Video Article

Static Adhesion Assay for the Study of Integrin Activation in T Lymphocytes

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Abstract

T lymphocyte adhesion is required for multiple T cell functions, including migration to sites of inflammation and formation of immunological synapses with antigen presenting cells. T cells accomplish regulated adhesion by controlling the adhesive properties of integrins, a class of cell adhesion molecules consisting of heterodimeric pairs of transmembrane proteins that interact with target molecules on partner cells or extracellular matrix. The most prominent T cell integrin is lymphocyte function associated antigen (LFA)-1, composed of subunits αL and β2, whose target is the intracellular adhesion molecule (ICAM)-1. The ability of a T cell to control adhesion derives from the ability to regulate the affinity states of individual integrins. Inside-out signaling describes the process whereby signals inside a cell cause the external domains of integrins to assume an activated state. Much of our knowledge of these complex phenomena is based on mechanistic studies performed in simplified *in vitro* model systems. The T lymphocyte adhesion assay described here is an excellent tool that allows T cells to adhere to target molecules, under static conditions, and then utilizes a fluorescent plate reader to quantify adhesiveness. This assay has been useful in defining adhesion-stimulatory or inhibitory substances that act on lymphocytes, as well as characterizing the signaling events involved. Although described here for LFA-1 - ICAM-1 mediated adhesion; this assay can be readily adapted to allow for the study of other adhesive interactions (*e.g.* VLA-4 - fibronectin).

Video Link

The video component of this article can be found at http://www.jove.com/video/51646/

Introduction

T lymphocyte adhesion is a fundamental process in the immune response[1]. It is required for T cell interaction with endothelial cells decorating the capillary walls, for scanning antigen presenting cells (APC) within lymph nodes, and for the formation of immunological synapses (IS) with target cells[2]. These requirements are functionally and kinetically distinct. The process of lymphocytes extravasation consists of chemoattraction, rolling, firm adhesion, and transmigration. The transition from rolling to firm adhesion requires T cells to respond to a G protein coupled receptor signal rapidly. This response yields an integrin ligand interaction that slows and arrests the rolling cell[3]. An immediate change in integrin avidity mediates this process. Migration requires dynamic interactions betweenTcells and endothelial cells with the formation of adhesions at the 'front end' and breakage of adhesions at the 'rear end' with an interval of about a minute between forming and breaking[4]. The IS forms inminutes, but needs to remain intact for hours[6].

Interestingly, one adhesion molecule, the integrin family member lymphocyte function associated antigen (LFA)- 1, is essential for all these processes[5]. LFA-1 mediates adhesion through interactions with several members of the immunoglobulin superfamily. The most extensively studied ligand with the highest affinity to LFA-1 is the intercellular adhesion molecule (ICAM)-1. Non-activated circulating lymphocytes express low affinity LFA-1 on the cell surface, and therefore are unable to adhere to ICAM-1-coated surfaces. LFA-1 affinity is variable and regulated by several signaling events such as G protein coupled receptor activation, cytokine stimulation, and signals mediated by T cell receptors (TCR).The resulting high affinity form of LFA-1 conveys intracellular activation to the extracellular space through interactions with ICAM-1. This pathway is termed inside-out signaling[7]. Likewise, signaling through LFA-1 from the extracellular space is called outside-in signaling.

The intracellular signaling cascades involved in inside-out and outside-in signaling are a major focus of current research. The small GTPase Rap1 has recently emerged as the key component of inside-out signaling that is common to both TCR ligation and cytokine signaling[8]. The critical role of Rap1 in integrin activation is highlighted by the discovery that overexpression of Rap1 stimulates integrin-dependent adhesion of T cells, whereas T cell adhesion is blocked by expression of dominant negative Rap1[9]. These advances in our understanding of integrin regulation by Rap1 have been accomplished using *in vitro* tools. Among them is the static adhesion assay described here.

