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Multistage T Cell–Dendritic Cell Interactions Control Optimal CD4 T Cell Activation through the ADAP-SKAP55–Signaling Module

Jason S. Mitchell,* Brandon J. Burbach,* Rupa Srivastava,* Brian T. Fife, † and Yoji Shimizu*

The Ag-specific interactions between T cells and dendritic cells progress through dynamic contact stages in vivo consisting of early long-term stable contacts and later confined, yet motile, short-lived contacts. The signaling pathways that control in vivo interaction dynamics between T cells and dendritic cells during priming remain undefined. Adhesion and degranulation promoting adapter protein (ADAP) is a multifunctional adapter that regulates “inside-out” signaling from the TCR to integrins. Using two-photon microscopy, we demonstrate that, in the absence of ADAP, CD4 T cells make fewer early-stage stable contacts with Ag-laden dendritic cells, and the interactions are characterized by brief repetitive contacts. Furthermore, ADAP-deficient T cells show reduced contacts at the late motile contact phase and display less confinement around dendritic cells. The altered T cell interaction dynamics in the absence of ADAP are associated with defective early proliferation and attenuated TCR signaling in vivo. Regulation of multistage contact behaviors and optimal T cell signaling involves the interaction of ADAP with the adapter src kinase–associated phosphoprotein of 55 kDa (SKAP55). Thus, integrin activation by the ADAP-SKAP55–signaling module controls the stability and duration of T cell–dendritic cell contacts during the progressive phases necessary for optimal T cell activation. The Journal of Immunology, 2013, 191: 2372–2383.

Initiation of the T cell immune response involves recognition by the Ag-specific TCR complex of a combination of an antigenic peptide and a self-MHC protein expressed on the surface of dendritic cells (DCs). T cell activation requires physical contact between the T cell and the DC that is facilitated by adhesion molecules, such as the integrin LFA-1 (1). T cell interactions with APCs that lead to T cell activation and clonal expansion are highly dynamic in vivo (2, 3). In the absence of Ag encounter, naive T cells move rapidly through the T cell zone of lymph nodes (LNs), exhibiting a random walk migration. When contacting an Ag-laden DC, Ag-specific T cells reduce their rate of motility and eventually form prolonged contacts with DCs (4, 5). This stable phase of contact persists for hours and while the T cells maintain dynamic movement over the DC, they are highly confined to the DC site (6). After ~24 h, a time point at which the T cell begins to proliferate, velocity increases and the cells regain motility that is characterized by “swarming” behavior around the DCs, making brief and sometimes, repeated, contacts (5).

These temporal phases of T cell contact with DCs during initial T cell activation have been observed for both CD4 and CD8 T cells (5, 6). Several studies suggested that disruption of the stable contact phase can lead to changes in the quality of the ensuing T cell response. Ab-mediated disruption of TCR signaling on CD4 T cells with an anti-MHC class II Ab during the early stable contact phase (6 h) results in transient successive T cell–DC contacts and a pronounced defect in early T cell proliferation and effector differentiation (7). In contrast, disruption of T cell signaling at the later “swarming” phase (24 h) does not alter early T cell activation. Imaging studies suggest that one mechanism of action of inhibitory receptors, such as CTLA-4 and programmed death-1 (PD-1), is alteration of T cell contacts with DCs through disruption of the TCR stop signal (8, 9). An analysis of CD8 T cells revealed a loss of stable T cell–DC contacts when the DC lacks expression of ICAM-1, a ligand for the LFA-1 integrin (10). The loss of these stable contacts resulted in impaired priming and survival of CD8 T cells. Overall, these studies suggest an important role for the initial stable contact phase of T cell–DC interactions for T cell activation in vivo. However, little is known about the intracellular signals emanating from the TCR that control these dynamic intracellular interactions in vivo.

The hematopoietic-specific adapter molecule adhesion and degranulation promoting adapter protein (ADAP; also known as Fyb or SLAP-120/130) regulates the rapid and transient activation of LFA-1 that occurs upon TCR engagement (11, 12). As a result, ADAP-deficient CD4 T cells exhibit a reduced ability to interact with Ag-pulsed APCs in vitro (13). Regulation of TCR signaling to integrins by ADAP requires the constitutive association of ADAP with another adapter, src kinase–associated phosphoprotein of 55 kDa (SKAP55) (14–16). The ADAP-SKAP55–signaling complex regulates TCR-mediated adhesion by targeting ADAP-SKAP55 to β2 integrin sites by the SKAP55 pleckstrin homology.
domain (17, 18). A distinct biochemical pool of ADAP that is not associated with SKAP55 can bind in a TCR-inducible fashion with the CARMA1 scaffold and, thus, participates in the regulated assembly of the CARMA1-Bcl10-Malt1 complex that is critical for NF-κB activation (15, 17). Although ADAP-deficient T cells exhibit impaired adhesion to APCs in vitro and impaired T cell proliferation both in vitro and in vivo (13, 15, 19), little is known about the role that ADAP and, in particular, ADAP-dependent signals to integrins play in regulating T cell–DC contact in vivo. In this study, we used two-photon laser scanning microscopy (TPLSM) to examine the function of ADAP in regulating the multiphase interactions of CD4 T cells with Ag-laden DCs during the first 24 h of T cell activation. These studies revealed that impaired CD4 T cell activation in the absence of ADAP is associated with changes in all three phases of T cell–DC contact in vivo, including increased early motility in the presence of Ag, conversion of the stable contact phase to a period of repetitive transient contacts, and a failure to maintain confined swarming behavior at later time points. The latter two behaviors are regulated by the interaction of ADAP with SKAP55, implicating TCR-mediated integrin activation signals in the control of T cell–DC contact duration that dictates optimal T cell activation in vivo.

