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Single cell force spectroscopy of T cells recognizing a myelin-derived peptide on antigen presenting cells ¹

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Abstract

T-cell recognition of peptide-MHC complexes on APCs requires cell-cell interactions. The molecular events leading to T-cell activation have been extensively investigated, but the underlying physical binding forces between T-cells and APCs are largely unknown. We used single cell force spectroscopy for quantitation of interaction forces between T-cells and APCs presenting a tolerogenic peptide derived from myelin basic protein. When T-cells were brought into contact with peptide-loaded APCs, interaction forces increased with time from about 0.5 nN after 10 seconds interaction to about 15 nN after 30 minutes. In the absence of antigen, or when ICAM-1-negative APC were used, no increase in binding forces was observed. The temporal development of interaction forces correlated with the kinetics of immune synapse formation, as determined by LFA-1 and TCR enrichment at the interface of T-cell/APC conjugates using high throughput multispectral imaging flow cytometry. Together, these results suggest that ICAM-1/LFA-1 redistribution to the contact area is mainly responsible for development of strong interaction forces. High forces will keep T-cells and APCs in tight contact, thereby providing a platform for optimal interaction between TCRs and peptide-MHC complexes.

Keywords

Antigen presentation; atomic force microscopy; cellular interaction forces; intercellular adhesion molecule-1 (ICAM-1)

¹Abbreviations used in this paper: AFM, atomic force microscopy; SCFS, single cell force spectroscopy; MIFC, multispectral imaging flow cytometry; APC, antigen presenting cell; LFA-1, lymphocyte function antigen-1; ICAM-1, intercellular adhesion molecule-1; pMHC, peptide-MHC complex

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1. Introduction

Initiation of immune responses often requires cellular interactions, but the underlying molecular mechanism and physical binding forces of the respective cell adhesion processes are only poorly understood. A notable example is the interaction between T lymphocytes and antigen presenting cells (APC)³. During their search for cognate antigen T cells continuously travel through the body, in particular through the secondary lymphoid organs such as lymph nodes and spleen. There, they move through the dense network formed by the dendritic cell (DC) dendrites, and scan the DCs for the presence of antigen, as revealed by 2-photon studies [1],[2]. In the absence of antigen numerous brief and presumably low-avidity interactions with DCs take place. However, these weak interactions are not without consequences, as indicated by recent studies showing that in the absence of antigen T cells are continuously stimulated by TCR-mediated recognition of self MHC molecules on DCs, thereby inducing a basic T cell signalling level that is required for rapid responsiveness to foreign antigen [3]. In contrast, upon recognition of cognate antigen the T cells are arrested and form tight contacts with DC, lasting for more than 1 hour, resulting in the formation of a so-called immune synapse (IS) and activation of the T cell [4],[5]. Long-lasting contacts occur also between T cells and B cells which then can move as cell-cell conjugates through the lymph node [6], indicating that considerable binding forces must keep the cell couples together.

IS formation is triggered by interactions between TCR and MHC/peptide complexes resulting in an orchestrated assembly of signalling, adhesive, and scaffolding molecules at the cell contact zone [7]. A mature IS is characterized by a central part of the supramolecular activation cluster (c-SMAC), enriched in MHC and signalling molecules such as TCR and CD3. The c-SMAC is surrounded by a peripheral part (p-SMAC) enriched in adhesion molecules like ICAM-1 on the APC side, and its ligand LFA-1 on the T cell side. This clustering of membrane proteins is accompanied by re-organization of cytoskeletal proteins such as actin [8]. The $\alpha_L \beta_2$ integrin LFA-1 is present on resting T cells in a so-called closed and low-affinity form. Interaction of the TCR with MHC/peptide complexes will induce LFA-1 by inside-out signalling to undergo a conformational change and to adopt an open and high-affinity conformation. The high affinity LFA-1 molecules are then able to bind strongly to ICAM-1 molecules on APCs, thereby inducing the above-mentioned clustering of ICAM-1/LFA-1 complexes [9],[10]. Sustained LFA-1 clustering and prolonged T-cell/APC interactions are then driven by calmodulin and the actin-binding protein L-plastin [11]. This mechanism of LFA-1 activation ensures that T cells bind firmly only to those APCs which present a cognate antigen.

