T lymphocytes need less than 3 min to discriminate between peptide MHCs with similar TCR-binding parameters

Alexandre Brodovitch\textsuperscript{1,2,3}, Eugene Shenderov\textsuperscript{4}, Vincenzo Cerundolo\textsuperscript{4}, Pierre Bongrand\textsuperscript{1,2,3,5}, Anne Pierres\textsuperscript{1,2,3} and Philip Anton van der Merwe\textsuperscript{6}

\textsuperscript{1} Lab Adhesion Cellulaire and Inflammation, Aix-Marseille Université, France
\textsuperscript{2} INSERM U1067, France
\textsuperscript{3} CNRS, U7333, France
\textsuperscript{4} MRC Human Immunology Unit, Weatherall Institute for Molecular Medicine, University of Oxford, Oxford, UK
\textsuperscript{5} Assistance Publique, Hôpitaux de Marseille, France
\textsuperscript{6} Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

T lymphocytes need to detect rare cognate foreign peptides among numerous foreign and self-peptides. This discrimination seems to be based on the kinetics of TCRs binding to their peptide–MHC (pMHC) ligands, but there is little direct information on the minimum time required for processing elementary signaling events and deciding to initiate activation. Here, we used interference reflection microscopy to study the early interaction between transfected human Jurkat T cells expressing the 1G4 TCR and surfaces coated with five different pMHC ligands of 1G4. The pMHC concentration required for inducing 50\% maximal IFN-\(\gamma\) production by T cells, and 1G4-pMHC dissociation rates measured in soluble phase or on surface-bound molecules, displayed six- to sevenfold variation among pMHCs. When T cells were dropped onto pMHC-coated surfaces, rapid spreading occurred after a 2-min lag. The initial spreading rate measured during the first 45 s, and the contact area, were strongly dependent on the encountered TCR ligand. However, the lag duration did not significantly depend on encountered ligand. In addition, spreading appeared to be an all-or-none process, and the fraction of spreading cells was tightly correlated to the spreading rate and spreading area. Thus, T cells can discriminate between fairly similar TCR ligands within 2 min.

Keywords: Affinity · Early T-cell activation · Interference reflection microscopy · Kinetics · pMHC · Spreading · TCR

Introduction

An essential step of adaptive immune responses is the recognition by T lymphocytes of a cognate antigen exposed as a peptide–MHC (pMHC) complex on an antigen-presenting cell (APC). This recognition possesses remarkable sensitivity, speed, and selectivity [1]. Indeed, some T cells can detect a few and perhaps a single pMHC [2–4]. T-cell/APC encounters may trigger a signaling event, such as phosphorylation or transient calcium rise, within a few seconds [5] and a physiological response, such as arrest...
Typical images of spreading Jurkat cells on pMHC-coated surfaces. 1G4-transfected Jurkat T cells were sedimented onto pMHC-coated surfaces under microscopic observation and recording (One image per second). (A to C, G) Sequential IRM images of a typical cell. (D to F, H) Computer-calculated contact areas (shown as black pixels on a gray area). (A) Initial aspect of sedimenting cells with concentric rings indicative of a fairly spherical shape. (B and E) Point-like contact (0.03 μm²) that appeared 10 s later. (C and F) Display two contact spots with a total area of 1.1 μm² (time = 26 s). (G and H) Represent a more extensive contact (4150 pixels corresponding to 5.9 μm²; time = 379 s). Bar = 2 μm. Images are representative of 24 independent experiments performed, with about 20 studied individual cells per experiment.

Figure 1. Typical images of spreading Jurkat cells on pMHC-coated surfaces. 1G4-transfected Jurkat T cells were sedimented onto pMHC-coated surfaces under microscopic observation and recording (One image per second). (A to C, G) Sequential IRM images of a typical cell. (D to F, H) Computer-calculated contact areas (shown as black pixels on a gray area). (A) Initial aspect of sedimenting cells with concentric rings indicative of a fairly spherical shape. (B and E) Point-like contact (0.03 μm²) that appeared 10 s later. (C and F) Display two contact spots with a total area of 1.1 μm² (time = 26 s). (G and H) Represent a more extensive contact (4150 pixels corresponding to 5.9 μm²; time = 379 s). Bar = 2 μm. Images are representative of 24 independent experiments performed, with about 20 studied individual cells per experiment.