Materials and Methods

Mice

DO11.10 wild-type and ADAP−/− mice (11), crossed to transgenic hCAR mice (20) on the BALB/c background, were generated as previously described (16). DO11.10 ADAP−/− Rag−/− Thy 1.1/2.2 mice were also generated by crossing with DO11.10 Rag−/− Thy 1.1 mice. Recipient BALB/c mice were purchased from the National Cancer Institute. Mice were housed in specific pathogen-free facilities and were used between 8 and 12 wk of age. All experimental protocols involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Cell labeling

DO11.10 T cells were labeled with different intravitreal cell dyes for cell visualization using the following modified protocols (21, 22). Cells labeled with CellTrace Violet (Invitrogen) were resuspended in PBS with 10% FBS at 1 × 10^7 cells/ml. CTV diluted in PBS was added 1:1 to the cells (7.5 and 3.75 μM final concentration for TPSM and flow cytometry, respectively) and incubated at 37°C for 20 min. For CFSE labeling (Invitrogen), cells were resuspended in PBS with 5% FBS at 2 × 10^7 cells/ml. CFSE diluted in PBS was added 1:1 to the cells (2.5 μM final concentration) and incubated at 25°C for 5 min. Cells labeled with CellTracker Orange (CTO; Invitrogen) were resuspended in DMEM with 1% FBS at 4 × 10^7 cells/ml CTO diluted in DMEM with 1% FBS was added 1:1 to the cells (2.5 μM final concentration) and incubated at 37°C for 30 min. After dye incubation, all labeled cells were quenched by washing twice with complete T cell media (RPMI 1640, 10% FBS, 100 U/ml penicillin-streptomycin, 1 mM sodium pyruvate, 55 μM 2-ME, 2 mM l-glutamine). Dye switching was used in all assays to confirm that there were no adverse effects of the different labeling conditions.

In vivo T cell activation

We used a previously described in vivo ear priming model in this study (5). Briefly, chicken OVA protein was emulsified in IFA (Sigma) using two glass syringes and an emulsifying hub. When the emulsion was used for imaging endogenous DCs, CFSE was incorporated at a final concentration of 1 mM. Mice were anesthetized with an i.p. ketamine injection, and 10 μl of emulsion containing 2 μg OVA (unless otherwise stated) was injected s.c. into both ears. At 24–72 h after injection, either unlabeled or CFSE-labeled Thy1.1 wild-type DO11.10 and Thy1.1/1.2 ADAP−/− DO11.10 T cells were cotransferred by i.v. injection, and the ear-draining cervical LNs were harvested at the indicated time points for analysis. For ICAM-1–blocking experiments, transferred cells were allowed to home to LNs for 1 h before 200 μg anti–ICAM-1 Ab (clone YN1.7.4; Bio X Cell) was injected i.v. Cell suspensions were stained for the transferred T cells and PD-1 expression with the following anti-mouse Abs: FITC DO11.10 TCR (KJ1-26), PE PD-1, allophycocyanin Thy 1.1, Pacific Blue Thy 1.2, allophycocyanin–eFluor 780 CD4, or PerCP-Cy5.5 dump (negative gating strategy using B220, CD8, CD11b, F4/80) (eBioscience), and proliferation was assessed by CTV dilution. In ADAP-reconstitution experiments, wild-type and ADAP−/− hCAR+ DO11.10 T cells were purified and transduced with adenovirus, as previously described (15–17, 19, 23), and cotransfected into primed recipients. To distinguish the cotransfected populations, ADAP−/−-reconstituted cells were labeled with CTV, and wild-type cells were labeled with CFSE.

For analysis of T cell–signaling responses, draining LNs were harvested and immediately dissociated into 10 ml 1.6% paraformaldehyde in PBS and fixed for 15 min at 25°C; subsequently, 40 ml 20°C methanol was added to the cells, and they were incubated on ice for 20 min, as previously described (24). The cells were then stained for the transferred T cells, as described above, as well as for phosphorylated c-Jun (Santa Cruz; #SC-822) and phosphorylated ribosomal S6 protein (Cell Signaling; #4854).

All samples were acquired on a LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Short-term in vivo cohoming assays

In vivo homing assays were performed using previously described methods (25). Briefly, recipient mice were primed in one ear with 1 μg OVA and IFA and in the contralateral ear with IFA only. One day later, an equal number of CTV-labeled Thy1.2 wild-type DO11.10 T cells and CFSE-labeled Thy1.1/1.2 ADAP−/− DO11.10 T cells were transferred by i.v. injection. Draining cervical LNs and spleens were harvested at 4, 8, and 24 h after transfer, and the single-cell suspensions were labeled with FITC DO11.10 TCR (KJ1-26), allophycocyanin Thy 1.1, allophycocyanin–eFluor 780 CD4, or PerCP-Cy5.5 dump (B220, CD8, CD11b, F4/80) (eBioscience) prior to flow cytometry. The homing index was calculated as previously described (26).

