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T Lymphocytes Sense Antigens within Seconds and Make a Decision within One Minute

Alexandre Brodovitch,*†‡ Pierre Bongrand,*†‡ and Anne Pierres*†‡

Adaptive immune responses are triggered by the rapid and sensitive detection of MHC-bound peptides by TCRs. The kinetics of early TCR/APC contacts are incompletely known. In this study, we used total internal reflection fluorescence microscopy to image human T cell membranes near model surfaces: contact was mediated by mobile protrusions of <0.4 μm diameter. The mean lifetime of contacts with a neutral surface was 8.6 s. Adhesive interactions increased mean contact time to 27.6 s. Additional presence of TCR ligands dramatically decreased contact to 13.7 s, thus evidencing TCR-mediated triggering of a pulling motion within seconds after ligand encounter. After an interaction typically involving 30–40 contacts formed during a 1-min observation period, TCR stimulation triggered a rapid and active cell spreading. Pulling events and cell spreading were mimicked by pharmacological phospholipase Cy1 activation, and they were prevented by phospholipase Cy1 inhibition. These results provide a quantitative basis for elucidating the earliest cell response to the detection of foreign Ags.

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A key step for the initiation of adaptive immune responses is the detection by T lymphocytes of foreign material exposed by professional APCs or even any virus-infected cell. Self- or foreign proteins are split into oligopeptides (p) that may be bound by cell membrane proteins encoded by the MHC. This recognition step is a daunting task in view of at least four highly demanding requirements (1, 2): 1) because a T lymphocyte bears a single TCR species, T cells of a given specificity may be vastly outnumbered by APCs. Detection of a foreign structure must therefore be very rapid, and indeed Ag detection on a single cell must occur within a few minutes as shown in vitro (3) or in vivo (4). 2) Because thousands of proteins may be used to generate the hundreds of peptide–MHC molecule complexes (pMHCs) exposed on a cell membrane, a T cell must be able to recognize a few and perhaps a single cognate pMHC species on a given cell. This was indeed experimentally demonstrated (5, 6). In addition to the requirement for an exquisite T cell sensitivity, this requires that all APC-exposed pMHCs visit the T cell/APC contact area during an encounter lasting a few minutes. This is very close to the limit set by diffusion (1). 3) Because all pMHCs exposed by an APC may differ by a few and even a single amino acid, TCR/pMHC interaction must be highly specific. According to the kinetic proofreading model (7), this may be achieved if T cells are sensitive to the duration of individual TCR/pMHC interactions, but this may be difficult to reconcile with aforementioned rapidity requirements. Recent experimental (8, 9) and theoretical (10, 11) results are consistent with the hypothesis that T cells might repeatedly test a single pMHC within a reasonable amount of time by using membrane movements to decrease bond lifetime. 4) Because the total length of TCR/pMHC complex is only ∼14 nm, molecular interaction between TCR and pMHC requires a tight membrane interaction at the nanometer scale, which is strongly dependent on T cell submicrometer-scale membrane movements.

Thus, two key pieces of information are required to understand the Ag detection process at the molecular level. First, we need know the precise frequency and duration of T cell/APC membrane tight contacts. Second, we need know whether and how membrane motions are altered when a TCR has first detected a cognate pMHC. Extensive studies were done to explore the mechanisms by which TCRs generate signals and T cells process these signals into a decision. Thus, it has been shown that the outcome of TCR/pMHC encounter is dependent on the lifetime of molecular binding events at the second and subsecond scale (2). TCR engagement generates within seconds many events like receptor clustering (12), calcium transients, and tyrosine phosphorylation (13). T cells then initiate bulk membrane movements involving active actin remodeling, spreading, and formation of an extensive contact area within minutes (14, 15). This may be viewed as a global event involving the whole cell (16) and a hallmark of analog-to-digital signal processing (17, 18). Also, strong evidence supports the hypothesis that extensive morphological changes may act as early reporters of the triggering of a specific activation program (19, 20). However, the precise time scale of and relationship among membrane motion, membrane receptor engagement, and triggering of a coordinated mechanical response remain incompletely understood.

