Corrections

NEUROSCIENCE

The authors note that the author contributions footnote should be revised to reflect the following. Itzhak Nissim should be credited with designing the research and writing the paper. He should not be credited with performing the research. The corrected author contributions footnote appears below.

Author contributions: J.T.C., A.V., I.N. and A.S.C. designed research; J.T.C., C.M.M., S.K., and J.A.E. performed research; J.T.C. and A.S.C. analyzed data; and J.T.C., I.N., and A.S.C. wrote the paper.

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Immune synapse formation determines interaction forces between T cells and antigen-presenting cells measured by atomic force microscopy

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During adaptive immune responses, T lymphocytes recognize antigenic peptides presented by MHC molecules on antigen-presenting cells (APCs). This recognition results in the formation of a so-called immune synapse (IS) at the T-cell/APC interface, which is crucial for T-cell activation. The molecular composition of the IS has been extensively studied, but little is known about the biophysics and interaction forces between T cells and APCs. Here, we report the measurement of interaction forces between T cells and APCs employing atomic force microscopy (AFM). For these investigations, specific T cells were selected that recognize an antigenic peptide presented by MHC-class II molecules on APCs. Dynamic analysis of T-cell/APC interaction by AFM revealed that in the presence of antigen interaction forces increased from 1 to 2 nN at early time-points to a maximum of ~14 nN after 30 min and decreased again after 60 min. These data correlate with the kinetics of synapse formation that also reached a maximum after 30 min, as determined by high-throughput multispectral imaging flow cytometry. Because the integrin lymphocyte function antigen-1 (LFA-1) and its counterpart intercellular adhesion molecule-1 (ICAM-1) are prominent members of a mature IS, the effect of a small molecular inhibitor for LFA-1, BIRT377, was investigated. BIRT377 almost completely abolishes the interaction forces, emphasizing the importance of LFA-1/ICAM-1-interactions for firm T-cell/APC adhesion. In conclusion, using biophysical measurements, this study provides precise values for the interaction forces between T cells and APCs and demonstrates that these forces develop over time and are highest when synapse formation is maximal.

Cell-cell contacts play a crucial role in triggering the body’s immune system. During adaptive immune responses, antigen-presenting cells (APCs) process foreign antigens into peptides, which are loaded into major histocompatibility complex (MHC) molecules. T cells patrolling the body scan APC and establish intercellular contacts when their antigen-specific T-cell receptors (TCR) recognize a foreign peptide/MHC complex on the APC. Dynamic studies in biological systems, and single-cell force spectroscopy (SCFS) by AFM has been established as an important tool for the study of cell adhesion (14).

The recent years have seen a significant increase of AFM-related studies in biological systems, and single-cell force spectroscopy (SCFS) by AFM has been established as an important tool for the study of cell adhesion (14). This technique allows for the analysis of adhesion processes and adhesion forces under near-physiological conditions. To the best of our knowledge, the interaction forces between T cells and APC have not yet been investigated by SCFS. In the present study, we have modified and adjusted SCFS techniques for the measurement of long-time interaction forces between T cells and APC. These force spectroscopy measurements were complemented by conjugate and high-throughput fluorescence assays relating the kinetics of IS formation to the development of interaction forces between T cells and APCs.

Results

Conjugate Formation Between T Cells and APCs. For the present work, a well-studied T-cell/APC pair was selected, namely the
Conjugates were allowed to form for 30 min in the presence (black bars) or absence (white bars) of HEL35–45 peptide. Fig. 1 shows the expression of surface markers such as Ak, ICAM-1, LFA-1, CD3, and CD43, which are important for the T-cell/B-cell interface and, concomitantly, disturbed mature IS formation. The complete inhibition curves for Fig. 1D are presented in Fig. S2. Together, the above results show that the 3B11/LK35.2 cell pair efficiently forms conjugates and hence is suitable for AFM studies. However, this conjugate assay does not provide information on immune synapses and interaction forces, which were analyzed in the following set of experiments.

Immune Synapse Formation. T cells accumulate a multitude of their surface receptors, intracellular signaling and scaffolding molecules in the contact zone with APCs, resulting in the formation of a mature immune synapse. The IS is defined by a cluster of the TCR/CD3 complex in the center of the contact zone (central supramolecular activation cluster, cSMAC), which is surrounded by a second cluster containing LFA-1 (peripheral SMAC, pSMAC) (18). To study the kinetics of receptor accumulation and IS maturation at the contact zone of 3B11 T cells and LK35.2 B cells, all cell couples were analyzed by multispectral imaging flow cytometry (MIFC), using the Image Stream system. MIFC is a method that combines the advantages of a high-throughput flow cytometer and fluorescence microscopy (19), thereby allowing rapid quantitative and objective analysis of proteins in the contact zone between T cells and APCs for all T-cell/APC pairs within a given population.