TPLSM acquisition and analysis

A Leica TCS MP resonance scanning microscope with four nondescanned photomultiplier detectors and a Mai Tai DeepSee two-photon laser (15 W; Spectra-Physics) was used for in vivo imaging. Explanted draining LNs were immobilized on coverslips, with the hilium facing away from the objective, and maintained at 37°C using a heated chamber with circulated RPMI 1640 media bubbled with 95% O2; 5% CO2. Samples were excited with the two-photon laser at 840 nm, and emission wavelengths of 450–490 nm (for CTV), 500–560 nm (for CFSE), and 584–676 nm (for CTO) were collected in the detectors. The x-y acquisition dimensions were 0.28 μm. Z-stacks of 100–150 μm at a 1.98-μm step size were acquired every 30 s for 30 min, at a minimum of 40 μm below the capsule (identified by second harmonic generation) and proximal to the afferent lymphatics, as determined by drainage of the CFSE/IFA emulsion. Image analysis used the identified and tracked cell tracking software performed using Imaris 5.2.6x64 software (Bitplane). Contact time was determined by manually examining each T cell–DC interaction; no contact was scored if black pixels were visualized between the cells. Cell displacement was calculated using a Microsoft Excel–written macro program, written in Microsoft Visual Basic for Applications, as previously described (8). The postcellular DC contact confinement ratio (CR) was determined by first creating cell tracks that began at the point of T cell–DC contact termination; only tracks that were visible for ≥5 min were analyzed. The CR was then created by dividing the cell displacement from origin (contact termination) by the total track length (27).

Results

Impaired Ag-specific proliferation of ADAP-deficient CD4 T cells in vivo

To examine the role of ADAP in regulating T cell–DC contacts during T cell activation in vivo, we used an activation system that was previously used to define the three distinct stages of CD4 T cell motility during the initial 24 h of T cell activation (4, 5). In this system, adoptively transferred CD4 T cells expressing the OVA-specific transgenic TCR DO11.10 were stimulated in naive BALB/c recipients by an Ag-emulsion depot in the ear with OVA protein and IFA. As previously described, this ear priming results in Ag acquisition by skin-resident DCs and the subsequent migration of Ag-bearing activated DCs to the draining LN (5). Under these conditions, we observed dramatically impaired proliferation of ADAP-deficient DO11.10 T cells compared with wild-type DO11.10 T cells responding to Ag in the same mouse at all time.
points examined (Fig. 1A) and at multiple Ag doses (Fig. 1B). Clonal expansion was also impaired with ADAP-deficient DO11.10 T cells (Fig. 1C). The activation phenotype of ADAP-deficient DO11.10 T cells differed from wild-type DO11.10 T cells, because expression of the inhibitory receptor PD-1, which is rapidly upregulated upon TCR stimulation (28), was reduced in the ADAP-deficient T cells (Fig. 1D). Thus, ADAP-deficient T cells exhibit an attenuated proliferative response to Ag in vivo in this system.

We next assessed the Ag-specific activation of ADAP-deficient T cells during the first 24 h of stimulation in vivo. As early as 4 h after Ag stimulation in vivo, wild-type DO11.10 T cells exhibited robust phosphorylation of c-jun that was sustained at 12 and 24 h after challenge (Fig. 2). In contrast, Ag challenge of ADAP-deficient DO11.10 T cells resulted in much lower levels of c-jun phosphorylation at all of the time points examined. These results are consistent with our in vitro studies demonstrating that TCR-dependent phosphorylation of c-jun is regulated by ADAP (19). To determine whether other pathways downstream of the TCR were also impaired following Ag challenge of ADAP-deficient T cells in vivo, we examined phosphorylation of the S6 ribosomal protein (Fig. 2), which is dependent on activation of PI3K, mammalian target of rapamycin, and MAPK (29). Although these pathways are not directly dependent on ADAP (11, 15), we also observed reduced S6 phosphorylation following activation of ADAP-deficient DO11.10 T cells in vivo. Together, these results suggest that multiple signaling pathways downstream of the TCR are attenuated in vivo in the absence of ADAP.

**T cell basal motility is not dependent on ADAP**

Because we observed that ADAP controls T cell proliferation and downstream TCR signaling in vivo, we tested the hypothesis that these deficits in activation are due to the loss of stable ADAP-mediated T cell–DC contacts. We used TPLSM to examine the motility of wild-type and ADAP-deficient CD4 T cells in the absence and presence of Ag. To visualize Ag-laden DCs, we immunized mice with the dye CFSE incorporated into the IFA emulsion during ear priming to label endogenous DCs that have acquired Ag (5). Wild-type and ADAP-deficient DO11.10 T cells were labeled with either CTO or CTV prior to transfer into mice.
With the use of these three dyes, the interactions of both wild-type and ADAP-deficient DO11.10 T cells with DCs in the same LN could be visualized. We initially examined the motility of wild-type and ADAP-deficient CD4 T cells in the absence of Ag challenge. At 4 h after transfer, both wild-type and ADAP−/− DO11.10 T cells moved at similar average velocities of 10.81 and 10.29 μm/min, respectively (Fig. 3A, Supplemental Video 1). Both T cell populations displayed randomly, consistent with previously reported studies (5), suggesting that ADAP is dispensable for normal scanning behavior (Fig. 3B). These results indicate that ADAP is not required for basal motility of T cells in LNs.