Recently, we used quantitative interference reflection microscopy (IRM) to monitor individual human CD4+ T lymphocytes sedimenting on model planar surfaces with 1-s temporal resolution and 3-nm vertical spatial resolution (21). Cells falling on passivated neutral surfaces (i.e., Ig-coated coverslips) did not establish any contact during several minutes. In contrast, encounter with surfaces made adherent to lymphocytes with anti–HLA class I Abs resulted in the slow growth of molecular contacts (∼0.2 μm²/s) after a lag of ∼1 min. When surfaces were coated with anti-CD3 Abs, thus inducing strong delayed proliferation (20, 21), the 1-min
lag was followed by a spreading response that was ~8-fold more rapid than that found on anti-HLA-coated surfaces (~1.5 μm/s) and generated ~4-fold higher contact area 5 min later. Thus, the cell decision to undergo rapid spreading was a good reporter of the detection of a minimal amount of TCR ligands performed during a 1-min observation period. However, the resolution of our study was not sufficient to dissect the events occurring during this 1-min period. Other authors used total internal reflection fluorescence microscopy (TIRFM) to image the contact area between fibroblasts (22) or T lymphocytes (22, 23) and specifically coated substrates within ~200 nm of these surfaces. These studies revealed spontaneous protrusions and retractions of the contact boundary with a period on the order of 1 min. T cells deposited on lipid bilayers containing ICAM-1, CD80, and pMHCs exhibited contractile oscillations with a periodicity of ~0.42 s (23). However, these authors did not focus on the earliest interactions occurring during the first minutes following cell-to-substrate contact.

In this study, we used TIRFM to obtain quantitative information on this key initial step. Our experiments yielded several major pieces of information. First, T cells generated transient second-scale contacts with highly mobile microvilli of submicrometer diameter. Second, TCR ligand detection generated a pulling motion within 10 s. Third, accumulation of a few tens of such recognition pieces of information. First, T cells generated transient second-scale contacts with highly mobile microvilli of submicrometer diameter. Second, TCR ligand detection generated a pulling motion within 10 s. Third, accumulation of a few tens of such recognition events within 1 min generated an extensive and active spreading. Fourth, phospholipase Cγ1 (PLCγ1) activation was involved in both rapid and localized retraction events and resulting extensive spreading. Taken together, these results provide important insight in the mechanism of Ag detection and signal processing by T lymphocytes.

Materials and Methods

**CD4+ T cell isolation and treatment**

Peripheral blood from healthy volunteers was provided by the French Blood Bank. PBMCs were purified by density gradient centrifugation using Lymphocyte Separation Medium (Eurobio, Les Ulis, France) following standard techniques. CD4+ T lymphocytes were isolated by negative selection using magnetic cell sorting (Milteny Biotech) (20, 21).

For PLCγ1 activation or inhibition experiments, 5 × 10^5 cells were treated for 10 min at 37°C with 25 μM PLCγ1 activator (m-3M3FB; Sigma-Aldrich) or 2 μM PLCγ1 inhibitor (U-73122; Sigma-Aldrich) in RPMI 1640 culture medium supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin.

**Plasma membrane staining**

A total of 10^6 CD4+ T lymphocytes were incubated with 15 μg/ml 4-(4-dihexadecylaminostyryl)-N-methylpyridinium iodide (DiA; Molecular Probes, Invitrogen; 460 nm excitation, 580 nm emission) in PBS/2% FBS and generated ~4-fold higher contact area 5 min later. This decision to undergo rapid spreading was a good reporter of the detection of a minimal amount of TCR ligands performed during a 1-min observation period. However, the resolution of our study was not sufficient to dissect the events occurring during this 1-min period. Other authors used total internal reflection fluorescence microscopy (TIRFM) to image the contact area between fibroblasts (22) or T lymphocytes (22, 23) and specifically coated substrates within ~200 nm of these surfaces. These studies revealed spontaneous protrusions and retractions of the contact boundary with a period on the order of 1 min. T cells deposited on lipid bilayers containing ICAM-1, CD80, and pMHCs exhibited contractile oscillations with a periodicity of ~0.42 s (23). However, these authors did not focus on the earliest interactions occurring during the first minutes following cell-to-substrate contact.