The overall goal of this method is to study T cell adhesiveness to ICAM-1 coated surfaces. More specifically, it is used to objectively measure and quantify LFA-1 affinity toward its counter ligands in live cells at real time, under different conditions. This technique uses polystyrene wells coated with ICAM-1 to mimic the cellular surfaces that the T cells interact with. Many previously described static T cell adhesion assays were experimentally complex. These assays often required for T cells to be radioactively labeled, utilized cultured bovine corneal cells to create an extracellular matrix as the substrate for T cell adhesion, or called for non-physiological T cell stimulation over an extended duration to promote

T cell adhesion[10]. The use of fluorometric measurement to quantify T cells following the adhesion incubation is a more sensitive and accurate method of quantification as compared to flow cytometry and microscopy, as are utilized in many other assay systems[11]. Additionally, single cell microscopic analysis of integrin localization does not allow for the broad, population based analysis in the same way as the fluorometric measurement. While activation state-specific LFA-1 antibodies are commercially available, these antibodies offer low sensitivity relative to the method outlined here. The main advantage over alternative techniques is its simplicity and the ability to examine multiple experimental conditions simultaneously. When considering this method for a specific application, one should take into account that T cells should be negatively selected, freshly isolated, and labeled with a fluorescent marker.

Protocol

1. Coating the Microplate Wells

Note: The goal of this step is to coat polystyrene surfaces with ICAM-1 to serve as ligands for T cell LFA-1.

1. Prepare the following solutions:
    1. Prepare the coating solution by resuspending recombinant ICAM-1 with phosphate buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) enriched with calcium (1 mM $CaCl_2$) and magnesium (2 nM $MgCl_2$). Make sure that the final concentration of ICAM-1 is 10 µg/ml, made from a 0.7 mg/ml stock solution and kept in a -80 °C freezer.
    2. Prepare the adhesion solution by enriching PBS with 0.5% human (or bovine) serum albumin (HSA/BSA), 2 mM $MgCl_2$, and 1 mM $CaCl_2$.
    3. Prepare the wash solution by adding 2 mM $MgCl_2$ and 1 mM $CaCl_2$ to PBS. Warm all solutions to 37 °C before use.
2. Wash 24 wells with 50 µl of wash solution. This will include unwashed wells, positive (PMA treated cells), and negative (uncoated wells) controls. For each condition set at least three wells (triplicate).
3. Add 50 µl of coating solution to each well. Do not add to the uncoated control wells. Incubate for one hour inside 37 °C incubator.
4. Aspirate the coating solution gently, without allowing the tip to touch the bottom of the wells. Wash once with 50 µl of wash solution.
5. Add 50 µl of adhesion solution and incubate the microplate for one hr inside 37 °C incubator.
6. Aspirate gently and wash once with 50 µl wash solution. Use the plate on the same day, however it is possible to keep it at 4 °C over night, and use it the following day.

2. T Cell Isolation from Blood

1. Add human T cell enrichment cocktail at 50 µl/ml of whole blood (*e.g.* for 3 ml of anticoagulant (heparin, EDTA, or citrate) whole blood, add 150 µl of cocktail). Mix well.
2. Incubate 20 min at room temperature.
3. To a 50 ml centrifuge tube add 3 ml of treated blood and an equal volume of PBS (without calcium and magnesium) enriched with 1% D-glucose at room temperature. Mix gently with a Pasteur pipette.
4. Add 15 ml Ficoll-Paque PLUS to the centrifuge tube.
5. Carefully layer the 6 ml diluted blood sample on the lymphocyte isolation medium.
6. Centrifuge at 400 x g for 20 min at 20 °C with break off.
7. Draw off the upper layer using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at the interface. *The upper layer of plasma may be saved for later use*. Using a clean Pasteur pipette transfer the lymphocyte layer to a clean centrifuge tube.
8. Add 3 volumes (9 ml) of PBS to the lymphocytes. Suspend the cells by gently drawing them in and out of a Pasteur pipette.
9. Centrifuge at 400 x g for 5 min at 20 °C.
10. Remove the supernatant. The lymphocytes should now be suspended in the medium appropriate to the application. Transfer the cells to a T-75 culture flask in 20 ml RPMI 1640 media containing 10% FBS, 1% penicillin/streptomycin.

3. Preparation of the Cells

Note: A prerequisite is for the cells to be labeled with a fluorescent reagent. In this protocol use carboxy fluorescein succinimidyl ester (CFSE), however green fluorescent protein (GFP) expressing cells are an acceptable alternative.