**ADAP-deficient T cells do not form stable contacts with Ag-bearing DCs**

We next examined the effect of Ag on the motility and interaction dynamics of wild-type and ADAP−/− CD4 T cells. At 4 h after T cell transfer, the presence of Ag-bearing DCs resulted in a significant reduction in the average velocities of wild-type (3.3 μm/min) and ADAP-deficient (4.3 μm/min) T cells (Fig. 3A, Supplemental Videos 2, 3). Although both T cell populations reduced their average velocity, we consistently observed a slightly higher velocity for ADAP-deficient T cells. This was not due to fewer ADAP−/− T cells encountering and initiating contact with DCs, because the percentage of T cell contacts with DCs was similar between wild-type and ADAP−/− T cells (data not shown). Indeed, ADAP−/− T cells making contacts with DCs also exhibited a statistically higher velocity than did wild-type T cells making DC contacts (Fig. 3A) that did not result in significantly greater displacement (Fig. 3B). At the 4-h time point, we frequently observed wild-type DO11.10 T cells stably engaged with the body of the DC (Supplemental Videos 2, 3). In contrast, ADAP−/− T cells engaged the periphery of the DC, and contacts were transient and sometimes repetitive. Fig. 3C depicts the interactions of two wild-type T cells and two ADAP−/− T cells with a single DC using a kymograph format (Supplemental Video 2). This analysis shows that the wild-type T cells maintained a stable interaction with the DC during the entire 30 min of imaging, whereas the ADAP−/− T cells displayed unstable, transient interactions. Analysis of additional wild-type and ADAP−/− T cells revealed a similar difference in behavior with multiple DCs (Fig. 3D, Supplemental Video 3). When we assessed the total contact time with a DC, wild-type T cells had a 2-fold greater average total contact time compared with ADAP−/− T cells (25.0 min versus 12.3 min) (Fig. 3E). We also observed that the total contact time for an individual ADAP−/− T cell was typically either very low (<10 min) or very high (>20 min). In the absence of Ag, CFSE-labeled DCs could still be identified in the draining LN, but the transferred CD4 T cells did not form stable contacts with the DCs (Fig. 3E, Supplemental Video 1). Because a role for ADAP in T cell signaling may vary with the strength of the TCR signal, we also examined T cell–DC contacts at 4 h after challenge with a high dose of Ag (5 μg). We increased the imaging time from 30 to 60 min to detect potential differences between wild-type and ADAP−/− T cells at this higher Ag dose. We found that increasing the Ag dose resulted in increased total average contact time for both wild-type and ADAP−/− T cells (Fig. 3F). However, the average contact time remained higher for wild-type T cells (46.0 min for wild-type T cells versus 30.7 min for ADAP−/− T cells). These results indicate that ADAP is critical for long-lasting stable contacts with Ag-laden DCs at early time points.

Altered trafficking of naive ADAP−/− T cells into LNs (30) might alter the kinetics of the interaction of ADAP−/− T cells with Ag-laden DCs in vivo. To examine this issue, we performed short-term in vivo cocohoming assays (31). In the presence of IFA but no Ag, we observed a modest (~15–20%) reduction in the homing of ADAP−/− T cells to the draining cervical LN that reached statistical significance only at 8 and 24 h after T cell transfer (Supplemental Fig. 1). Similar results were observed in the presence of Ag, although homing of wild-type and ADAP−/− T cells was now comparable at the 24-h time point. There was no defect in the trafficking of ADAP−/− T cells to the spleen (Supplemental Fig. 1). Because we observed some reduction in the trafficking of ADAP−/− T cells to LNs in the presence of Ag at the early time points (4, 8 h), we examined T cell–DC contacts at a slightly later time point of 8 h after T cell transfer. Consistent with what we observed at 4 h (Fig. 3E), wild-type T cells again displayed a higher total average contact time compared with ADAP−/− T cells (23.0 min versus 12.3 min) (Fig. 3G). In addition, analysis of individual T cells demonstrated that, although most ADAP−/− T cells had low total contact times, a small number of ADAP−/− T cells had high levels of contact time comparable to many of the wild-type T cells.

**ADAP regulates contact time with DCs at later time points**

We next examined the interaction dynamics of wild-type and ADAP−/− T cells at a later time point: 24 h after transfer. At this time point, activated DO11.10 T cells begin blasting, regain motility, and make infrequent short contacts with DCs (5). We also observed that both wild-type DO11.10 T cells (8.0 μm/min) and ADAP−/− T cells (8.6 μm/min) had a higher average velocity at 24 h that was similar to previous reports of late-stage velocity...
FIGURE 3. ADAP-deficient T cells do not form early stable contacts with cognate DCs in vivo. CTV-labeled wild-type (blue) and CTO-labeled ADAP<sup>−/−</sup> (red) DO11.10 T cells (3 × 10<sup>6</sup> cells each) were cotransferred into CFSE/IFA ear-primed recipients, with or without 1 μg OVA Ag, and draining LNs were harvested 4 h after transfer and imaged by TPLSM. Average track velocity (A) and cell displacement (B) of individual videos are representative of at least five independent experiments. (C) Three-dimensional images and corresponding kymograph of a single CFSE-labeled DC (green) interacting with two wild-type (blue) and two ADAP<sup>−/−</sup> (red) DO11 T cells. Original magnification ×20 with ×4 scan zoom. (D) Kymograph of T cell–DC interactions with multiple DCs from a single acquired video. (E) Pooled cumulative T cell–DC contact time at 4 h after Ag challenge from three separate videos. (F) Pooled cumulative T cell–DC contact time at 4 h after challenge with a high dose of Ag (5 μg) from three separate videos. (G) Pooled cumulative T cell–DC contact time at 8 h after challenge with 1 μg OVA from three separate videos. *p < 0.05, unpaired t test.
(Fig. 4A). The slightly higher average velocity of the ADAP−/− T cells compared with wild-type T cells in the presence of Ag at the 24-h time point was not statistically significant. However, we continued to see differences in contact time with DCs between wild-type and ADAP−/− T cells at this later time point (Fig. 4B, Supplemental Video 4). Although the average contact time for wild-type T cells decreased from the earlier stable phase to 9.1 min, the average contact time for ADAP−/− T cells was even lower.

