were adsorbed. Following stimulation, cells were lysed and luciferase assays were performed. Results are expressed as means  $\pm$  SEM of the percent maximum NF- $\kappa$ B luciferase activity for three independent experiments.

activation of NF- $\kappa$ B activity in both clustered and unclustered cells. These results demonstrate that RhoA acts downstream of the Cbl-b signal.

## Discussion

T cell activation has long been known to require co-stimulation of the TCR and CD28 molecules. Here, we show that in addition to these signals, homotypic cell-to-cell clustering significantly enhances T cell activation. We propose that in addition to the co-stimulatory step that occurs between a T cell and an antigen presenting cell, a clustering step significantly enhances NF- $\kappa$ B activation. Furthermore, this clustering-mediated activation appears to involve the downregulation of Cbl-b activity, activation of PI3K, and downstream RhoA signaling (Fig. 6).

Cbl-b prevents the aggregation of T cell receptors on the T cell surface and attenuates the formation of the immunological synapse [22]. Recently, several studies have implicated Cbl-b in the regulation of the CD28 signal and its affect on T cell activation [20,21,23,24]. Cbl-b has been shown to negatively recruit the p85 subunit of PI3K to CD28 [21] and thereby diminishing the signal from CD28. In addition, Cbl-b also negatively regulates T cell activation by promoting the clearance of engaged T cell receptors from the cell surface [25]. Our work suggests that the increase in NF- $\kappa$ B activation seen following T cell-to-T cell clustering may involve the inactivation of Cbl-b. The decrease in NF- $\kappa$ B activity in clustered T cells following blockade of PI3K with wortmannin further supports the involvement of the Cbl-b and PI3K pathways in clustering-induced T cell activation. Further studies will be required to define more clearly the relationship between co-receptor based and aggregation based T cell activation.

The involvement of Rho signaling in this clustering pathway is consistent with its known role in T cell activa-

tion. The guanine nucleotide exchange factors of the Vav family are instrumental in the transition of Rho GTPases from inactive to active states [26] and Cbl-b is known to negatively regulate Vav via PI3K [20]. Upon tyrosine phosphorylation, Vav becomes part of a unique protein signaling complex thought to facilitate the physical proximity of RhoA to an area of cytoskeletal reorganization [27]. Furthermore, there is overwhelming evidence utilizing pharmacological inhibitors of PI3K, signaling mutants that prevent PI3K activation, and constitutively active PI3K that supports the control by PI3K of Rho mediated changes in the cytoskeleton [8]. Our study shows that the presence of dominant negative Rho inhibits the increase in NF- $\kappa$ B activity seen following T cell-to-T cell clustering, and that this initiation occurs downstream of Cbl-b signaling. Thus, the clustering signal may enhance these signals necessary for T cell activation, and perhaps support the downstream changes in cytoskeletal restructuring following activation.

This report is among the few published studies that have addressed the role of cell clustering or crowding in T cell activity. Metcalf et al. [13] showed that cellular clustering or crowding results in an increase in colony stimulating factor and IL-3 in murine spleen cells, but not in thymus or marrow cells. In addition to the paracrine effects of crowding, direct cell-to-cell contact may also be involved. Recent reports implicate PI3K in the induction of endothelial cell proliferation to cells with direct cell-to-cell contact [28] and blockade of Rho results in inhibition of cadherin-induced proliferation signal [29]. Our work in T cells is consistent with these studies.

The increased activation of NF- $\kappa$ B seen when T cells cluster together may be important in a number of normal and abnormal immune system responses. These range from altering the initial clonal expansion of a single T cell to attenuating the inflammatory response in specific tissues. As this report is confined to over-expression experiments in T cell lines, further studies are needed to examine this effect in more physiologically relevant systems.

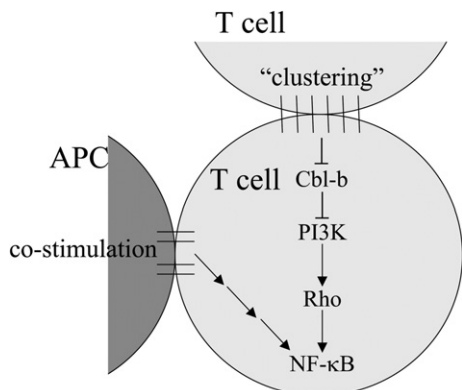


Fig. 6. Model proposing co-stimulatory and clustering signals in NF- $\kappa$ B activation. A clustering step between T cells leads to abrogation of the Cbl-b inhibition of PI3K and activation of Rho resulting in activation of NF- $\kappa$ B at levels greater than T cell co-stimulation alone.

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