1. Count 2.4 x 10^6 T cells (1 x 10^5 for each well) using a hemocytometer.
2. Resuspend the cells in culture media lacking serum (serum starvation). Incubate the cells for 2 hr in the 37 °C incubator.
3. Centrifuge the cells for 5 min (400 x g). Aspirate the media and resuspend in 1 ml PBS.
4. Add CFSE at 1:1,000 dilution (stock made to 5 mM). Cover from light and incubate for 8 min at room temperature.
5. Stop the reaction by adding 10 ml of 37 °C PBS and spin at 400 x g for 5 min.
6. Re-suspend cell pellet with 1.2 ml of pre-warmed adhesion solution (1 x 10^5 cells per 50 µl).

4. Stimulation of the Cells

Note: In order to initiate adhesion, cells must be stimulated. In the following experiment the cells are stimulated via the TCR using anti-CD3 crosslinking antibodies, however soluble SDF-1 could serve as an alternative. Depending on the goal of the experiments, various pharmacological reagents could be added before or during stimulation to study the impact on cellular adhesion. Phorbol myristate acetate (PMA) is a phorbol ester that is structurally similar to the second messenger diacyl glycerol (DAG) and therefore activates multiple kinases downstream the TCR (mainly Protein kinase C); here it is used as a positive control.

1. Warm the microplate to 37 °C.
2. Divide the cells into 8 empty 1.5 ml tubes (150 µl in each tube), one tube for each condition.
3. Treat the cells accordingly:
    1. Stimulate one tube with PMA at 10 ng/ml to serve as positive control. Keep three tubes untreated, to serve as control for stimulation ("no stimulation"), unwashed loading control ("no wash"), and control for ICAM-1 coating ("uncoated").
    2. Stimulate four tubes with different concentrations of anti-CD3 antibodies (0.1, 1, 5, and 10 µg/ml). Continue to the next step without delays.
4. Aliquot 50 µl of stimulated cell mix into each empty well. The final number of cells per well is 1 x 10^5.
5. Place the microplate in the 37 °C incubator for 15 min.
   Note: Some T cell lines require shorter stimulation time. During this time, the cells will settle down to the bottom of the wells and adhere to the target ligands.

5. Washing Away Non-adherent Cells

Note: The goal of this step is to remove cells that were not able to form tight contacts with the ligand-coated surfaces. Use a multichannel pipette for this step in order to ensure that the same physical forces are applied to all the wells. It is important to keep the indicated control wells unwashed to assist in the calculation of the percentage of adherent cells.

1. Add 150 µl of warm adhesion solution to each wells. Shake the plate gently for few seconds before removing the medium from the wells. Do not touch the bottom of the wells with the tips.
2. Repeat this step three times. Change the direction of the pipette when pipetting the buffer in and out.

6. Determining the Percentage of Adherent Cells

Note: In this step a fluorescent plate reader is used to measure the fluorescent intensity within each well. The intensity is in direct correlation with the number of the adherent cells.

1. Turn on the plate reader. Open the plate reader software by clicking the shortcut icon on the desktop.
    1. From the "Task" menu click "New" to set up a new protocol.
    2. From the "Actions" menu choose the "Read" option. Click "Fluorescent intensity" and hit the "Ok" tab.
    3. From the drop down menu select "Excitation wavelength 485" and "Emission wavelength 528". Hit the "Ok" tab.
    4. In "Optic" option menu select "Bottom" and hit the "Ok" tab. Go back to the "Actions" menu and set the temperature to 37 °C and click "Ok".
    5. Insert the microplate into the plate reader and click "Run". The result will be exported into excel spreadsheet.
2. Calculate the percentage of adherent cells using the following formula: *Percentage of adherent cells = (average fluorescent intensity read in the washed wells)/(average fluorescent intensity read in the unwashed cells) x 100%.*

Representative Results

Below is an example of adhesion assay using primary T cells stimulated with various concentrations of anti-CD3 antibodies. It is useful to know the fluorescence of unwashed cells (*i.e.* total loading). Unstimulated cells serve as a negative control and PMA treated cells are the positive control for anti-CD3 antibodies. Cells plated in uncoated wells serve as a control for ICAM-1. In a typical experiment the percentage of adherent PMA treated cells is between 40% to 50% while the percentage of unstimulated cells is between 5% to 10%. An alternative method to quantify cellular adhesiveness is by calculation fold increase of fluorescent intensity in each condition over that of unstimulated cells.