**FIGURE 4.** ADAP-deficient T cells are not confined near DC contact sites at the late swarming contact phase. CTV-labeled wild-type (blue) and CTO-labeled ADAP−/− (red) DO11.10 T cells (1 × 10⁶ cells each) were cotransferred into CFSE/IFA ear–primed recipients, with or without OVA Ag, and draining LNs were harvested 24 h after transfer and imaged by TPLSM. (A) Average track velocity of individual videos from three independent experiments. (B) Pooled cumulative T cell–DC contact time from three independent experiments. (C) Three-dimensional images of CFSE-labeled DCs (green) interacting with wild-type (blue) and ADAP−/− (red) DO11.10 T cells. The images show the initial T cell–DC contact, the contact termination or post–T cell–DC contact, and the end point displacement of the cell after 5 or 20 min. Original magnification ×20 with ×4 scan zoom. (D) The pooled CR of the tracks described in (C) from three independent experiments. *p < 0.05, **p = 0.00, unpaired t test.
(3.3 min) (Fig. 4B, Supplemental Video 4). Thus, although both wild-type and ADAP−/− T cells have transitioned to the late-stage swarming-type interactions exemplified by an increased velocity at 24 h, ADAP−/− T cells continue to establish more transient contacts with DCs than do wild-type T cells.

ADAP−/− T cells do not confine their movement around a DC

Additional analysis of the motility behavior of control wild-type DO11.10 T cells at the 24-h time point revealed that initial contact with a DC was followed by a confinement of the movement of the T cell to a region where the contact was terminated. Tracking analysis showed that a wild-type T cell interacted with a DC, then broke contact and migrated away, only to return to a region close to the DC (Fig. 4C, Supplemental Video 5). In contrast, ADAP−/− T cells generally migrated in a linear path away from the DC following initial contact (Fig. 4C, Supplemental Video 6). Because we could not determine the contact history of the T cell prior to its first observed contact with a DC, we analyzed T cell behavior only after the contact with the DC was observed. In addition, we focused our analysis on the first 5 min following termination of contact, because this was the minimum amount of time required for a T cell to return to a contact site. Many of the ADAP−/− T cells often migrated out of the field of view during the observation period, suggesting random walking behavior. When we applied these analysis parameters, we calculated the CR of the initial DC contact, where the total distance from the origin (terminated contact point) is divided by the total track length. Thus, a T cell moving in a straight line away from the origin will have a CR of 1 (32). When the 5-min postinitial DC contact tracks of wild-type T cells were analyzed, the mean CR was 0.488 ± 0.037. In contrast, ADAP−/− T cells had a significantly higher mean CR of 0.640 ± 0.048 (Fig. 4D). However, many wild-type T cells exhibited a more circuitous confinement and, thus, would often take longer than 5 min to return to the contact site (Fig. 4C, Supplemental Video 7). Thus, we also determined the CR at the end point of each track in the 30-min video. The end point CR was also higher for ADAP−/− T cells (0.523 ± 0.048) compared with wild-type T cells (0.316 ± 0.025) (Fig. 4D). Thus, ADAP−/− T cells exhibited less confinement to the regions in the LN where they had initially made contact with a DC.

ADAP-dependent regulation of CD4 T cell activation in response to Ag in vivo involves the SKAP55-binding region of ADAP

We previously demonstrated that different regions of ADAP control the regulation of integrin function and CARMA1-dependent signaling pathways following TCR stimulation (15, 16, 19, 23). To test the hypothesis that the impaired T cell–proliferative responses of ADAP−/− T cells in vivo are specifically due to ADAP-dependent regulation of T cell–DC contacts in vivo, we examined the functional effect of re-expressing wild-type ADAP and an ADAP mutant with a small deletion of the proline-rich region of ADAP (aa 338–358) in primary DO11.10 ADAP−/− T cells. The proline-rich region of ADAP mediates the association of ADAP with SKAP55, but it does not affect the TCR-inducible association of ADAP with CARMA1 and TAK1 (15, 16). As a result, expression of this ADAP mutant (ADAPΔ338) in ADAP−/− T cells does not restore the integrin-mediated T cell–APC conjugate defect, but it does restore ADAP-dependent signaling downstream of CARMA1 (16). We infected wild-type or ADAP−/− DO11.10 T cells expressing the hCAR transgene with a control adenovirus construct expressing a Thy1.1 marker gene alone or adenovirus constructs expressing Thy1.1 and either wild-type ADAP or the ADAPΔ338 mutant (Supplemental Fig. 2). We showed previously that expression of wild-type ADAP, but not the ADAPΔ338 mutant, in ADAP−/− T cells results in the rescue of endogenous SKAP55 expression (16, 17). These T cell populations were transferred together with wild-type DO11.10 T cells infected with the control adenovirus. This population served as an internal control in our experiments. Fig. 5A shows that ADAP−/− T cells infected with the control adenovirus retained an impaired proliferative response to OVA/IFA challenge in vivo compared with wild-type T cells expressing the control adenovirus. In contrast, expression of wild-type ADAP in ADAP−/− T cells resulted in Ag-dependent proliferation that was comparable to the wild-type control T cell population. This was not observed when we expressed the ADAPΔ338 mutant in ADAP−/− T cells. Similar results were observed when we examined Ag-dependent expression of PD-1 (Fig. 5A).