IS formation is assumed to result in strong interaction forces between cells. Interaction forces can be precisely determined by atomic force microscopy (AFM) [12]. Being an extremely sensitive force sensor, AFM has been used to characterize the interaction force between individual molecules [13],[14],[15]. When applied as single cell force spectroscopy (SCFS) it also allows measuring the overall adhesion force between cells and substrate [13],[16],[17],[18] or between pairs of cells [19],[20], with the advantage that interaction of receptors and ligands can be studied in their physiological environment at the cell surface. In a previous study we have adapted AFM to the analysis of APC-T cell interactions and observed that IS development between APCs and T cells recognizing a hen-egg lysozyme (HEL) peptide was paralleled by an increase in binding forces between APCs and T cells [21]. Since these studies were the first ones to determine the interaction forces between T cells and APC, it was important to investigate another pair of T cells and APC. Whereas in the former study the T cells were specific for an immunogenetic HEL peptide, we selected here T cells recognizing a tolerogenic myelin-derived peptide, MBP Ac1-11.4Y. The natural MBP peptide Ac1-11 is known to induce experimental autoimmune encephalitis (EAE) in

mice [22], the murine model for the autoimmune disease multiple sclerosis in humans. The altered peptide ligand Ac1-11.4Y contains at position 4 a lysine to tyrosine substitution which increases the affinity of the peptide for the MHC class-II molecule Au, and converts it into a tolerogenic peptide that can induce suppression of EAE [23],[24]. As APC, Au-transfected fibroblasts were used that expressed or not ICAM-1, thereby allowing to determine the contribution of ICAM-1 to binding forces. Despite the fact that here and in the former study [21] very different T cell/APC pairs were employed, we obtained remarkably similar results with regard to the development of interaction forces.

2. Materials and Methods

2.1. Cells and reagents

L.Au cells are Ltk⁻ cells transfected with Au.MHC class II α and β chains, generously provided by the late C. Janeway. L.Au cells were supertransfected with a pABES-puro plasmid encoding murine ICAM-1 obtained from D. Vestweber and G. Cagua to generate L.Au.ICAM-1 cells. The T cell hybridoma 1934.4 expressing the Tg4 TCR with specificity for the myelin basic protein-derived peptide Ac1-11 was kindly provided by D. Wraith [22]. Cells were maintained in complete RPMI 1640 tissue culture medium, supplemented with 10% FCS. The anti-LFA-1 antibody M17.4 was purchased from BioXcell (USA). The MBP peptide Ac1-11 (ASQKRPSQRHG) and the high affinity altered peptide ligand Ac1-11.4Y (ASQYRPSQRHG) were produced by the peptide synthesis unit of the German Cancer Research Center.

2.2. Antigen presentation and cell conjugate assays

Antigen presentation assays were performed as described [25]. Briefly, 5×10^4 APCs were pulsed with peptide for 2 hours, and incubated with 5×10^4 1934.4 T cells in triplicate in 96-well plates. After 40 hours, IL-2 released by the T cells was measured by an europium-based fluorescence immunoassay. Cell conjugate assays were performed as reported [26]. Briefly, 1934.4 T cells were stained for 10 min by 37°C with 0.5 μ mol of CFSE (Molecular Probes). APCs were pulsed with 50 μ g/ml of Ac1-11 peptide, followed by labelling with 5 μ mol of SNARF (Invitrogen). 10^5 cells of each population were mixed in 100 μ l tissue culture medium, centrifuged for 1 minute, and fixed after indicated times with 2% paraformaldehyde. Analysis was performed with a FACS Calibur instrument.