Conjugates were stained for CD3 (cSMAC), LFA-1 (pSMAC), and F-actin (pSMAC) (20), and analyzed by MIFC. In the absence of HEL peptide, most T cells displayed a fairly equal distribution of CD3 and LFA-1 on the cell surface (Fig. 2A Upper Left), or randomized clusters of CD3 (see Fig. S3 for additional MIFC pictures). In contrast, in the presence of HEL peptide, strong polarization of both CD3 and LFA-1 in the contact zone was observed (Fig. 2A Upper Right). Quantitation of T cells showing an enrichment of both CD3 and LFA-1 in the contact zone—and thus display a mature synapse—revealed that the peak number of mature immune synapses was reached after ~30-min incubation time at 37 °C in the presence of HEL peptide (Fig. 2B). Thereafter, the number of T cells displaying a mature synapse started to decrease, but stayed above the level of values obtained without HEL peptide by at least a factor of 3. Interestingly, the amount of F-actin in the contact zone, which is crucial for the avidity regulation of integrins, increased over time (Fig. 2B). As a control, cells were preincubated with EDTA, which inhibits both integrin function and TCR/CD3-dependent calcium influx (16, 21). Hence, EDTA should abolish formation of a mature immune synapse. Fig. 2 shows that EDTA indeed inhibited both the maturation of the immune synapses and the accumulation of F-actin in the contact zone (Fig. 2R). Next, we analyzed whether the LFA-1 inhibitor, BIRT377, would affect the accumulation of LFA-1 and CD3 in the IS. Indeed, BIRT377 interfered with the accumulation of both LFA-1 and CD3 at the T-cell/B-cell interface and, concomitantly, disturbed mature IS formation (Fig. 2A Lower and C). Accumulation of F-actin in the contact zone was also strongly inhibited in the presence of BIRT377, demonstrating that activation of LFA-1 is required for both IS formation and stable accumulation of F-actin in the IS.
Interaction Forces Measured by AFM. The interaction forces between T cells and APC were measured in an AFM system equipped with a tissue-culture chamber for live cell experimentation. In orientating studies we determined that 3B11 T cells bound well to cantilevers coated with anti-CD43 antibodies, whereas the CD43-negative LK35.2 B cells adhere well to glass plates coated with poly-L-lysine (PLL) without any signs of toxicity for the cells. AFM experiments were performed using custom-made Petri dishes divided into two chambers by a barrier. B cells were allowed to adhere to a PLL-coated glass plate placed into one chamber, whereas T cells were added to the other chamber. Using manual controls, the anti-CD43-coated cantilever was placed above a T cell whose viability had been confirmed by propidium iodide in the assay medium. The cantilever was advanced stepwise toward the T cell, and upon tapping the T cell adhered to the anti-CD43-coated cantilever without exerting high forces on the cell. The T cell was then hauled up, moved above a B cell (Fig. 3A), and lowered down for establishment of contact between the two cells. To allow for conjugate formation, we used forces in the range of 1–2 nN to press cells against each other, thereby ensuring that the T cell was in contact with the B cell. After various contact times, TC, the cells were separated by retracting the cantilever over a range of \( \pm 100 \, \mu \text{m} \). Laser deflection correlating to separation force measurement a fresh pair of T cells and B cells. This process was used in orientating studies the contact times were usually short. Peptide-dependent interaction forces between T cells and APCs are probably special, because it has been shown that upon contact MHC-containing membrane fragments are pulled out from the APCs and remain because it has been shown that upon contact MHC-containing membrane fragments are pulled out from the APCs and remain bound to the T cell, a process termed trogocytosis that can operate in both directions (25). To avoid potential complications of the lowest point of the spectrum and the value when the cantilever is fully retracted. In the examples shown in Fig. 3B, the interaction force for \( T_C = 30 \, \text{min} \) in the absence of antigen is 0.8 nN, and in the presence of antigen is 6.7 nN. Additional interaction force curves are shown in Fig. 3C. Further hallmarks of AFM spectra can be seen in Fig. 3B. So-called \( j \)-events (22) are short force curve segments with a negative slope. They resemble jumps in the cantilever retraction and are usually correlated to breakages of single molecules or whole molecular clusters. They appear in the central section of spectra and have a short breakaway. In contrast, so-called \( t \)-events are discernible as step-like structures at large Piezo positions when the cell bodies are already separated but still connected through viscous membrane nanotubes that carry one or several adhesion proteins on their tip. These nanotubes (also known as tethers) are viscously, that is, without force application, pulled out of the membrane reservoir.