Table 1 shows the fluorescence intensity of CFSE labeled primary T cells stimulated with different doses of soluble anti-CD3 antibodies and plated on ICAM-1 coated wells. **Figure 1** shows representative images of similar experiment. The cells were negatively selected from peripheral blood. After serum starvation for 2 hr, 2.4 x 10^6 cells were labeled with CFSE followed by stimulation with soluble anti-CD3 antibodies at various concentrations. Stimulated cells were plated into ICAM-1 coated wells and incubated for 15 min in the dark. After incubation the non-adherent cells were washed out three times and the fluorescence intensity was measured using a plate reader at 485 nm. PMA treated and unstimulated cells were used as controls. Note that the first column (1A-1C) contains cells that were not washed (total loading, *e.g.* 100%).

Figure 2 shows the percent of T cell adhesion as calculated based on the fluorescent intensities reported in **Table 1**. For every condition the average intensity of the three wells (triplicate) was calculated and converted into relative percentage out of total cells loaded (**Table 1**; Column 1). Standard error of the mean was calculated for each condition. As shown in the figure, the percentage of adherent cells is greater with higher concentration of anti-CD3 antibodies used for stimulation.

	No Wash	Uncoated	Unstimulated	PMA	Anti-CD3 0.1 µg/ml	Anti-CD3 1 µg/ml	Anti-CD3 5 µg/ml	Anti-CD3 10 µg/ml
	1	2	3	4	5	6	7	8
A	89652	611	5219	45873	8964	20157	37972	37972
B	90248	320	6049	44698	7568	25486	32549	32549
C	88321	409	5456	42697	8542	24568	32892	3289

Table 1. Fluorescence intensity values of CFSE labeled primary T cells stimulated with varying concentrations of soluble anti-CD3 antibodies. The freshly harvested cells were serum starved for two hours and subsequently labeled with CFSE at concentration of 5 µM. Next, the cells were stimulated with a low (0.1 µg/ml), medium (1 µg/ml), high (5 µg/ml), and very high (10 µg/ml) concentrations of soluble anti-CD3 antibodies and 1 x 10^5 cells were plated in each well (pre-coated with ICAM-1). After 15 min the non-adherent cells were removed by three serial washes. The number of adherent cells was measured with a plate reader as shown in this table. Wells 1A-1C: unwashed cells; 2A-2C: uncoated wells; 3A-3C: unstimulated cells; 4A-4C: cells stimulated with 10 ng/ml PMA; 5A-5C: cells stimulated with low dose anti-CD3 antibodies; 6A-6C: cells stimulated with medium dose of anti-CD3 antibodies; 7A-7C: cells stimulated with high dose anti-CD3 antibodies; 8A-8C: cells stimulated with very high dose anti-CD3 antibodies.

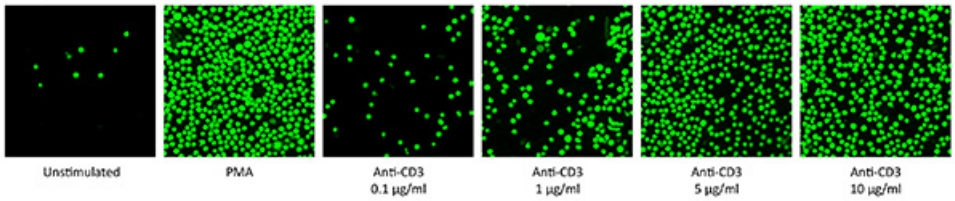

Figure 1. Images of primary T cells stimulated with varying concentrations of soluble anti-CD3 antibodies. Freshly harvested cells were serum starved for two hours and subsequently labeled with CFSE at concentration of 5 µM. Next, the cells were stimulated with PMA (10 ng/ml) or various concentrations of anti-CD3 antibodies (0.1, 1, 5, and 10 µg/ml) and plated on ICAM-1 coated surfaces. Representative images were taken with Zeiss 700 confocal microscopy using 20X magnification. Please click here to view a larger version of this figure.