2.3. Single cell force spectroscopy

The basic principles of atomic force microscopy (AFM) and single cell force spectroscopy (SCFS) have been described elsewhere [12],[19,21] The results presented here were obtained by using a NanoWizard II AFM equipped with a CellhesionTM module (JPK-Instruments, Berlin, Germany) which was operated on an inverted optical microscope (Axiovert 200, Zeiss, Göttingen, Germany). One day before the experiment, the L-cell APCs were seeded onto round glass coverslips with a diameter of 22 mm and incubated over night to allow firm adhesion. Ac1-11.4Y peptide loading was performed at least 2 hours before use with a concentration of 50 μ g/ml. Coverslips were then washed and mounted into the temperature controlled perfusion chamber (Biocell, JPK-Instruments) of the AFM and overlaid with Hank's buffered saline supplemented with 25 mM HEPES, pH 7.4. All AFM measurements were performed at 37°C. Gold-coated arrow-shaped TL-1 cantilevers with a nominal spring constant of 0.03 N/m (Nanoworld, Neuchâtel, Switzerland) were functionalized prior to use as follows: Cantilevers were plasma-cleaned and coated with 0.5 mg/ml biotinylated BSA in 0.1 M NaHCO₃ pH 8.8 over night at 37°C. After washing three times with PBS they were incubated in 0.5 mg/ml streptavidin for 1 hour at 37°C. Cantilevers were washed again and incubated with 10 μ g/ml of biotinylated anti-CD43 antibody for 1 hour at 37°C. Each coated cantilever was calibrated individually with the

thermal-noise method using the AFM's built-in software routines. 1934.4 T-cells were washed twice with Hank's buffered saline supplemented with 25 mM HEPES and flushed into the Biocell. After sedimentation, a T cell was selected and attached to the anti-CD43 functionalized cantilever by touching it with a force of 1 nN for 10 seconds. Subsequently the cantilever with the attached T cell was retracted and then lowered onto an APC until a contact force of 1 nN was reached. Contact was maintained for different lengths of time. The retraction speed was set to 1 $\mu\text{m/s}$. For interaction times longer than 10 seconds, new T cells were attached to a cantilever for each measurement. Measurements were carried out in closed loop and constant height mode. Analysis of the resulting force-distance plots was done using the JPK-IP V. 3.3.4 software package (JPK-Instruments). Several experiments were performed with a NanoWizard I AFM as described previously (12) with comparable results.

2.4. Multi-spectral imaging flow cytometry

The APCs (L.Au or L.Au.ICAM-1) were loaded with 0 or 50 $\mu\text{g/ml}$ MBP-peptide (Ac1-11.4Y) for 2 hours at 37°C. Thereafter, unbound peptide was removed and cells were resuspended in 250 μl culture medium at a density of $4 \times 10^6/\text{ml}$. 1934.4 T cells ($4 \times 10^6/\text{ml}$) were added in an equal volume, mixed and centrifuged briefly (200 $\times$ g for 1 minute). After removal of the supernatant the cells were resuspended in 150 μl culture medium and incubated in a controlled atmosphere (37°C/ 5% CO_2) for the indicated time points. Thereafter, cells were fixed with 1.5% PFA and stained for LFA-1 (CD18-FITC) and TCR (TCR -biotin plus Streptavidin-PE-TXred), respectively. To highlight the nucleus, the cells were incubated with Hoechst 33342 in the presence of 0.05% saponin. After extensive washing, the cells were analyzed using MIFC as described previously [11] [21], [27]. Briefly, 25,000 events were acquired using an ImageStream™ analyzer and INSPIRE software (Amnis, Seattle, USA). Image data were analyzed in a batch operation using IDEAS 3.0 software (Amnis). For analysis of the contact zone between 1934.4 T-cells and L.Au or L.Au.ICAM-1 cells, total events were gated on true cell pairs according to the DNA staining with Hoechst 33342. T-T, L.Au-L.Au or L.Au.ICAM-1-L.Au.ICAM-1 cell couples were excluded according to the TCR -staining pattern. Thereafter, a valley mask, dependent on the Hoechst 33342 dye, was defined between coupled cells. The valley mask was combined with a T-cell mask that utilizes the TCR staining, resulting in the so-called IS mask. Thereafter, protein accumulation was calculated as the ratio between the mean pixel intensity (MPI) of the respective protein, namely TCR or CD18 in the IS mask and the MPI of the same protein in the T cells mask [11] [21],[27]. Values of 1 correspond to TCR and LFA-1 distribution in conjugates obtained in the absence of peptide, whereas values >1 indicate the peptide/MHC-induced fold enrichment in the contact area between APCs and T cells. Multispectral imaging flow cytometry was also used to measure the diameter of cells. In the ImageStream™ analyzer the image pixel size is fixed to $0.5 \times 0.5 \mu\text{m}$, which allows the determination of the cell diameter in micrometer.