Results

T-cell contact with a surface bearing TCR ligands induces an all or none response within 3 min

CD8-negative Jurkat T cells bearing 1G4 TCR were injected into a chamber coated with pMHCs and a random field was monitored with IRM for at least 5 min. Images were recorded and processed for contact area determination. Typical images are displayed in Figure 1: cells first appeared as fairly circular structures with concentric rings, in accordance with the expected image of spherical objects in suspension (Fig. 1A). They displayed typical Brownian motion, with a minimum calculated distance to the surface ranging between 40 and 70 nm. When pMHCs were 1G4 TCR...
ligands, a fraction of cells ranging between 26 and 81% stopped and formed contacts with the substratum (Fig. 1B and C). After a variable lag, the contact area exhibited a rapid increase and reached a maximum of 10–100 μm² (Fig. 1G and H) within 2–3 min. A slow decrease of the contact area followed during the next tens of minutes. A representative movie is shown (see Supporting Information). As expected, no spreading response was observed when 1G4 ligand was replaced with an irrelevant pMHC complex (not shown).

**T-cell spreading is preceded by an observation period involving transient cell to surface contacts**

The spreading kinetics of a total of 495 cells was obtained on surfaces presenting a total number of five pMHC species and four surface concentrations each. This observation revealed the following behavioral patterns, as exemplified in Figure 2:

(i) The most frequent response (27% of cases) consisted of a rapid increase of contact area with a spreading rate on the order of several micrometer square per second (Fig. 2A). Interestingly, small area fluctuations with a period in the order of 10 s were frequently visible on the ascending part.

(ii) In about 63% of these cells, the robust spreading response was preceded by several (mean: 3.8 per cell) transient contacts (Fig. 2B) with a mean duration of 5.9 s ± 9.2 SD (n = 345 arrests). The distribution of these transient contact durations followed a power law with an exponent of −0.19 (Fig. 3).

(iii) Ten percent of cells displayed a less clear-cut response with delayed (Fig. 2C) or no definitive spreading (Fig. 2D).

(iv) Average responses of cells deposited on lower (Fig. 2E and F) or higher (Fig. 2G and H) concentrations of more active (3A, Fig. 2E and G) or less active (3Y, Fig. 2F and H) pMHC are also shown.

**Cellular spreading is strongly influenced by the quality of pMHCs**

First, the contact area measured 15–20 min after cell deposition was on surfaces coated with five different pMHC species. As summarized in Table 1, the contact area displayed 13-fold variation when the pMHC was varied, thus showing that cells efficiently discriminated between these TCR ligands within 20 min. The dependence of spreading on pMHC concentration was also studied: As shown in Figure 4, results allowed clear-cut discrimination between 2 groups of pMHCs. Thus, the pMHCs 3A, H74, and 9V yielded a higher contact area, even after a fourfold dilution, than 3Y and 9L. They also displayed a higher capacity to stimulate interferon-γ production, and formed more durable bonds with 1G4 TCR, as measured under both 3D and 2D conditions (Table 1). However, no single binding or spreading parameter was fully correlated with interferon-γ production, thus excluding the simple hypothesis that the initial discrimination between different pMHCs fully determines T-cell behavior for several hours following initial antigen detection.