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**Surface preparation**

Glass coverslips were dipped in sulphuric acid (Sigma-Aldrich) for 4 h, rinsed in water, and dried. They were then treated with (3-aminopropyl)triethoxysilane (Sigma-Aldrich) for 4 min on ice, washed, and incubated with 1% glutaraldehyde (Sigma-Aldrich) for 30 min at room temperature. They were rinsed with PBS and coated with 20 μg/ml monoclonal anti-CD3 (clone UCHT1), anti–HLA-ABC, or IgG1 control (all from Beckman Coulter) for 2 h at room temperature. For experiments with both anti-CD3 (clone UCHT1), anti–HLA-ABC, or IgG1 control (all from Beckman Coulter) as previously done by many authors (26). Initial contact analysis was done on the first 300 images seen after cell deposition. Tracking of every contact was done manually on a stack of 60 images (1 out of 5) using the ImageJ manual tracking plugin (National Institutes of Health). This allowed us to determine mean contact duration (~300 contacts/condition) and cumulated number of appearing contacts for 10 cells.

Samples labeled with fluorescent Abs were washed and observed with an Axiovert 200M inverted microscope (Zeiss). Images were collected by a cooled tri-CCD C7780 camera (Hanamatsu) using Aquacosmos software. Fluorescence analysis was done with ImageJ (National Institutes of Health).

**Statistics**

Statistical significances were calculated with GraphPad software (GraphPad, San Diego, CA) using ANOVA and Bonferroni post test as indicated in figure legends.

**Results**

**TIRFM can overcome IRM limitations and allow real-time detection of submicrometer-scale contacts**

We needed to monitor cell-surface interaction with a sensitivity matching the size of microvilli (i.e., a few hundreds of nanometers diameter) and time-scale of transverse membrane displacements (i.e., ~1 s) (25). Although IRM approaches these requirements (25), there are two limitations: first, contrast is fairly low, because molecular contact areas appear as zones where light intensity is lower than the background level by little more than three times the SD of background illumination (Fig. 1A–F). Reliable contact detection thus requires significant filtering, which decreases spatial and/or temporal resolution (21, 23). Second, the intensity/distance relationship is not monotonous, making it difficult to define unambiguously regions where the cell-to-surface distance is ~20–30 nm (Fig. 1G–Q). This limitation was overcome with TIRFM. This technique allows localized illumination of a space region of a few hundreds of nanometers thickness above a planar surface. When cells were labeled with a lipophilic membrane probe (DiA) and made to sediment on glass coverslips, initial cell-to-surface approach was detected much more efficiently than with IRM: the brightness of membrane areas close to the surface was frequently higher than the background brightness by ~12 times the SD (Fig. 1G–I). In addition, using microspheres to estimate the intensity/distance relationship (Fig. 1R, 1S) using the ImageJ manual tracking plugin (National Institutes of Health). This allowed us to determine mean contact duration (~300 contacts/condition) and cumulated number of appearing contacts for 10 cells.

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cells, pixels with brightness higher than average brightness by >5 SD appeared isolated (with a frequency of $\sim 2 \times 10^{-4}$) in empty fields. In contrast, initial images of falling cells were spots of $\sim 4$ pixel (320 nm) width (mean area 15.9 pixels $\pm 8.6$ SD; $n = 15$) that were easily detected by visual examination (Fig. 1M, 1N).

**TIRFM reveals that whole-cell spreading is preceded by the formation of small and transient interaction spots during a 1-min observation period**

When TIRFM was used to quantify T lymphocyte spreading on adhesive nonactivating or activating surfaces, we obtained the following conclusions: 1) measured spreading areas after 5 min contact were comparable to those obtained with IRM (Fig. 2A, 2B); 2) interaction between cells and surfaces was detected $\sim 1$ min earlier with TIRFM than with IRM, and this initial interaction appeared as limited spots of <400 nm thickness (Figs. 1M, 1N, 3A, Supplemental Video 1), matching the section area of microvilli; and 3) surprisingly, the rate of contact growth during this first minute was higher on anti-HLA–coated surfaces than on anti-CD3–coated ones (Fig. 2B, 2C).

