

# Mapping the polarity for T cell activation with an optical trap

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## ABSTRACT

In the body, individual T helper lymphocytes need to be activated first by physical contact with antigen-presenting cells (APC). T-cell contact with APCs initiates an activation cascade which includes an increase in T-cell intracellular calcium and leads to T-cell proliferation and differentiation. We have combined fluorescence spectroscopy and imaging with optical manipulation to investigate the physical properties of T-cell activation. We study cell-cell contact requirements for T-cell activation using optical tweezers to control the orientation of T-cell/APC pairs and fluorescence microscopy to measure the subsequent T-cell intracellular calcium level ( $[Ca^{2+}]_i$ ) response. APCs or beads coated with antibodies to the T-cell receptor are trapped with a near-infrared titanium-sapphire laser and placed at different locations along the T-cell, which has a polarized appearance defined by the shape and direction of crawling. T cells which are presented with antigen at the leading edge have a higher probability of responding and a shorter latency of response than those contacting APCs or beads with their trailing end. Alterations in antibody density and bead size are used to determine the spatial requirements for T cell activation and the minimum number of receptors which must be engaged in order to transmit a positive signal.

**Keywords:** Optical laser trapping, fluorescence detection, optical manipulation, T-cell activation, T cell receptor, calcium imaging, antibody-coated bead

## 1. INTRODUCTION

All adaptive immune responses are mediated by lymphocytes. Lymphocytes have cell-surface receptors for antigen that are encoded in rearranging gene segments. There are two main classes of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells), which mediate humoral and cell-mediated immunity respectively. Millions of newly formed lymphocytes leave the bone marrow daily. Each of these cells either multiplies and differentiates or undergoes apoptosis, depending on the nature, timing and location of interactions with other cells of the immune system. Specific recognition of foreign antigen by cell surface immunoglobulin induces B cells to proliferate either into antibody-producing cells or into memory B cells. This process, however, requires help from activated T cells (T helpers). Individual antigen-specific T helpers need to be activated first by physical contact with antigen-presenting cells (APCs), which can themselves be B cells.

The APC internalizes, degrades, and complexes a peptide fragment of the native antigen with a class II major histocompatibility complex (MHC II) molecule. MHC II-antigen complexes are presented on the surface of the APC, where they can be recognized by T cell receptors (TCR) on the T helper surface. The interaction between TCR and MHC-peptide elicits a series of intracellular biochemical events, including the activation of the tyrosine kinases *lck* and *fyn*, resulting in phosphorylation and activation of phospholipase C (PLC). Both 1,4,5 inositol triphosphate ( $IP_3$ ) and diacylglycerol (DAG) are generated, resulting in the activation of protein kinase C and release of  $Ca^{2+}$  from internal stores. In addition,  $Ca^{2+}$  influx from the external medium is triggered through voltage-independent  $I_{crac}$  channels. This series of signaling events continues, transferring signals from the T cell surface to the nucleus, where genes necessary for T cell activation are transcribed.

Typical T cell activation assays are based on populations of T cells. Although these types of population studies provide valuable information on T cell activation, they cannot provide insights into dynamic events of early T cell activation upon T cell-APC interactions. Recent advances in optical techniques have enabled a single-cell approach to immunology. Video-microscope techniques, together with the development of new fluorescent probes, are helping to clarify events during

antigen presentation, calcium signaling and effector functions including gene activation, cell proliferation, apoptosis, anergy, and secretion of cytokines, antibodies and channel-forming perforin molecules. There are three primary advantages of the single-cell approach: 1) Unambiguous and rapid identification of cell phenotype and response (no confounding issue of contaminating cell types); 2) Dynamic and quantitative measurement capability (on-line time course as opposed to taking samples and getting the answer hours later); 3) The ability to correlate responses and to perform kinetic analysis (averaged response from population measurement measurements including biochemical assays and optical measurements of cell suspensions in cuvettes inherently blur the actual responses of individual cells). Oscillations in  $[Ca^{2+}]_i$  and all or none gene expression are two examples of discoveries we have made by investigating single T cells using video imaging techniques.