We also examined Ag-mediated changes in S6 ribosomal protein and c-jun phosphorylation in ADAP−/− T cells expressing either wild-type ADAP or the ADAPΔ338 mutant. Compared with the wild-type control T cell population in each recipient mouse, Ag challenge in vivo of ADAP−/− T cells expressing the control adenovirus or the ADAPΔ338 mutant resulted in impaired c-jun phosphorylation and S6 phosphorylation (Fig. 5B). In contrast, expression of wild-type ADAP restored c-jun and S6 phosphorylation after Ag challenge to levels similar to those seen in the control wild-type T cells. We further quantitated this result by normalizing phosphorylation to the response observed with the cotransfected wild-type T cells expressing the control adenovirus (Fig. 5C). Together, these results suggest a critical role for the SKAP55-binding site in ADAP in optimal T cell activation following Ag challenge in vivo.

The SKAP55-binding site in ADAP regulates stable T cell–DC contacts and T cell–DC contact time

We next examined the interaction dynamics of ADAP−/− T cells expressing wild-type ADAP or the ADAPΔ338 mutant. ADAP−/− T cells expressing either the control adenovirus or wild-type ADAP had a similar average velocity (~10 µm/min) in the absence of Ag (Fig. 6A). At the 4-h time point, Ag challenge resulted in a dramatic decrease in average velocity for both of these T cell populations. However, as observed with wild-type and ADAP−/− T cells, the control ADAP−/− T cells had a statistically higher average velocity than did ADAP−/− T cells expressing wild-type ADAP (5.6 µm/min versus 3.7 µm/min). ADAP−/− T cells infected with the control adenovirus exhibited a contact behavior similar to uninfected ADAP−/− T cells (Fig. 6B, Supplemental Video 8). These T cells typically engaged in short repetitive contacts at the periphery of the DC (red spot–marked cells). Reconstitution of ADAP−/− T cells with wild-type ADAP restored stable long-term contacts, because these T cells (marked with a blue dot) rarely detached from the DC during the 30-min imaging period. Furthermore, expression of wild-type ADAP in ADAP−/− T cells resulted in an increase in total mean T cell–DC contact time from 11.6 min for control infected ADAP−/− T cells to 24.5 min for ADAP−/− T cells expressing wild-type ADAP (Fig. 6A). These contact times are almost identical to what was observed when we analyzed wild-type and ADAP−/− T cells (Fig. 3E). Thus, expression of wild-type ADAP in ADAP−/− T cells recapitulates the stable contact behavior of wild-type CD4 T cells.

Analysis of ADAP−/− T cells expressing the ADAPΔ338 mutant revealed that, in the absence of Ag, ADAP−/− T cells expressing wild-type ADAP or the ADAPΔ338 mutant displayed similar mean velocities (Fig. 6C). In addition, both T cell populations had similar low mean velocities at the 4-h time point following Ag challenge. This suggests that the higher mean ve-
The SKAP55-binding region in ADAP regulates early T cell activation in vivo. Primary ADAP−/− DO11.10 hCAR T cells were transduced with control adenovirus expressing Thy1.1 or adenovirus expressing Thy1.1 and either wild-type ADAP or the ADAPΔ338 mutant, as described in Materials and Methods. ADAP−/− reconstituted cells were cotransferred with wild-type DO11.10 hCAR T cells transduced with control adenovirus expressing Thy1.1 into OVA/IFA-primed mice. (A) Proliferation in draining LNs was determined by CFSE (Ctrl + Thy; wild-type DO11.10 T cells transduced with control adenovirus expressing Thy1.1 and either wild-type ADAP or the ADAPΔ338 mutant) dilution 48 h after transfer. Cell samples were also stained for PD-1 expression. (B) Draining LNs were harvested at 4 h after transfer, fixed, and stained for phosphorylated c-Jun and S6. (C) Intracellular staining for phosphorylated c-Jun and S6 from three independent experiments, as in (B), normalized to the maximum response in each mouse (response of wild-type DO11.10 T cells transduced with control adenovirus). *p < 0.05 unpaired t test.