A number of cell separation experiments failed because of technical difficulties. Failure rates increased especially at longer contact times (30 and 60 min). Although the cells were fixed to the cantilever or PLL-coated plate, respectively, the major part of the cells’ bodies were free to move, often resulting in separation or sliding into a parallel position so that the AFM cantilever would touch both cells. In AFM studies, investigators have often used the same cell attached to the cantilever for a large number of measurements (11, 23, 24). However, in these studies the contact times were usually short. Peptide-dependent interactions between T cells and APCs are probably special, because it has been shown that upon contact MHC-containing membrane fragments are pulled out from the APCs and remain bound to the T cell, a process termed trogocytosis that can operate in both directions (25). To avoid potential complications by such modified cells, we decided to select for each interaction force measurement a fresh pair of T cells and B cells. This
tedious and time-consuming procedure limited very much the number of measurements that could be performed.

In Fig. 4, the interaction force dynamics of 3B11/LK35.2 cell conjugates in the presence and absence of HEL peptide is shown. Fig. 4 includes a total of 92 successful measurements. At TC = 0 min, cells were pressed against each other until the setpoint was reached and separated immediately afterward, resulting in a contact time of 1–3 s. Interaction forces for all zero min time points yielded similar values for peptide pulsed and unpulsed cell pairs, namely 1.02 ± 0.46 nN and 1.08 ± 0.83 nN. Binding values remained similar after 2 min of contact. After TC = 15 min, both pulsed and control pairs showed higher separation forces at 4.38 ± 3.37 nN and 2.48 ± 1.71 nN, respectively. At TC = 30 min, the interaction force reached a maximum of 14.31 ± 3.64 nN for pulsed pairs, which represents a >10-fold increase compared with 1.28 ± 0.90 nN for control pairs. After 60 min contact time, pulsed values dropped back to 5.36 ± 4.25 nN and unpulsed to 0.95 ± 0.54 nN, demonstrating a decrease in the interaction forces after prolonged contact time. In five experiments with pulsed B cells, we observed forces >20 nN. However, we could not calculate the precise values because the T cell detached from the cantilever during separation due to the high forces. These experiments are not included in Fig. 4.

Our results, presented in Figs. 1 and 3, show that the LFA-1 antagonist BIRT377 interfered with maturation of the immune synapse and conjugate formation. Therefore, we decided to test the effect of BIRT377 on the interaction forces. Fig. 5 shows that in the presence of BIRT the peptide-dependent interaction force value decreased from 14.3 nN to 0.4 nN. In the absence of peptides, the interaction force was also inhibited and dropped from 1.3 nN to 0.3 nN. These results establish the LFA-1/ICAM-1 pair of adhesion molecules as a critical component in the development of strong adhesion forces between T cells and APC. Because the force of interaction will be influenced by the number of ICAM-1 and LFA-1 molecules on the cell surface, it should be noted that the respective expression levels on the LK35.2 B cells and 3B11 T cells used here are within the normal range and comparable to primary activated B and T cells, to a number of B cell lines and T hybridomas tested (data not shown).

Discussion

In the past, AFM has been used to characterize interaction forces and kinetics in molecule-molecule binding. To that end, AFM cantilevers covered with immobilized ligands were contacted...
with receptor-coated surfaces and unbinding forces measured during the retraction phase. For antibody-antigen binding, an interaction force of 244 ± 22 pN was reported (26), which is comparable in strength to the well studied streptavidin-biotin bond with an interaction force of 257 ± 25 pN (27). For investigation of membrane bound adhesion molecules in their native environment, cell-substrate studies have been conducted with various cell-ligand pairs. Moy and coworkers probed immobilized 3A9 (24) and Jurkat T cells (28) against ICAM-1-coated surfaces. Because the focus in those studies was the assessment of the bond strength of individual LFA-1/ICAM-1 complexes rather than overall forces on the cellular level, the cells were allowed to touch the substrates only for a few seconds, which is assumed to result in the formation of only one single binding. Benoît and coworkers pioneered the field of AFM based cell–cell adhesion measurements with Dictyostelium discoideum cells and found force values in the low nN regime for short contact times (11). In another study, Moy and coworkers examined interaction forces between human umbilical vein endothelial cells (HUVECs) and Jurkat T cells over a period of 10 s, yielding values in the range of 100 pN (13). Thus, so far, the majority of adhesion-related SCFS studies performed by AFM concentrated on short-time effects.

T cells need to switch effectively between different modes of adhesion. For example, adhesion properties change when T cells circulating in the blood stream start to migrate across the endothelial walls of blood vessels for infiltration into tissues. Likewise, for T-cell activation, intensive interactions with APCs are required. Because many of these adhesion events depend on integrins, T cells must tightly control their integrin activity. Hence, binding properties that build up over time have to be considered in cell–cell adhesion assays. It has been discussed that AFM may not be an appropriate tool for the investigation of long-term adhesion, because cells are motile and drifts may prevent experimental stability (12). However, despite these difficulties we have successfully measured the long-term dynamics of interaction forces in T-cell/APC conjugates with AFM-based SCFS in the present study. The number of successful measurements was limited by a substantial failure rate, particularly in the long-term measurements, despite the fact that cloned cell lines were used, which can be considered to be fairly homogenous. It is not clear whether these failures were due to high motility of the cells or other technical problems, or whether even cloned T cells and APCs may differ in their ability to form tight conjugates and immune synapses, for example depending on their cell cycle status. In preliminary AFM experiments with another T-cell hybridoma, we observed at Tc = 30 min an average interaction force of ~12 nN in the presence of antigen (data not shown), which is comparable with the 14.3 nN found for the Lk35.2/3B11 pair. However, more cell pairs need to be tested for determination of the range of interaction forces.