T cell polarity following contact with an APC has been well characterized on the basis on plasma membrane protein clustering, cytoskeletal and organellar reorganization, and cytokine secretion. These events last hours and contribute the activation of specific cells in crowded environments such as lymph nodes, where most antigen is detected. In addition to reorganization triggered by cell-cell contact, T cells are motile and therefore possess some inherent polarity even before they engage the APC. The role of inherent polarity in T cell activation has not been determined.

Although several single Thp-APC studies have been reported, however, some of these studies have dealt with fixed cells. And also, it is time-consuming to get spontaneous Thp-APC conjugates and contact quality is difficult to evaluate. The recent use of the laser-based optical trap has provided insights into T cell polarity to antigen and contact requirements for T cell activation with APC. Laser tweezers are highly focused beams of laser light that use the forces of radiation pressure to grab and manipulate microscopic objects, such as bacteria, cellular organelles and polystyrene. Taking advantage of relatively non-invasive optical trap, we are examining T cell/B cell orientation and T cell activation processes. First, APCs are placed by a titanium-sapphire laser trap at various locations along a single T cell, which has a "head" and "tail" defined by the shape and the direction of crawling. Next, fluorescence detection of functional indicators, T cell intracellular calcium, monitors activation. Thus, we are able to quantify the response of individual T cells either to complementary antigen-presenting B cells or to antibody-coated beads.

## 2. METHODS

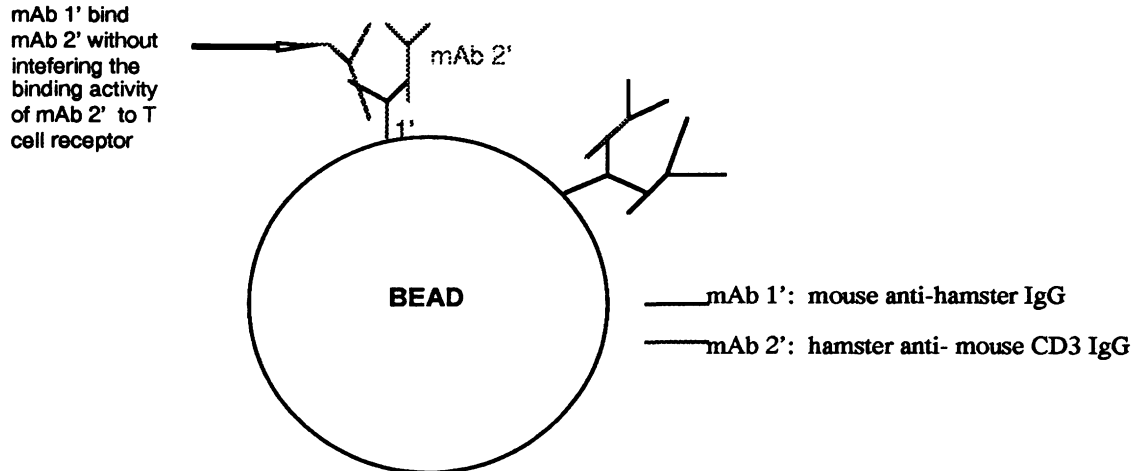
*Cell Culture.* The murine hen egg lysozyme (HEL)-restricted, CD4<sup>+</sup> T cell (IE5) and MHC II-restricted B cell (2PK3) hybridomas were grown in RPMI 1640 containing 10% fetal bovine serum (RPMI/FBS) 10 mM HEPES and 1% NEAA, glutamine, and sodium pyruvate. Cells were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>/95% air. IE5 cells were moderately adherent to plastic flasks at 37°C and were resuspended for collection by gentle shaking at room temperature. Antigen presenting 2PK3 cells were incubated with 10 µg/ml HEL for between 3 and 12 hours. This protocol produced a maximal response from IE5 T cells as judged by a contact-dependent  $[Ca^{2+}]_i$  response about 70% of cells. T cells were also probed with antibody-coated latex microspheres. We used 6 µm diameter polystyrene microspheres stabilized with sulfate charges (IDC, Portland Or). 100 µg/ml mouse-α hamster IgG in 10% PBS was adsorbed to beads for 8 hours at room temperature, centrifuged and washed twice with 10% PBS and then conjugated with 50 µg/ml hamster α-mouse CD3e for 3 hours. Beads were centrifuged and washed twice before use.