2.5. Statistics

Statistical significance between experimental groups was determined using one-way analysis of variance (ANOVA). A p -value <0.05 was considered significant.

3. Results

3.1. Stimulation of Ac1-11-specific T cells depends on ICAM-1 expression on APCs

For investigation by single cell force spectroscopy of the interaction forces between T cells and APCs presenting a myelin-derived peptide, the 1934.4 T hybridoma was used. 1934.4 T cells express the Tg4 TCR specific for the basic myelin protein (MBP) peptide Ac1-11 [22]. This TCR displays the fine specificity pattern of the majority of T cell clones obtained after

immunization of PL/J mice with Ac1-11 peptide [22],[28]. The APCs used were Ltk⁻ cells transfected with MHC class II Au and chains (L.Au cells), and genetically engineered to express in addition the adhesion molecule ICAM-1 (L.Au.ICAM-1 cells), so that the effect of ICAM-1 on T cell-APC interactions could be studied.

Fig. 1A shows MHC-II Au expression as well as the expression of ICAM-1 on transfected L.Au.ICAM-1 cells and expression of LFA-1 on 1934.4 T cells. As antigen, the altered peptide ligand Ac1-11.4Y was used which displays a high affinity for Au without alterations in the quality of interaction with the Tg4 TCR ([22]. This peptide is known to induce tolerance in PL/J mice, thereby preventing induction of EAE with the natural Ac1-11 peptide [24]. In the presence of Ac1-11.4Y peptide the L.Au.ICAM-1 APCs were clearly more efficient in activating the 1934.4 T cells than L.Au APCs, demonstrating the requirement of ICAM-1/LFA-1 interaction for optimal T cell activation (Fig. 1B). No T cell activation was observed with Ltk⁻ cells. In contrast to the tolerogenic Ac1-11.4Y peptide, the natural Ac1-11 peptide exhibits a low affinity for Au MHC class II molecules [24] and rapidly dissociates from peptide-pulsed APC. Indeed, Ac1-11-pulsed APCs displayed a low stimulatory capacity for 1934.4 T cells (suppl. Fig. 1), and in orientating SCFS experiments no significant binding forces above background were observed (data not shown). Therefore, the Ac1-11 peptide was not included in the further studies.

3.2. Conjugate formation between T cells and APCs measured by flow cytometry

The capacity of peptide-pulsed APCs to form conjugates with 1934.4 T cells was examined following labelling of APCs with the red dye SNARF, and of 1934.4 T cells with the green dye CFSE. Labelled cells were mixed, briefly centrifuged, incubated for various times at 37°C and then fixed with paraformaldehyde (PFA) for analysis by flow cytometry. A typical FACS plot is depicted in Fig. 2A. Double-stained conjugates are located in the upper right-hand quadrant. Peptide-pulsed L.Au.ICAM-1 cells efficiently formed conjugates with 1934.4 T cells and reached a maximum of about 50% after 15-30 minutes incubation time, whereas in the absence of peptide the number of conjugates was low and reached only about 18% (Figs. 2B,C). L.Au cells formed only about 10% conjugates regardless of the presence of the peptide (Figs. 2D,E). Conjugate formation by L.Au.ICAM-1 cells could be markedly inhibited by antibody M17.4 against LFA-1 (Fig. 2B), whereas conjugates formed with L.Au cells were not inhibited (Figs. 2D,E), demonstrating the importance of ICAM-1 interaction with LFA-1 for APC adhesion to T cells. Interestingly, in the absence of peptide, conjugates between L.Au.ICAM-1 APCs and 1934.4 T cells were also blocked by anti-LFA-1. Ltk⁻ cells lacking Au also formed about 10% conjugates with 1934.4 T cells (Fig. 2F), suggesting that molecules other than MHC class II or ICAM-1 contribute to this “basal” level of conjugate formation.