**Peptide discrimination is clearly visible within 2 min after initial stimulation**

It was important to determine whether the 15- to 20-min period between initial encounter and contact area measurement was required to allow peptide discrimination. We addressed this question by monitoring the beginning of contact formation on
Table 1. Relationship between pMHC and T-cell spreading

| Peptide | EC50 (μg/mL) | 3D k_{off} (s^{-1}) | 2D k_{off} (s^{-1}) | Contact area (μm²) | Initial spreading rate (μm²/s) | Maximum spreading rate (μm²/s) | Lag before max spreading (s) |
|---------|--------------|---------------------|---------------------|-------------------|-----------------------------|-------------------------------|--------------------------|
| 3A      | 70           | 0.11                | 0.076               | 62.7 ± 0.011 SEM  | 2.13 ± 0.23 SD              | 2.58 ± 0.23 SEM               | 130 ± 19 SEM             |
| H74     | 107          | 0.13                | 0.133               | 40.1 ± 0.011 SEM  | 2.04 ± 0.50 SD              | 3.02 ± 0.43 SEM               | 73 ± 18 SEM              |
| 9V      | 180          | 0.09                | 0.271               | 70.2 ± 0.013 SEM  | 2.23 ± 0.14 SD              | 2.64 ± 0.06 SEM               | 75 ± 5 SEM               |
| 3Y      | 240          | 0.61                | 0.477               | 13.6 ± 0.007 SEM  | 1.22 ± 0.22 SD              | 1.70 ± 0.07 SEM               | 120 ± 7 SEM              |
| 9L      | 426          | 0.37                | 0.512               | 4.9 ± 0.005 SEM   | 1.24 ± 0.12 SD              | 1.52 ± 0.28 SEM               | 90 ± 33 SEM              |

Significance: *p < 10^{-10}*. Contact area was measured between 15 and 20 min after cell deposition. Initial spreading moment was determined by visual examination of spreading curves and calculation of the average slope was done on the period of time ranging between 15 and 20 min after cell deposition. The maximum spreading rate and the period between initial contact and the beginning of the 45-s period with maximum spreading rate were calculated on a subpopulation of cells that could be observed before any contact formation with the surface. The significance of difference between peptides was calculated with analysis of variance and Satterwaith correction for unequal samples.

Fraction remaining

Fraction remaining of transient contacts. The frequency distribution of transient contacts such as exemplified in Figure 2B is represented as a survival plot revealing a typical power law with an exponent of −0.19. The curve was built out of 345 recorded values of contact duration. Data are representative of 24 independent experiments.

Figure 3

Maximum Area (μm²)

Figure 4. Dependence of spreading on deposited peptide-MHC. 1G4-transfected Jurkat cells were deposited on surfaces coated with varying amounts of MHC-coupled peptide 3A (diamond), H74 (square), 9V (crosses), 3Y (triangles), or 9L (circles) and the average spreading area was determined in the period of time ranging between 15 and 20 min after deposition. (A) Each point represents a mean of 249–1096 values. (B) Each point represents the average spreading rate of cells that displayed an active response. A total number of 495 cells were studied. Vertical bar length = twice the SD. Data shown are representative of 24 independent experiments.

Individual cells. As shown in Table 1, the pMHCs that induced higher contact areas also induced more rapid initial spreading, although the initial spreading rate displayed only 1.8-fold variations of spreading fraction closely matched spreading area.

Figure 5

Spreading rate, contact area, and spreading fraction are tightly correlated. 1G4-transfected Jurkat cells were deposited on surfaces coated with varying amounts of MHC-coupled peptide 3A (diamond), H74 (square), 9V (crosses), 3Y (triangles), or 9L (circles). The fraction of cells with a spreading response (13 574 cells), the contact area of cells that displayed a spreading response (6622 cells), and the initial spreading rate of cells that displayed a spreading response were measured. (A) The correlation coefficient between spreading rate and contact area was 0.8915 and (B) the correlation coefficient between the spreading fraction and the contact area was 0.9362. Data shown are representative of 24 independent experiments, with an average of 20 samples/group.
Figure 6. Dependence of spreading fraction and maximum slope on activating surfaces. 1G4-transfected Jurkat cells were deposited on surfaces coated with varying amounts of the 3A (diamond), H74 (square), 9V (crosses), 3Y (triangles), or 9L (circles) pMHCs. (A) The fraction of cells that displayed an active spreading response was determined. (B) The maximum spreading rate was determined in the period of time ranging between 0 and 10 min after deposition. A total number of 297 cells that could be monitored for 10 min and that did not display any contact at time zero were studied. (A and B) Vertical bar length = twice the SEM. Data shown are representative of 24 independent experiments, with an average of 20 samples/group.