*Optical Trapping.* The geometry of T cell-B cell contact was manipulated using a tunable, near infrared titanium:sapphire laser producing a trapping beam at about 760 nm (Berns et al., 1992). The trapping laser was introduced via the TV port of a Zeiss Laser Scanning Confocal microscope (LSM 410). A short-pass (720 nm) dichroic reflector was used to separate trapping and fluorescence excitation beams. A 100X 1.3 NA Neofluor objective and focused the near infrared and visible beams, resulting in 60 mW trapping power at the focal plane. This arrangement allowed trapping and fluorescence-based  $[Ca^{2+}]_i$  measurements on the same cells.

*[Ca<sup>2+</sup>]<sub>i</sub> imaging.* To measure T-cell  $[Ca^{2+}]_i$  on the LSM, IE5 cells were co-loaded with a combination of fura-red/AM (5 µM) and oregon-green/AM (2 µM), two long-wavelength Ca<sup>2+</sup> indicators which respond to the 488-nm excitation line of the argon laser. Cells loaded for 1.5 hr at 37°C produced a red to green shift when  $[Ca^{2+}]_i$  was elevated. This shift was quantified by scanning cells with the argon laser and dividing the fluorescence intensity signals from two photomultipliers with emission bands of 520-570 nm (green) and >610 nm (red). In these experiments a single, 2PK3 cell or antibody-coated bead was held in the trap on a heated stage and positioned so that it made contact with a particular region of a dye-loaded T cell. Once the cells were positioned, the trapping beam was cut off and 488 nm laser excitation were performed. A third

photomultiplier collected a  $\text{Ca}^{2+}$ -insensitive blue emission band (400–480) from incandescent illumination which was used to produce a brightfield image. 30–40 scans at 10 s intervals were made to determine whether a  $[\text{Ca}^{2+}]_i$  increase occurred in the T cell following contact with APC. T cells not responding within 400 s were scored as unresponsive.

*Antibody coating on beads.* Various sizes of sulfate polystyrene beads were coated with 100  $\mu\text{g}/\text{ml}$  of anti-hamster IgG monoclonal antibodies (mAbs) first and then incubated with various densities of hamster anti-murine TCR:CD3 mAbs. The anti-hamster mAbs bind the Fc portion of hamster anti-TCR:CD3 mAbs so that all of the binding sites of the hamster anti-murine TCR:CD3 mAbs are free (figure 1). Well-coated beads are manipulated with an optical trap and placed at different sites of T cells (leading edge, mid-section, or tail region).