The lifetime of cell-surface interaction is increased by nonspecific adhesion and decreased by TCR stimulation

To solve the apparent discrepancy between second- and minute-scale adhesion behavior of cells deposited on activating and nonactivating surfaces, we investigated the dynamics of contact formation. First, we measured the frequency of contact formation: interestingly, cells bound to surfaces with either anti-HLA or anti-CD3 displayed similar frequency of contact formation with 30–40 spots/min (Fig. 3B). Assuming that contacts were formed as a consequence of random membrane movements, this suggested that overall membrane dynamics were not strongly influenced by...
TCR engagement during the first minute of interaction. Secondly, we measured the duration of observed contacts. As shown in Fig. 3C and 3D, contact survival displayed fairly exponential decay during the first 20 s following their formation, and mean contact time (±SEM) on neutral, nonadhesive surfaces was 8.6 ± 0.4 s. As expected, adding adhesive forces by coating surfaces with anti-HLA increased contact duration to 27.6 ± 1.0 s, and similar kinetics were obtained after 2- or 4-fold dilution of anti-HLA (not shown). Third, mean contact duration was only 11.9 ± 0.8 s on adhesive/activating anti-CD3–coated surfaces. It was important to know whether this was due to a lower adhesion efficiency on anti-CD3 or an active cell response. Thus, we measured the duration of contacts formed between cells and surfaces coated with both anti-CD3 and anti-HLA. As shown in Fig. 3C and 3D, anti-CD3 reduced contact duration. It is concluded that TCR engagement was detected and translated into a mechanical response within <13.7 ± 0.8 s.

Both TCR-triggered local microvillus retraction and global spreading are correlated to PLCγ1 activation

It was of obvious interest to identify a biochemical substratum of the functional events revealed by our microscopic study. Phosphatidylinositol 4,5-bisphosphates (PIP2) are known as prominent actors of membrane–cytoskeletal interaction and regulation of microfilament behavior (27), and PIP2 hydrolysis following PLCγ1 activation is an early and well-known consequence of TCR engagement. As shown in Fig. 4A–C, T lymphocytes displayed both more extensive spreading and phosphorylation of PLCγ1 when surfaces were coated with anti-CD3 rather than anti-HLA. Interestingly, PLCγ1 activation (with m3M3FBS) strongly increased spreading on anti-HLA, and PLCγ1 inhibition (with U73122) strongly decreased spreading on anti-CD3 after 5-min contact (Fig. 4A–C).

Next, we found that PLCγ1 modulation was also involved in determining the lifetime of transient contacts. As shown in Fig. 4D and 4E, the survival of contacts formed between T lymphocytes and activating surfaces (anti-CD3) was increased by PLCγ1 inhibition, thus resembling interaction with nonactivating surfaces. Conversely, the duration of contacts formed with nonactivating surfaces was decreased by PLCγ1 activation, thus matching contacts formed with activating surfaces. Thus, the short-term pulling and later active spreading triggered by anti-CD3 could both be mimicked by pharmacological activation of PLCγ1. However, PLCγ1 modulation only weakly influenced the duration of contacts formed on nonadhesive, nonactivating IgG1-coated surfaces because the contact time was, respectively, 9.6 ± 0.6 (222 contacts) and 6.6 ± 0.3 s (229 contacts) when cells were treated with a PLCγ1 inhibitor and activator, as compared with 8.6 ± 0.4 s on controls.

Discussion

The main purpose of our work was to provide quantitative information on the kinetics of T cell/antigen-presenting cell membrane interaction to gain some insight on the conditions of molecular encounters between allospecific TCR and pMHC and help interpret experimental data on the link between TCR/pMHC molecular interaction parameters and T cell response.

A first unambiguous conclusion is that in our model, early TCR/ pMHC interactions must be restricted on localized areas corresponding to the tip of filopodium or microvillus-like protrusions. This is consistent with the well-established hypothesis that filopodia are exploratory structures produced by many cell types (28–30).

A second conclusion concerns microvillus dynamics: the mean lifetime of contacts formed between T cells and neutral (nonadhesive, nonactivating) surfaces was 8.6 s. This is consistent with the concept that cell surfaces display spontaneous and continual protrusion/retraction processes with a characteristic time scale often ranging between 1 and 100 s (22, 23, 31). Adhesive interactions resulted in a >3-fold increase of this lifetime (from 8.6 to 27.6 s). This increase may be ascribed to a combination of both passive inhibition of spontaneous retraction (by adhesive interactions) and active cell response to the adhesive force or engagement of a specific membrane receptor, which might either increase or decrease retraction. Indeed, the cell capacity to probe the mechanical properties of their surroundings has been abundantly demonstrated during the last decade (31). The key result of our study is that addition of TCR engagement to anti-HLA–mediated adhesion resulted in a 2-fold decrease of contact lifetime (from 27.6 to 13.7 s). It may be concluded that the time period of 13.7 s is an upper bound for the total duration of two sequential events: ligand recognition by at least one TCR molecule and triggering of a retraction motion. It must be emphasized that this is a very localized phenomenon, in contrast to the bulk pulling phase recently reported to occur a lag on the order of 1 min after T cell stimulation (32). An important finding supporting the concept of a local response is that the frequency of contact formation was similar during the first minute of contact between a T cell and an activating or a nonactivating surface (Fig. 3B), thus ruling out a bulk change of membrane dynamics. Also, visual examination of TIRFM images revealed that contact appearance and disappearance could occur independently in a localized area of a few micrometers squared. The hypothesis that sequential microvillus protrusion and retraction involves at least partially local coordination is consistent with earlier reports on the capacity of cytoplasmic neutrophil fragments to display autonomous motion (33) as well as more recent demonstration that many signaling responses are spatially restricted (34, 35). This concept is highly relevant to the rapid phenomena described in this report: a membrane protein