Figure 7. Relationship between surface coating and duration of analysis. 1G4-transfected Jurkat cells were deposited on surfaces coated with varying amounts of the 3A (diamond), H74 (square), 9V (crosses), 3Y (triangles), or 9L (circles) pMHCs and the duration of the lag between initial contact and beginning of spreading at maximal rate was determined on 297 cells that displayed a spreading response and were not in contact with the surface at the onset of the observation period. (A) Average value of the lag between initial contact and spreading at maximum rate. Vertical bar length = twice the SEM. (B) Individual values of the lag between initial contact and spreading at the maximum rate. Data shown are representative of 24 independent experiments, with an average of 20 samples/group.

Discussion

The purpose of this work was to determine the minimum time needed by T cells to discriminate between two slightly different pMHC ligands. We used cell spreading as an early and robust reporter of the cell decision to initiate an activation program [7, 24, 25]. Our results show that clear-cut discrimination between pMHCs agonists that display only quantitative differences in their capacity to induce interferon-γ production [13] is completed within a 2-minute period preceding the spreading burst. The probability (fraction of responding cells) and intensity (initial spreading rate and maximum spreading area) were highly correlated, suggesting that they were part of the same cell decision and functional program.

There are some key advantages to using IRM to monitor cell-surface contacts. First, it allows clear-cut detection of effective molecular contacts. Studies done with electron microscopy have long shown that the apparent contact area revealed by conventional light microscopy between lymphocytes and their targets markedly exceeds the true contact area [27]. Second, it is not necessary to label the cells with fluorescent probes or illuminate them with intense light, reducing the likelihood of artifacts. Third, the range of IRM analysis is ideally suited to monitor molecular contacts. The maximum contrast is seen when membrane-to-surface gap varies between about 0 and 100 nm, and the length of typical ligand–receptor couples ranges between about 14 nm (e.g., TCR-pMHC, CD2-CD58, or CD28/CD80-CD86) and 40 nm (LFA-1-ICAM1) or even 80 nm (P-selectin-PSGL-1). The dominant glycocalyx molecules, such as CD43 or CD45, are approximately 30–50 nm.

While calculating the distance using Equation (2) is an approximation [28, 29], the cell surface is too complex to warrant more detailed analysis. Furthermore, as previously emphasized [26, 30], Equation (2) is fairly robust and calculations are not affected by image processing, such as background subtraction or contrast...
Interestingly, the spreading fraction is also quantitatively related to the final contact area of cells that have decided to spread \((r = 9362, \text{Fig. 6B})\).

A long-standing difficulty in understanding the specificity of T cell recognition is to reconcile the hypothesis that T-cell activation is determined by the lifetime of TCR-pMHC bond and the finding that widely different responses are induced by pMHCs that are bound with dissociation rates differing by a factor lower than 10. Possible ways of resolving this paradox were the so-called proof-reading mechanism [20], with a need for a TCR/pMHC interaction to be sufficiently long to be productive, or the rapid summation of multiple interactions [22], or the hypothesis that forces generated at the T-cell/APC interface might increase the difference between bond lifetime involving agonist and antagonist pMHCs [16–18]. The (nonexclusive) mechanisms suggested by our results would be that T cells might sum the TCR–pMHC interaction occurring during a fairly fixed period of time of about 2 min and take a decision accordingly. This period is consistent with the results of Liu et al. [18] and matches well the order of magnitude of the duration of TCR/APC interaction occurring in lymph nodes under physiologic conditions [37, 38]. Our experimental model provides a suitable tool for studying the possible mechanism of this putative summation process.