**Figure 1. Antibody coating on bead diagram**

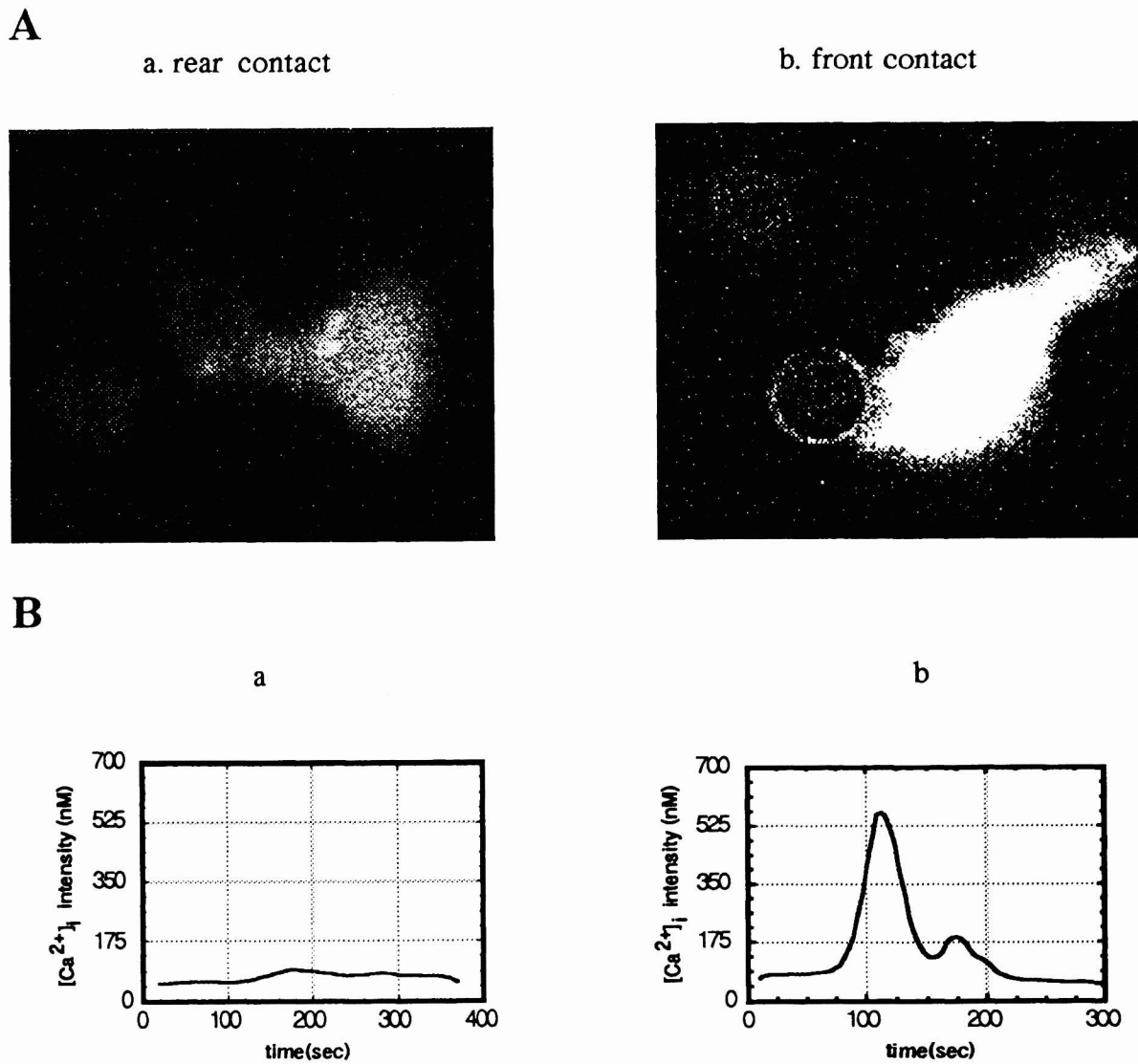
Notice that mAb 1' bind mAb 2' without interfering the binding activity of mAb 2' to T cell receptor so that all of the binding sites of mAbs 2' are free .