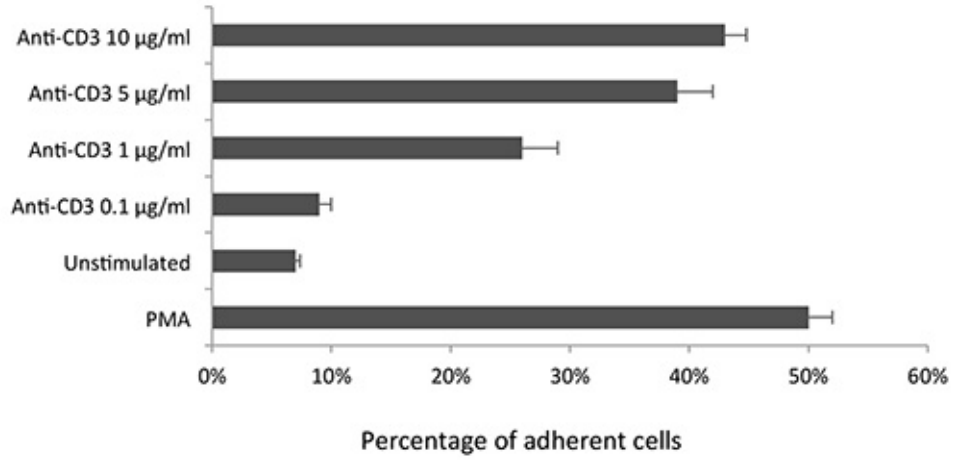

Figure 2. Stimulation of T cells with high dose anti-CD3 antibodies results in increased adhesion. Graphed representation of the percent of T cell adhesion as calculated from the shown in **Table 1**. The average percentage of adherent cells was calculated relative to unwashed cells that represent 100%. The histograms present the results (means ± SEM) of at least 3 wells. Please click here to view a larger version of this figure.

Discussion

There are several assays to study signals to LFA-1 activation and T cell adhesion[12]. Flow cytometry is used to measure LFA-1 affinity states in living cells using monoclonal antibodies that bind selectively to either bended or extended LFA-1. One of the limitations of this method is that it does not take into account the integrins' avidity. Migration assays are useful tools but they measure migration, and not measly adhesion at a specific time point. Mouse models to study T lymphocyte migration (*e.g.* cutaneous hypersensitivity model) are physiologically relevant, however utilization of these models is multifactorial and complicated to execute. The main strength of the static adhesion assay described here is its ability to measure both avidity and affinity in a simple manner. Another advantage of this method is its ability to detect a small number of cells quickly and accurately. When compared to other methods, it is much easier to manipulate the cells and treat them with different reagents in a functional assay as the one we describe. Moreover, the adherent cells are counted objectively with a plate reader, elimination bias associated with manual counts. This method can be used to screen the effect of multiple drugs or genes manipulation on the adhesion process.

However this technique is not free of limitations. One potential weakness is the fact that the percentage of adherent cells is a relative number, which may vary from one experiment to the other. Another weakness is the fact that blast lymphocytes cannot be used, as these must be freshly isolated. Moreover, adhesion studies with cells recovered from frozen peripheral blood mononuclear cells are not consistent. It is important to mention that PMA should work consistently, and we recommend using it in every experiment. A positive and a negative control are the first steps in troubleshooting unsuccessful experiments. In case of lack of augmented adhesion in stimulated cells, the concentration of the target ligand covering the wells should be checked. In addition it is required to validate that more than 95% of the cells are viable, and in case of a cell line, their growth phase should be calculated. In case of significant variability within the same condition, it is recommended to use more than three identical wells (more than triplicate).

In order to appreciate this assay it is useful to understand that some steps in the protocol such as the incubation time and the number of seeded cells must be uniform among all conditions. Small differences in incubation time may result is inaccurate measurements. It is also vital to aliquot

exactly the same number of cells is each well. Future applications of this technique, improving its accuracy would be an automated version that would load the cells and perform the washes in a more objective manner. In addition, an automated version will enable us to perform large-scale screens.

Disclosures

The authors have nothing to disclose.

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