3.3. Cellular binding forces determined by single cell force spectroscopy

Conjugate formation as tested above fails to provide information on intercellular adhesion forces. Therefore, single cell force spectroscopy was employed for the assessment of the binding forces between APCs and 1934.4 T cells. The AFM system used was equipped with a tissue culture chamber, and mounted on an inverted microscope (see *Materials and Methods*). The 1934.4 T cells were bound to the cantilevers via anti-CD43 antibodies, whereas firm adherence of the L cell-derived APCs to the glass cover slips was achieved by overnight incubation. Thereafter, the APCs were loaded with peptide and the cover slips placed into the tissue culture chamber of the AFM instrument. The T cell bound to the cantilever was then lowered onto an APC with a force of 1 nN to allow for conjugate formation (Fig. 3A,B). After various interaction times the cantilever was retracted. Retraction results in bending of the cantilever until molecular bonds between the cells begin to rupture. From the reflection of a laser beam (Fig. 3A) and the spring constant of the

cantilever the disruption or unbinding forces can be determined. Owing to variations in the production process of cantilevers, the spring constant and cantilever sensitivity must be determined individually for each measurement. The AFM software provides a respective built-in calibration procedure. Typical AFM retraction curves obtained after 30 minutes interaction of 1934.4 T cells with L.Au.ICAM-1 cells in the presence and absence of cognate peptide and for L.Au cells with peptide are presented in Figs. 3C. The numerous “steps” in the retraction curve reflect the disruption of individual or groups of molecular bonds [29], [12]. The lowest point of the retraction curve indicates the maximum unbinding force, which equals the overall cellular interaction force. In the examples shown, with peptide-pulsed L.Au.ICAM-1 cells after 30 minutes an interaction force of 10.4 nN was measured, whereas in the absence of peptide a force of 2 nN was measured. For L.Au cells with peptide a force of 0.56 nN was found. Fig. 4 shows the cumulative data for temporal development of the interaction forces between 1934.4 T cells and APCs. After 10 seconds contact time between L.Au.ICAM-1-APCs and 1934.4 T cells the mean binding forces were about 0.7 nN in the presence of Ac1-11.4Y peptide, and slightly increased after 2 minutes to 2 nN. At 10 minutes contact time in the presence of peptide, the binding force was significantly increased to an average of 7.5 nN, and after 30 minutes an average value of 14.6 nN was reached. In the absence of peptide, binding forces were low (around 0.5 nN) and no significant increase with time was observed. Measurements for more than 30 minutes were very difficult to perform due to the high motility of the 1934.4 T cells. When L.Au cells lacking ICAM-1 were used as APCs, values remained low between 0.2 and 0.7 nN, regardless of the presence of the peptide. Together, these results demonstrate the importance of peptide and ICAM-1 for the development of strong adhesion forces between T cells and APC.

3.4. Maturation of immune synapses

In order to see whether the development of binding forces would correlate with maturation of IS, the kinetics of IS formation were determined. As a measure of IS formation, the kinetics of accumulation of TCR and LFA-1 at the interphase of conjugates between 1934.4 T cells and APCs were investigated by multi-spectral imaging flow cytometry, using the high throughput ImageStream system. L.Au and L.Au.ICAM-1 cells were loaded or not with peptide, mixed with 1934.4 T cells, briefly centrifuged, and incubated to allow for IS formation. After the indicated time points, the cells were fixed with PFA and stained for LFA-1 and TCR. Nuclei were stained with the Hoechst dye 33342. A total of 25.000 cells was analyzed by MIFC for each time point. Extensive redistribution of LFA-1 and TCR was observed on L.Au.ICAM-1/1934.4 conjugates in the presence of peptide. Typical examples are presented in Fig. 5A. In the absence of peptide, LFA-1 and TCR remained evenly distributed over the T cell surface. Likewise, on conjugates between peptide-pulsed L.Au cells and 1934.4 T cells no redistribution of LFA-1 and TCR molecules to the contact zone was observed. Kinetic studies revealed an increase of mature IS at 15 minutes post conjugate formation (Fig. 5B). Mature IS formation could be inhibited with anti-LFA-1 antibody (Fig. 5A, lower left hand panel, and 5C). Thus, the kinetics of IS formation and dependency on LFA-1 correlate with the development of binding forces.