![FIGURE 2](http://www.jimmunol.org/) - T cell–spreading kinetics on activating and nonactivating surfaces. (A–C) Mean contact area as a function of time. Naive CD4+ T cells were allowed to spread for 5 min (A, B) on activating (anti-CD3 Abs at 20 μg/ml; red curve) and nonactivating (anti–HLA class I Abs at 20 μg/ml; black curve) surfaces. Contact area was observed using IRM (A) and TIRFM (B, C). (C) Magnified image of the boxed area in (B). Before TIRFM experiments, T cell membrane was stained with DiA. Curves in (A)–(C) show mean area ± SEM of 10 cells.
is expected to move by no more than a few micrometers during a 10-s period (36). However, this does not exclude the possibility that some features such as retraction frequency might be driven by a global cell parameter. It must be emphasized that the hypothesis that TCR stimulation by agonist pMHC might trigger a rapid retraction event is consistent with previous reports demonstrating that: 1) TCR–pMHC interaction on cell membranes is shorter than previously measured on soluble molecules (8, 37); and 2) comparing the interaction between a TCR and several pMHCs under two-dimensional conditions (involving T cell–bound TCRs) or three-dimensional conditions (involving soluble molecules), it was found that the most potent pMHCs exhibited longer three-dimensional association and shorter two-dimensional association with TCRs (37). This is consistent with the hypothesis that pMHC forming more productive TCR engagement might trigger a more efficient pulling motion, resulting in more efficient reduction of binding lifetime.

Third, it would be attractive to speculate that the motion we observed might play a role in TCR-mediated signal generation. Indeed, TCR was suggested to act as a mechanotransducer (38–40), and T lymphocytes were recently reported to sense substratum stiffness (41). Thus, they might use forces to probe the presence of...
FIGURE 4. Both contact spot formation and T cell spreading involve PLCγ1. (A) T cells were deposited on anti-CD3- and anti-HLA–coated surfaces. Five minutes later, they were fixed and stained with anti–p-PLCγ1 followed by Alexa Fluor 488 anti-rabbit F(ab')2 mAb. Contact area (IRM, left panel) and fluorescent labeling (right panel) are shown after 5-min spreading. Scale bars, 5 μm. Relative area (B) and fluorescence intensity (C) after 10-min treatment with PLCγ1 activator (m-3M3FBS; 25 μM) and PLCγ1 inhibitor (U73122; 2 μM). On anti-CD3, contact area and PLCγ1 phosphorylation are 2-fold higher than on anti-HLA. Treatment with PLCγ1 activator increased contact area (+20%) and PLCγ1 phosphorylation (35.4%) on anti-HLA. On the contrary, inhibition of PLCγ1 decreased both spreading area (−50%) and PLCγ1 phosphorylation (−27%) on anti-CD3. (D) Frequency distribution of contact duration after PLCγ1 modulation. (E) Mean contact duration during the first minute of cell spreading. Inhibition of PLCγ1 lead to an increase of contact duration on anti-CD3 (22.2 ± 1.29 versus 11.9 ± 0.78 s). On anti-HLA, contact duration is shorter after PLCγ1 activation (14.51 ± 0.99 versus 27.62 ± 1.04 s). Bars represent mean contact duration ± SEM of >300 contacts (10 cells). ANOVA and Bonferroni posttest: *p < 0.05, ***p < 0.001. Ctrl, Control.

ligand on neighboring surfaces. This might be a powerful means of increasing the reliability of Ag analysis by allowing cells to shorten the time required to test individual bonds: applying a pulling force may result in rapid bond rupture, allowing repeated testing of a single bond within a short time (9). More generally, because transverse membrane movements were demonstrated on many cell types (31), they may provide a general mechanism for rapid environment probing. Thus, in analogy with higher organisms, cells would use a two-level process for environment sensing: first, finger-like membrane protrusions would react within seconds by adapting motion and sending signals; and second, global signal integration would be performed within tens of seconds by a whole-cell network anticipating more elaborate neural systems.

