Corrections

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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 (TCRs) recognize a foreign peptide/MHC complex on the APC. Elegant two-photon microscopy studies have revealed the dynamics of this process in lymph nodes. There, T cells move through the network of dendritic cells (DCs) and scan DCs for foreign antigen. In the absence of antigen brief transient interactions are observed, whereas upon recognition of a cognate antigen T cells are arrested and interactions prolonged to >1 h (1, 2). Similarly, during antibody responses, long-lasting antigen driven interactions between T helper cells and B cells have been observed in lymph nodes (3). Subsequently, at the contact zone between T cells and APC spatially organized molecular clusters develop, referred to as immune synapse (IS), which is crucial for T-cell activation and effector cells development (4).

Formation of an IS includes the coordinated translocation of several protein complexes, among others TCR and its ligand pMHC, and the integrin lymphocyte function-associated antigen-1 (LFA-1) and its counterpart intercellular adhesion molecule 1 (ICAM-1). This orchestrated reorganization of membrane proteins involves many cytoplasmic molecules and is presumably supported by cytoskeletal factors like actin (5). Although many important aspects of IS formation have been identified, little is known about the underlying biophysics and interaction forces between T cells and APCs. Integrins represent a family of major cell adhesion proteins used by cells to tune their adhesion propensity. This tuning is achieved by controlling the number of proteins present at the cell’s interaction face and by the activation state of the adhesion proteins themselves. Switch blade-type heterodimeric integrins are known to exist in different activation states, which are transmitted from the cytoplasmic tail to the extracellular domain (6). It is believed that activation state changes are triggered by inside-out-signaling, for instance when a TCR recognizes a peptide presented by MHC molecules (7). The activation of LFA-1 upon TCR-triggering is mainly mediated by PKC and the small GTPases Ras and Rap1 ([8] and references therein). The association of actin to LFA-1 accompanies this process. Subsequent motor protein motion yields a cytoskeleton contraction, which exerts low forces on LFA-1 to induce occupied integrin activation and to fully arrest the two cells for adhesion. By actio et reactio, this force has to be counterbalanced on the APC side, resulting in a high interaction force between T cells and APCs.

Cell–cell adhesion has been studied by micropipette aspiration techniques (9, 10) and atomic force microscopy (AFM) (11–13). 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


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3B11 T hybridoma and the LK35.2 B cell line (15), because in orientating experiments 3B11 cells could be attached well via antibodies to the cantilever of the AFM instrument. The 3B11 T hybridoma recognizes a peptide HEL35–45 derived from the model antigen hen egg lysozyme presented by Ak MHC class II-positive APC, such as LK35.2 B cells. Fig. 1D shows that LK35.2 cells loaded with HEL35–45 peptide stimulated IL-2-production by 3B11 T cells, whereas irrelevant peptide failed to stimulate 3B11 (data not shown). Because IL-2-secretion was highest at a 100 μmol peptide concentration, for further studies this concentration was used. Fig. S1 shows the expression of surface markers such as Ak, ICAM-1, LFA-1, CD3, and CD43, which are relevant for the present study.

Next, we analyzed whether 3B11 T cells and LK35.2 APCs would adhere to each other in the presence of peptide and form conjugates. For this purpose, 3B11 cells were labeled with the green dye CFSE and LK35.2 with the red dye SNARF (16). Cells were mixed at a 1:1 ratio, centrifuged briefly, and incubated for various times before fixation with paraformaldehyde. Conjugates were formed to form for 30 min in the presence (black bars) or absence (white bars) of HEL35–45 peptide. For inhibition, 100 μg/mL antibody was used. For detailed inhibition curves, see Fig. S2.

Fig. 1. Antigen presentation and conjugate formation between T cells and APCs. (A) Presentation of HEL35–45 by Lk35.2 APCs to 3B11 T cells results in IL-2 secretion. (B) and (C) Kinetics of conjugate formation between 3B11 T cells and LK35.2 APCs in the presence (B) and absence (C) of HEL35–45 peptide, and inhibition with 80 μM LFA-1 antagonist BIRT377 (triangles). (D) Inhibition of conjugate formation by antibodies against MHC-II, CD4, MHC-I, and BIRT377. Conjugates were allowed to form for 30 min in the presence (black bars) or absence (white bars) of HEL35–45 peptide. For inhibition, 10 μg/mL antibody were used. For detailed inhibition curves, see Fig. S2.

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Interactions 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 placed above a T cell whose viability had been confirmed by propidium iodide in the assay medium. The cantilever was placed above a T cell whose viability had been confirmed by propidium iodide in the assay medium. The cantilever was placed above a T cell whose viability had been confirmed by propidium iodide in the assay medium. The cantilever was placed above a T cell whose viability had been confirmed by propidium iodide in the assay medium. The cantilever was placed above a T cell whose viability had been confirmed by propidium iodide in the assay medium.

When the cantilever is retracted, it starts to bend until a certain point is reached after which molecular interactions begin to be disrupted. The total interaction force is defined as the difference 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 Tc = 30 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. S4. 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 f-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 viscous, 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. Benoist 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 an after various times with 2% paraformaldehyde. Conjugate formation was already observed after a few minutes. This finding is in contrast to the more informative MIFC and AFM studies, which reveal that maximal synapse formation and interaction forces require ~30 min for full development. Together, these observations suggest that weak interaction forces are sufficient to form conjugates, which are then stabilized by fixation with paraformaldehyde.

In conclusion, the present study provides precise biophysical values for the interaction forces between T cells and APCs, and correlates the kinetics of synapse formation with the development of interaction forces. Although full synapse formation does not always seem to be required for activation of T cells (32), it is likely that strong interaction forces will favor optimal T-cell activation. The approach described here will allow further studies on the interrelationship between molecules influencing interaction forces, synapse formation, and T-cell activation.
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, CD3 or CD18, in the IS mask and the MPI of the same protein in the T cells mask. 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 microscope system. Using a Matlab program, force curves were corrected for cantilever drift and different support positions. Data were analyzed with MATLAB (MathWorks). Silicon nitride tipless cantilevers (NSC 12 NoAl, μmach). Cantilever force constants ranged from 0.05–0.50 N/m as determined 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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