4. Discussion

Functional recognition of antigen by T cells requires intensive interaction with antigen-presenting cells. In the present study we have utilized single cell force spectroscopy for the measurement of the precise physical interaction forces between T cells and APCs presenting a tolerogenic, altered peptide ligand Ac1-11.4Y, derived from MBP. The experiments show that the binding forces build up with time. Between 10 and 120 seconds interaction time in the presence of peptide no significant increase in binding forces was evident, but after 10

minutes the forces increased to an average value of 7.5 nN, which further increased after 30 minutes to about 15 nN. Values for the latter time point ranged from 10-26 nN, indicating that the overall binding forces differ between individual cell pairs. These data demonstrate that the binding forces between T cells and APCs are of considerable strength, explaining why T cell-APC conjugates can migrate through densely packed lymph nodes without breaking apart [30]. The use of APCs that have been genetically modified to express ICAM-1 allowed us to demonstrate a crucial role for ICAM-1 interaction with LFA-1 on T cells in the adhesion process. In the absence of ICAM-1 the binding forces remained low, even after 30 min interaction time with peptide. Thus, the temporal development of binding forces appears to depend mainly on ICAM-1/LFA-1 interactions. These observations are in agreement with our data showing that 1934.4 T cells fail to respond well to peptide presented by ICAM-1-deficient APC (see Fig. 1D).

The increase in binding forces correlates well with the kinetics of IS formation as measured by the enrichment of LFA-1 and TCR at the contact zone between T cells and APC, which also reached a maximum after 15-30 minutes. It is likely that the high interaction forces obtained after this time are due to the clustering of adhesion molecules at the interphase, but at present it is not clear whether this represents an unidirectional process in which the clustering proceeds automatically once it is initiated by TCR recognition through inside-out signalling, or whether the gradually increasing adhesion forces also contribute in a reciprocal fashion to LFA-1 activation and reorganization. By the SCFS method used here it was not possible to measure the binding forces mediated by the interactions between the cell-bound TCR and pMHC-II molecules, probably because the respective weak forces are superimposed by the “basal” adhesion forces of about 0.5 nN, which can be measured between APCs and T cells in the absence of antigen. In principle, the AFM is sensitive enough for determination of binding forces between TCR and pMHC, but probably purified molecules have to be used for respective studies. Similar to the previous AFM study [21], interaction times lasting for more than 2 minutes were difficult to measure, owing to the high motility of the T cells. In many of our SCFS experiments the T cells had a tendency to move away from the APCs despite the presence of peptide, suggesting that in a given clonal T cell population only a fraction of cells may be in a state allowing the establishment of firm contacts with APCs. The molecular factors influencing the propensity of a T cell to form high stability contacts and a mature IS formation require further investigation.

Surprisingly, the results presented here were similar to our previous study [21], although different APCs and T cells were used, namely LK35.2 B cells as APC and a T cell-recognizing and immunogenic HEL peptide. In contrast, the present study employed a tolerogenic MBP peptide, and Au-transfected fibroblasts as APC. Despite these differences the maximal binding forces were about 15 nN in both systems and developed with similar kinetics which was paralleled by IS formation. LFA-1 and ICAM-1 expression in respective APCs and T cells were comparable, whereas MHC-classII expression seemed to be lower on the L.Au.ICAM-1 cells used here. Since the overall binding forces probably depend upon the size of the contact area between APC and T cells, we assume that cells of similar size will yield comparable binding forces. Thus, our findings suggest that there is not a general force threshold that dictates whether or not interactions between T cells and APC result in tolerance or immunity. In this context it will be of interest to see if altered peptide ligands with modified TCR contact residues will influence interaction forces. In another SCFS study, a bispecific antibody with specificity for the OKT3 molecule and the human EpCAM molecule was used to target Jurkat T cells to cancer cells. In this system, strong binding forces of about 5 nN were already observed after 1-2 minutes contact time, probably due to the high affinity of the antibody (S. Hoffmann et al., unpublished observation).