Fourth, our results may give some information on the way T cells integrate local events to make a decision. Previous experimental studies suggested that the initiation of an activation program might require the triggering of hundreds (42) to thousands (43) of TCRs, depending on the stage of T cells and conditions of TCR engagement. This would result in a combination of events including calcium rise, cytoskeletal reorganization, adhesion stabilization, and spreading with a time scale of minutes (15, 20, 21, 42, 44). We suggest that our experiments revealed the unitary recognitions events of a few second duration that preceded the coordinated global cell responses that are described in aforementioned reports.

Fifth, it would be of prominent interest to elucidate the biochemical basis for the TCR-induced second-scale retraction process we described. That activation of PLCγ1 mimicked second-scale contact termination and minute-scale spreading triggered by TCR engagement, whereas PLCγ1 inhibition reversed these events, made it an attractive hypothesis to consider PLCγ1 activation as a physiologically significant consequence of TCR signaling. Indeed, it is well known that PLCγ1 activation is an early consequence of TCR activation. Also, when we performed a series of preliminary experiments to monitor the phosphorylation of known components of TCR-triggered cascades with immunofluorescence (20), only PLCγ1 displayed robust (2-fold) phosphorylation increase in contrast with lck, ZAP-70, LAT, SLP-76, rac-1, cdc42, and Iκκ (not shown). Further, there are numerous potential links between PLCγ1 activation and the events we observed: indeed, membrane retraction might be a consequence of inactivation of ERM protein interaction with membranes (45) as a consequence of PIP2 elimination. Actin depolymerization may also involve an interaction with the actin binding protein villin (46).

Also, PLCγ1 might trigger a local calcium rise, resulting in active pulling through a myosin-based mechanism (47). Preliminary attempts at testing this possibility did not reveal any robust correlation between contact termination and calcium rise, as evidenced with Fluo-4 labeling (20), or actin depolymerization, as monitored with LifeAct-GFP (48) labeling (not shown). It must be emphasized that more extensive studies are required to explore these possibilities because local events are expected to be very weak. Indeed, although micromolar scale whole-cell calcium rises occurring minutes after stimulation may be easily detected on single cells (20, 49), transient rises of a few tens of nanomolar amplitude (47) may be more difficult to identify unambiguously. However, it must be emphasized that PLCγ1 cannot be the sole determinant of the phenomena described in this work. Indeed, adhesive forces are important because: 1) activating or inhibiting PLCγ1 induced only ~10% variation of contact duration on IgG1-coated surfaces, whereas anti-HLA Abs induced >3-fold increase...
of contact time; and 2) PLCγ1 activation did not induce any active spreading on nonadhesive surfaces (not shown).

A sixth point is about the physiological relevance of our experimental setup. First, our choice to image the first contact between cells and surfaces led us to monitor unpolarized cells, and it is likely that the sensitivity of pMHC recognition was lower than demonstrated on the lamellipodia of moving cells (42, 50). Also, our choice to study primary human blood cells led us to use anti-CD3 rather than pMHC as TCR ligands. Our rationale was to use our quantitative observations to derive a workable test of patients’ T lymphocyte function (20, 51). Finally, the choice of Ab-coated glass surfaces was done to ensure good optical conditions for observation and at the same time test a minimal stimulation model.

Finally, it must be pointed out that our setup might in principle allow more precise analysis of T cell membrane shape. This might include a controlled change of the angle of laser ray excitation (26, 52) as well as enhancing image quality with standard filtering procedures (20). However, this might result in some loss of spatial or temporal resolution, and this was not felt appropriate in the current study.

In conclusion, our work provided a quantitative description of the dynamics of initial encounter between human primary T lymphocytes and model activating or nonactivating surfaces. We feel that this kind of information is a prerequisite for elucidating the highly complex mechanisms of Ag detection and analysis by T cells at the molecular level.

Disclosures
The authors have no financial conflicts of interest.

References