Inhibition of ICAM-1 reduces T cell contact time with Ag-laden DCs

To provide further evidence that the reduced interactions of ADAP−/− T cells with DCs is mediated by ADAP-dependent regulation of LFA-1, we examined the interaction of wild-type and ADAP−/− DO11.10 T cells with Ag-laden DCs in the presence of an inhibitory anti–ICAM-1 Ab. Because short-term trafficking of naive T cells into LNs would likely be impaired with ICAM-1 blockade, we waited for an hour after T cell transfer before injecting the anti–ICAM-1 Ab and assessed T cell–DC contacts at 4 h after T cell transfer. Treatment with the anti–ICAM-1 Ab resulted in individual wild-type T cells detaching from a DC during the imaging period (Fig. 7A, Supplemental Video 10). In contrast, stable interactions of wild-type T cells with DCs were observed in the presence of an isotype control Ab (Fig. 7B, Supplemental Video 11). ADAP−/− DO11.10 T cells exhibited unstable, transient interactions with a DC in the presence of either the isotype-control Ab or the anti–ICAM-1 Ab (Fig. 7A, 7B). ICAM-1 blockade resulted in a reduction in the average contact time of wild-type T cells with DCs from 21.6 min to 13.1 min (Fig. 7C). The total contact time of wild-type T cells with DCs in the presence of anti–ICAM-1 was slightly higher than the contact time of ADAP−/− T cells with DCs in the presence of an isotype control Ab (10.2 min), but the difference was not statistically significant. The difference in contact time between wild-type T cells in the presence of anti–ICAM-1 and ADAP−/− T cells was due to a subset of wild-type T cells that maintained stable contacts with DCs for the entire 30-min imaging period (Fig. 7C). Interestingly, we also observed that ICAM-1 blockade resulted in a statistically significant reduction in the total average contact time of ADAP−/− T cells with DCs from 10.2 min to 6.8 min. These results are consistent with the hypothesis that T cell–DC contacts are dependent on ADAP-mediated regulation of LFA-1.
Discussion

The ADAP-SKAP55-signaling module mediates “inside-out” signaling from the TCR to integrins, thereby providing a mechanism by which Ag recognition can lead to enhanced integrin-dependent contacts between T cells and DCs. In this study, we demonstrate that the ADAP-SKAP55-signaling axis is critical for the dynamic motility and early (4 h) stable contacts between CD4 T cells and DCs, as well as the later (24 h) “swarming” contact phase and T cell confinement to DC contact sites during swarming. The changes in T cell contact interactions with DCs in the absence of
ADAP are associated with impaired early T cell–activation responses. Thus, ADAP-SKAP55 represents a key signaling pathway that controls the stability and duration of contact between CD4 T cells and DCs during the initial phases of T cell activation.

Other studies also demonstrated that cell contact duration affects the quality and magnitude of an immune response. The loss of germinal center responses in mice lacking the adapter protein SAP is due to impaired duration of interactions between activated SAP-deficient CD4 T cells and activated B cells (33). Although our work suggests that ADAP-SKAP55 controls cell contact dynamics during the early phases of activation of naïve CD4 T cells via regulation of integrin function, activated CD4 Th cells appear to use a distinct integrin-independent mechanism of adhesion to interact with activated B cells (34).

Imaging studies also provided evidence that inhibitory receptors, such as CTLA-4 (9) and PD-1 (8), attenuate T cell responses by inhibiting the duration of contact between T cells and DCs. Regulatory T cells have also been proposed to inhibit CD4 and CD8 T cell activation by disrupting the interaction of these T cells with DCs (22). Tolerogenic activation conditions were reported to result in more transient T cell contacts with DCs compared with T cells activated under immunogenic conditions (24, 35), although this difference in interaction dynamics between these distinct types of activation conditions has not been observed consistently (36).

Our studies are consistent with a model in which optimal CD4 T cell activation involves an early phase of ADAP-SKAP55-dependent stable contact with a DC. We suggest that this stable contact phase is initiated by TCR signals received during early transient contacts that result in increased functional activity of LFA-1, resulting in the ability of LFA-1 to mediate more stable contacts with DCs after a few hours of stimulation. In the absence of the ADAP-SKAP55 complex, T cell contacts with DCs remain transient, and the cumulative signals received by the TCR remain suboptimal during this critical early phase of T cell activation. Our analysis of the interaction of wild-type T cells with DCs in the presence of ICAM-1 blockade is consistent with this model, because the presence of the anti–ICAM-1 Ab reduced the contact time of wild-type T cells with DCs to a level similar to that seen with ADAP2/2 T cells. It is interesting to note that ICAM-1 blockade also caused a further decrease in the total contact time of ADAP2/2 T cells with DCs. This suggests the possibility that an additional pathway that regulates T cell–DC contacts in vivo is independent of ADAP but still dependent on ICAM-1. Our previous studies of T cell interactions with APCs in vitro also suggested the existence of an ADAP-independent pathway of integrin-mediated T cell adhesion. Similar to what we observed in these in vivo studies, ADAP2/2 DO11.10 T cells exhibit impaired adhesion to APCs in vitro. However, Ab-mediated inhibition of LFA-1 further inhibits conjugate formation between ADAP2/2 T cells and APCs (13).

There also appears to be additional heterogeneity in the interaction dynamics of T cells with DCs in vivo that was revealed by our study of ADAP2/2 T cells. In our analysis of T cell–DC contact time, we observed that the total average contact time of individual ADAP2/2 T cells with DCs was either very low or, less frequently, very high. This bimodal distribution of contact times

FIGURE 7. Inhibition of ICAM-1 reduces T cell contact time with Ag-laden DCs. CTV-labeled wild-type (blue) and CTO-labeled ADAP2/2 (red) DO11.10 T cells were cotransferred into CFSE/IFA ear–primed recipients with OVA Ag. One hour after T cell transfer, 200 μg of anti–ICAM-1 Ab or isotype-control Ab was administered by i.p. injection. Draining LNs were harvested 3 h after Ab treatment and imaged by TPLSM. Three-dimensional images and corresponding kymographs of a single DC interacting with transferred T cells in the presence of anti–ICAM-1 Ab (A) or isotype-control Ab (B). Colored spots match cell images to contacts on the kymograph. Original magnification ×20 with ×4 scan zoom. (C) Pooled cumulative T cell–DC contact time from three separate videos. *p < 0.05, unpaired t test.
was much more pronounced for ADAP−/− T cells than for wild-type T cells, and it was observed at 4 and 8 h after transfer and at low and high Ag doses. This result indicates that some T cells are able to maintain stable contacts with DCs in the absence of ADAP. Because our experimental system used a method of labeling endogenous DCs, it is possible that T cell interactions with different types of DCs may be differentially dependent on ADAP. Identification of molecular mediators that control T cell–DC contacts in vivo independently of ADAP will be an important area of future investigation.