### Materials and methods

#### Molecules and surfaces

Experimental procedures were as previously described [13, 15]. Briefly, pMHCs were HLA-A2 molecules in complex with the peptide SLLMWITQV (9V) and variants thereof that differed by a single amino acid in the peptide (3A, 3Y, 9L) or the MHC (H74). Glass surfaces \((1 \text{ cm}^2)\) were cleaned with a mix of 70% sulfuric acid and 30% H\(_2\)O\(_2\), then rinsed thoroughly and coated with poly-L-lysine \((150 \ 000—300 \ 000 \text{ Da})\), then incubated in glutaraldehyde for coating with biotinylated BSA. After blocking unreacted aldehyde groups with glycin, slides were coated with neutravidin before adding different amounts of biotinylated pMHCs. The surface density of pMHCs was determined by labeling with an excess of Alexa Fluor 488 labeled anti-HLA antibody (#311415, Biolegend, San Diego) and fluorescence determination. Absolute calibration was done as previously described [39] by measuring the fluorescence of a thin sheet of antibody solution.

#### Spreading experiments

Experiments were performed as previously described [24] in custom-made chambers made of pMHC-coated coverslips forming the floor \((1 \text{ cm}^2)\) of teflon-walled wells containing 0.5 mL of HEPES-buffered RPMI medium supplemented with 10% FCS. They were maintained at 37°C in a heating enclosure (TRZ 3700, Zeiss) mounted on an inverted microscope. About 250 000 cells suspended in warm medium were added, and an observation field
was selected for 10 min continual monitoring with video recording. A series of microscope field images were then acquired to determine the average contact area between 15 and 20 min after initial contact with better statistical accuracy. A total of 495 cells were thus followed for continual monitoring and a total of 13,574 instantaneous cell images were recorded. Data corresponding to a given condition (i.e., pMHC species + concentration) were a pool of four to six separate experiments.

**Image acquisition and processing**

IRM was performed as previously described [24, 26]. Briefly, cells were examined with an Axiovert 135 inverted microscope (Zeiss, Germany) using a 63 × Antiflex™ objective and 546 nm excitation wavelength. Images were obtained with an Orca C4742-95-10 camera (Hamamatsu, Japan) as stacks of typically 500–700 images of 8-bit depth and 1024 × 1024 pixel size captured with 1 Hz frequency for each monitored cell. Pixel size was 125 × 125 nm². Images were corrected by mean filtering and linear compensation for variations of background intensity. Cell/substratum distance \( d \) at each pixel was derived from illumination intensity \( I \) with the low incidence approximation [26, 40]:

\[
d = (\lambda/4\pi)\text{Arccosin}\left[\frac{(2I - I_m - I_M)}{(I_m - I_M)}\right].
\]

where \( \lambda \) is the light wavelength in aqueous medium, and \( I_m \) and \( I_M \) are, respectively, the minimum and maximum intensities corresponding to \( d = 0 \) and \( d = \lambda/4 \approx 100 \) nm, respectively. All calculations were performed with a custom-made image processing software written in C++ [30]. Molecular contact between cells and surfaces was assumed to occur when the calculated distance \( d \) was ≤34 nm on at least two pixels.

Initial spreading was defined as the first time point where contact was initiated and (i) contact was maintained for at least 45 s, and (ii) contact area reached a minimum level of 7.8 μm² (corresponding to 500 pixels). The maximum spreading rate was obtained by calculating the maximum average spreading rate during a 45-s period from the initial spreading to the end of the observation period.

**Statistics**

ANOVA was performed with Satterwaith’s correction for unequal samples [41].

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Abbreviations: IRM: interference reflection microscopy, pMHC: oligopeptide–MHC.

Full correspondence: Prof. Pierre Bongrand, LAI, Inserm U1067, Parc Scientifique de Luminy, Case 937, 13288 Marseille Cedex 09, France Fax: +33 491 82 88 51 e-mail: pierre.bongrand@inserm.fr

Additional correspondence: Prof. PA van der Merwe, Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK Fax: +44 (0)1865 2755 91 e-mail: anton.vandermerwe@path.ox.ac.uk

Additional correspondence: Anne Pierres, Lab Adhesion Cellulaire and Inflammation, Aix-Marseille Université, France e-mail: anne.pierres@inserm.fr

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