### 3. RESULTS AND DISCUSSION

We had previously found that T-cell hybridomas warmed to 37°C and placed on glass or plastic substrates assumed a polarized shape which correlated with their ability to crawl. In addition, observation of 68 random T-B interactions suggested that T cells which contacted B cells with their leading edge usually generated  $[\text{Ca}^{2+}]_i$  responses, while T cells contacting B cells with their tails had only a 17% chance (3/17) of progressing past the contact phase. The purpose of the present study was to directly determine whether morphological polarity and the ability of T cells to detect antigen were related. The laser-based optical trap was used to control T-cell B-cell contact geometry and T-cell  $[\text{Ca}^{2+}]_i$  was measured as an indicator of successful T-cell receptor activation. With the B cell placed at the T-cell tail, no response occurred, and the B cell detached from the T cell within two minutes. Trapping the loose B cell and placing it at the leading edge of the same T cell rapidly elicited a T-cell  $[\text{Ca}^{2+}]_i$  increase. We found that T cells were preferentially responsive to contact with B cells at their leading edge. T cells which were presented antigen at the leading edge (“head contact”) had a higher probability of responding (84% vs. 31%) and a shorter latency of response (42 s vs. 143 s) than those contacting B cells with their trailing end (“tail contact”).

T cell/B cell contact during antigen presentation involves intercellular interactions between a number of molecular pairs, any of which could contribute to the observed polarity. To investigate whether polarity could be observed via TCR engagement alone, we used beads coated with antibodies to the CD3 subunit of the TCR complex to mimic TCR engagement in the absence of any coreceptors and got similar results (figure 2 and table 1). Initial results showed more dramatic polarity

was observed using 6  $\mu\text{m}$  diameter anti-TCR: CD3 mAb coated polystyrene beads to stimulate T cells (87% for leading edge contact vs. 6% for tail contact) and implied increased TCR density at the leading edge of the T cell might account for the polarized response to antigen.



*Figure 2. Optical trapping of antibody-coated bead reveals functional polarity of T cell.*  
 (A) Bright field, fluorescence intensity overlays of T-cell-bead pairs with antibody-coated bead (round shape) trapped at either the tail (a) or leading edge (b) of the T cell. T cell intensity ratio generated from oregon-green and fura-red co-loaded into IE5 cells (dark, red=low [Ca<sup>2+</sup>]<sub>i</sub>; bright, green=high [Ca<sup>2+</sup>]<sub>i</sub>; --see Methods). (B) Time course of [Ca<sup>2+</sup>]<sub>i</sub> for cells shown in (A).

The results of 29 different T-B cell pairs and 109 bead-T cell pairs are summarized in Table 1. T cells which were presented either with antigen or anti-CD3 mAb at the leading edge (contact zone 1) had a higher probability of response and shorter latency of response than those contacting with their tail (contact zone 3).

**Table 1. Polarized T-cell Response to TCR Stimulation**

| contact zone<br>(on T cell) | cells responding (%) |               | latency (sec) |               |
|-----------------------------|----------------------|---------------|---------------|---------------|
|                             | B cell               | anti-CD3 bead | B cell        | anti-CD3 bead |
|                             | 82 (14/17)           | 87 (77/87)    | 42 ± 16       | 52 ± 15       |
|                             | 80 (4/5)             | 82 (14/17)    | 60 ± 22       | 78 ± 39       |
|                             | 31 (4/13)            | 6 (1/15)      | 146 ± 29      | 340           |

T cells were stimulated by either antigen-presenting B cells or anti-CD3-coated beads at the region indicated. Region 1 is defined as the leading edge. Latency indicates the delay between contact and a detectable  $[Ca^{2+}]_i$  increase in the responding population.

These findings show that T-cells are effectively polarized antigen sensors, a result which should further our understanding of cell activation, signal processing and the human immune response.

#### 4. SUMMARY

Optical laser trapping microscopy has emerged as a powerful tool not only for the optical manipulation of cells and macromolecules, but also for the study of cellular physiological responses via force transduction and fluorescence imaging. We describe here the most recent results from our laboratory in the use and application of laser trapping microscopy to study activation of T cells using receptor-specific microspheres delivered to different cellular regions via an optical trap. Here we show not only that T-cell is a polarized antigen sensor, but also that receptor stimulation can be directly correlated to a functional response. Such studies are expected to aid in the design of new therapies for promoting or inhibiting immune response within the human body.

#### 5. ACKNOWLEDGMENTS

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