Another hallmark of these early interaction dynamics is the significant reduction in T cell velocity that occurs in response to Ag. Similar to wild-type T cells, ADAP-deficient T cells exhibit this Ag-dependent reduction in velocity. TCR-mediated increases in intracellular calcium have been associated with this reduction in T cell motility (37, 38). Previous work showing normal calcium flux in TCR-stimulated ADAP−/− T cells (11) is consistent with ADAP not being a major regulator of this Ag-dependent reduction in motility. Although ADAP−/− T cells reduced their velocity in response to Ag, the overall reduction in average velocity was always less pronounced for the ADAP−/− T cells. ADAP appears to be critical for this difference in velocity, because ADAP−/− T cells expressing wild-type ADAP show a decrease in velocity in response to Ag that is similar to control wild-type T cells. ADAPΔD24 T cells expressing the ADAPΔ338 mutant also have a reduction in velocity similar to wild-type T cells, suggesting that a region of ADAP distinct from the SKAP55-binding site controls this differential velocity reduction in response to Ag.

At the later 24-h time point, T cells regain their motility and “swarm” around DCs with generally short-lived contacts. The role that this behavior has in promoting T cell activation remains unclear, although T cell signaling that occurs at this later time point was proposed to be important for cell cycle entry, sustained CD25 expression, and delayed type hypersensitivity responses (39, 40). The ADAP-SKAP55–signaling module also plays a role in regulating T cell contact behavior at this later time point. Although ADAP−/− T cells also resume their motility and engage DCs with transient contacts after 24 h, ADAP−/− T cells still exhibited reduced total cumulative contact time at this stage compared with wild-type T cells. Furthermore, ADAPΔD24 T cells exhibited reduced swarming. Because both T cell populations were moving at equal relatively fast velocities, we were able to compare how each of the cell types could “swarm” back to a DC after termination of the initial contact. This analysis revealed that ADAP−/− T cells showed significantly less confinement and more straight-line movement after initial DC encounter. The mechanism by which ADAP controls this “swarming” behavior is not clear. It is possible that this swarming requires T cells to receive a certain threshold of TCR signal that is not obtained by ADAP−/− T cells, as a result of the reduced ability of T cells to contact DCs in the absence of ADAP. Chemokines might also play a role in T cell swarming around DCs at the 24-h time point. Thus, it is possible that early signals received by a T cell that lead to expression of certain chemokine receptors may not occur in the absence of ADAP. Alternatively, ADAP might regulate the response of T cells to chemokine-mediated signals involved in T cell–DC interaction dynamics. CCR7-mediated regulation of LFA-1 was recently reported to be regulated by the ADAP-SKAP55–signaling module (30). Although this study reported a lower homeostatic velocity of naive T cells in LNs, we did not observe any difference in the basal velocity of naive ADAP−/− CD4 T cells compared with wild-type naive CD4 T cells. The reason for this discrepancy is not clear, although we note that the absolute velocity rates reported for wild-type and ADAP−/− CD4 T cells by Kliche et al. (30) in popliteal LNs are higher than we observed in our analysis of inflamed cervical LNs.

ADAP is a multifunctional adapter that exists in distinct biochemical pools defined by the association of a subset of ADAP molecules in a T cell with SKAP55. The pool of ADAP that is not associated with SKAP55 facilitates the TCR-inducible assembly of the CARMA1-Bcl10-Malt1 signalosome (15–17). Because the SKAP55 binding site in ADAP is not required for ADAP-dependent regulation of CARMA1-dependent signaling pathways (16), expression of the ADAPΔ338 mutant of ADAP in ADAP−/− T cells restores CARMA1-dependent signaling responses, such as NF-κB activation, but it does not result in restoration of optimal inside-out signaling from the TCR to integrins. We previously found that stimulation of purified ADAP−/− T cells with anti-CD3 and anti-CD28 Abs, an activation protocol that does not require integrin-dependent interactions with APCs, results in impaired c-jun phosphorylation that is dependent on the pool of ADAP that interacts with CARMA1 and not the ADAP-SKAP55–signaling module (19). However, in the context of an in vivo Ag challenge that is dependent on efficient T cell–DC interactions regulated by ADAP, we found that Ag-mediated phosphorylation of c-jun is dependent on the ADAP-SKAP55–signaling module. This suggests that global TCR signaling is likely impaired as a result of the disruption of stable contacts of ADAP−/− T cells with DCs in vivo.

In summary, we showed that optimal Ag-specific activation of CD4 T cells in vivo is dependent on ADAP-SKAP55–regulated stable contact of activated CD4 T cells with DCs and swarming of CD4 T cells around DCs. Thus, ADAP-SKAP55 represents a key signaling pathway that controls the stability and duration of contact between CD4 T cells and DCs during the initial phases of T cell priming, which ultimately dictates optimal T cell activation.

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Disclosures

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References