A notable result of the present SCFS study is the observation that in the presence of peptide the interaction forces between T cells and APCs increase with time and reach a maximum after ~30 min, whereas in the absence of peptide the binding forces remain low. This increase correlated well with the kinetics of IS formation, which also reached a maximum after ~30 min, in agreement with other studies on IS formation (29). This relationship suggests that the strength of interaction forces depends on the state of IS maturity. Our observation that the small molecule inhibitor BIRT377 inhibits both, the increase of interaction forces and IS formation, by blocking the activation of LFA-1, identifies LFA-1 as the major adhesion-inducing component in the IS investigated here. Moreover, this finding demonstrates that the interaction between TCR and pMHC is not responsible for the high interaction forces. Rather, our results indicate that after activation of TCR by pMHC T cells effectively switch between adhesion modes, whereby LFA-1 activation is triggered by an inside-out mechanism. This finding is in agreement with current views and shows how cells can control their adhesion propensity from the inside. Whether the spatial organization of LFA-1 within the contact zone influences the adhesion force, or vice versa, is not known at present.

Materials and Methods

Antigen Presentation and Cell Conjugate Assays. The murine T-cell hybridoma 3811, recognizing the HEL3 4–4 5peptide (15), and the B cell lymphoma LK35.2 were maintained in RPMI MEDIUM 1640 tissue culture medium. For antigen presentation assays (33), 5 × 10^5 LK35.2 cells were pulsed with peptide for 4 h and incubated with 5 × 10^4 3811 cells in triplicate in 96-well plates. After 40 h, IL-2 released by 3811 cells was measured by an Europium-based fluorescence immunoassay. Cell conjugate assays were performed as reported (16). Briefly, 3811 T cells were stained for 10 min at 37 °C with 0.3 μM of CFSE (Molecular Probes). LK35.2 cells were loaded with 100 μmol of HEL3 4–4 5 and labeled with 5 μmol of SNARF (Invitrogen). Cells of each population (10^6) were mixed in 100 μL medium, briefly centrifuged, fixed after various times with 2% paraformaldehyde, and analyzed with a FACs Calibur. BIRT377 was kindly provided by Terence Kelly (Boehringer Ingelheim).

Analysis of Immune Synapses by ImageStream (MIFC). For analysis of IS, conjugates were formed between 3811 T cells and HEL peptide-loaded LK35.2 cells as described in ref. 20. Briefly, 1 × 10^6 cells were centrifuged in 250 μL medium for formation of contacts and immediately resuspended in 50 μL medium. After incubation at 37 °C for various time points, cells were fixed with 2% paraformaldehyde and stained with anti-CD3-biotin plus streptavidin–TexasRed, anti-CD18–FITC (BD BioSciences), and nuclear dye (Hoehst33342; Invitrogen) as indicated. Data acquisition (20,000 cells per sample) was performed with an ImageStream system (Amnis), and data were analyzed with IDEAS 3.0 software. To find the contact zone between 3811 and LK35.2 cells, total events were gated on true T-cell/B-cell pairs and a Hoechst dye

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dependent valley mask was defined between these coupled cells. The valley
mask was combined with a T-cell mask that utilizes the CD3 staining. This
results in the IS mask. Thereafter, protein accumulation was calculated as the
ratio between the mean pixel intensity (MPI) of the respective protein, that is,
dependent valley mask was defined between these coupled cells. The valley
scope (Carl Zeiss). The AFM was placed in a CO2 incubator box controlled by
a CellHesion module (JPK Instruments AG) mounted on a AxioVert 200 micro-
scope for cantilever drift and different support positions. Data were analyzed with
MATLAB (MathWorks). Silicon nitride tipless cantilevers (NS 12 NoAL,
μmach). Cantilever force constants ranged from 0.05–0.50 Nm as deter-
mined by individual calibration of the cantilever using the thermal noise
method (34). For capture of T cells, cantilevers were coated with rat anti-
mouse CD43 antibody (BD Biosciences) (Fig. S5). For AFM studies, modified
Petri dishes were used as described in Fig. S5. Separation experiments were
performed at constant retraction velocity of 1 μm/s to ensure comparable
conditions.

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