The high interaction forces between T cells and APCs are likely to provide positional stability, thereby enabling productive interaction between TCRs and pMHCs. In solution, TCRs have been found to display low affinity, but recently it has been reported that TCRs at the contact zone dramatically increase their association and dissociation rates for pMHC, suggesting rapid sampling of pMHC and serial engagement of specific pMHCs in the sea of self peptides [31],[32]. Such a process will only take place if T cells and APCs are firmly held together.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
 ICAM-1 and LFA-1 expression contribute to T cell activation by APCs
 A, Surface expression of ICAM-1, LFA-I and Au on L.Au, L.Au.ICAM-1 cells, and 1934.4 T cells. B, Response of 1934.4 T cells to L.Au.ICAM-1, L.Au or Ltk⁻ cells pulsed with Ac1-11.4Y peptide.

Fig. 2.

Conjugate formation between APC and T cells

A, APC were labelled with the red dye SNARF, and 1934.4 T cells with the green dye CFSE. Following centrifugation, cells were incubated to allow for conjugate formation, and then fixed. The representative FACS plot shown was obtained after 30 min incubation time. B,C, Inhibition with anti-LFA-1 antibody of conjugates obtained with L.Au.ICAM-1 cells in the presence and absence of peptide. D,E, Anti-LFA-1 antibody does not inhibit conjugates obtained with L.Au APC. F, Frequency of conjugates obtained with L.Au-ICAM-1, L.Au and Ltk⁻ cells in the presence of peptide.

Fig. 3.

Principle of SCFS experiments analyzed by AFM

A, A T cell bound to the cantilever (I) is pressed onto an APC attached to a cover slip. (II): Upon retraction of the cantilever (III) it will bend according to the interaction forces and change the deflection of a laser beam which, together with the known spring constant of the cantilever, will allow precise assessment of interaction forces (adapted from 28). B, (i) shows an adherent L.Au.ICAM-1 cell on the cover slip, (ii) displays a 1934.4 T cell captured by the tip of the cantilever via anti-CD43 antibody, and (iii) shows the T cell after it has been brought into contact with the APC by lowering the cantilever. C, Typical retraction curves. The lowest point of the retraction curve indicates the maximum unbinding force which equals the overall cellular interaction force. From left to right: L.Au.ICAM-1 cells with peptide, 30 minutes interaction time; L.Au.ICAM-1 cells without peptide, 30 minutes; L.Au. cells with peptide, 30 minutes.

Fig. 4.

Time-dependent increase of interaction forces between T cells and APCs

For each time point between 6 and 20 measurement were performed. A, Time-dependent increase of interaction forces between 1934 T cells and L.Au.ICAM-1 APCs pulsed or not with Ac1-11.4Y peptide. B, Interaction forces between 1934 T cells and ICAM-1-negative L.Au APCs in the presence and absence of peptide. Note that binding forces remain low even after 30 minutes contact time. * $P < 0.05$ as indicated. C, Interaction forces between 1934.4 T cells with L.Au.ICAM-1 or L.Au cells in the absence of peptide. *** $P < 0.001$ as indicated.

Fig. 5.

Synapse formation in conjugates determined by multispectral imaging flow cytometry (MIFC)

A, Conjugates obtained with Ac-1-11.4Y peptide-loaded L.Au.ICAM-1 APCs display redistribution of LFA-1 and TCR into the contact zone (left hand side middle), whereas no such redistribution is seen in the absence of peptide. Likewise, conjugates with ICAM-1-negative L.Au cells fail to redistribute LFA-1 and TCR regardless of the presence or absence of peptide. B, Time-dependent formation of immune synapses. Conjugates were generated and quantified by MIFC, and the fold increase of conjugates displaying an immune synapse, as determined by enrichment of both CD3 and LFA-1 in the contact zone, was calculated as described in *Materials and Methods*. The graph shows the mean of three independent experiments. The values for 15 and 30 minutes are not statistically different. C, Conjugate formation with peptide-pulsed L.Au.ICAM-1 cells was inhibited by addition of 40µg/ml anti-LFA-1 antibody M17.4 to the conjugate assay. * $P < 0.05$ as indicated.