MOLECULAR AND CELLULAR BIOLOGY, Oct. 2007, p. 6863–6875 Vol. 27, No. 19
0270-7306/07/$08.00+0 doi:10.1128/MCB.00556-07

Functional Defects of SKAP-55-Deficient T Cells Identify a Regulatory Role for the Adaptor in LFA-1 Adhesion▽

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Received 29 March 2007/Returned for modification 1 May 2007/Accepted 17 July 2007

The ADAP–SKAP-55 module regulates T-cell receptor (TCR)-induced integrin clustering and adhesion in T cells. However, it has been unclear whether ADAP and/or SKAP-55 is an effector of the response. ADAP controls SKAP-55 expression such that *ADAP*$^{-/-}$ T cells are also deficient in SKAP-55 expression. In this study, we report the phenotype of the SKAP-55-deficient mouse. *SKAP-55*$^{-/-}$ T cells retain ADAP expression yet show defects in β1 and β2 integrin adhesion, leukocyte function-associated antigen 1 (LFA-1) clustering, production of the cytokines interleukin-2 and gamma interferon, and proliferation. This dependency was also reflected in more-transient conjugation times in response to the superantigen staphylococcal enterotoxin A on dendritic cells and a reduced number of cells with TCR/CD3 microcluster localization at the immunological synapse. *SKAP-55*$^{-/-}$ T cells showed the same general impairment of function as *ADAP*$^{-/-}$ T cells, indicating that SKAP-55 is an effector of the ADAP–SKAP-55 module. At the same time, the requirement for ADAP and SKAP-55 was not absolute, since a subset of peripheral T cells adhered with loss of expression of either adaptor. Further, dependency on SKAP-55 or ADAP differed with the strength of the TCR signal. As with the *ADAP*$^{-/-}$ mouse, SKAP-55-deficient mice showed no major effects on lymphoid development or the appearance of peripheral T cells, B cells, and NK cells. Our findings identify a clear effector role for SKAP-55 in LFA-1 adhesion in peripheral T cells and demonstrate that dependency on SKAP-55 and ADAP differs among T cells and differs with the strength of the TCR signal.

T-cell receptors (TCRs) undergo microclustering and supramolecular activation cluster formation at the immunological synapse (IS) during conjugation between T cells and antigen-presenting cells (APCs) (5, 21, 35). Microclustering can in turn activate GTP-binding proteins, protein kinases, phosphatases, and the phosphorylation of adaptor proteins. CD4- and CD8-p56lck activation leads to immunoreceptor tyrosine-based activation motif phosphorylation on the CD3 and TCRζ chains, the recruitment of ZAP-70 (zeta-associated protein-tyrosine kinase of 70 kDa), and the activation of TEC kinases ITK/RLK (interleukin-2-inducible/resting lymphocyte kinase) (1, 31–33, 41). Adaptor proteins possess binding sites and domains needed for complex-complex formation (31, 32, 41). Immune cell-specific adaptors include LAT (linker for activation of T cells), GADS (Grb-2-like adaptor downstream of Shc), SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), ADAP (adhesion- and degranulation-promoting adaptor protein; previously known as FYN T-binding protein/SLP-76-associated protein [FYB/SLAP]), and SKAP-55 (Src kinase-associated phosphoprotein of 55 kDa; also known as SCAP1) (3, 23, 24, 26, 31, 32, 40–42). Phosphorylation of LAT recruits phospholipase Cγ1, Grb-2, and GADS–SLP-76 and induces Ca^{2+} mobilization and cytokine transcription (32, 33, 41, 44).

Binding of leukocyte function-associated antigen 1 (LFA-1; also known as CD11/CD18 or $\alpha_L\beta_2$) to intercellular adhesion molecules 1 and 2 (ICAM-1 and -2) on APCs mediates T-cell–APC conjugation (5, 8, 11, 21, 37). Following initial adhesion, TCRζ/CD3 ligation induces signals (i.e., "inside-out signaling") that further activate integrin adhesion (2, 5, 8, 11, 37). Conversion of LFA-1 to intermediate- or higher-affinity forms involves changes in conformation and receptor clustering (11, 37). Multiple signaling proteins mediate this process. They include the GTP-binding protein Rap-1, its ligand RapL (regulator of cell adhesion and polarization enriched in lymphoid tissues), RIAM (Rap1-GTP-interacting adaptor molecule), the guanine nucleotide exchange factor Vav-1, and the adaptors SLP-76, ADAP, and SKAP-55 (6, 10, 13, 15, 16, 17, 18, 20, 22, 27, 28, 34). The protein-tyrosine kinase ZAP-70 phosphorylates YESP sites in SLP-76, which allows binding to the Src homology 2 (SH2) domain of Vav-1 (29), while the SH2 domain of SLP-76 binds to two YDDV sites in ADAP (30, 39). T-cell lines lacking SLP-76 show impaired superantigen-induced conjugation (44).

ADAP is an immune cell-specific adaptor with a unique N-terminal region, a proline-rich region, a canonical and a noncanonical SH3 domain, one Ena/VASP homology 1 (EVH1) binding domain, and two putative nuclear localization motifs (3, 4, 19, 26, 31). ADAP is preferentially phosphorylated by the Src kinase $p59^{fynT}$ (4, 25) and can cooperate with $p59^{fynT}$ and SLP-76 in amplifying TCR-induced interleukin-2 (IL-2) transcription (30). The adaptor can up-regulate integrin-mediated adhesion in certain basophilic cell lines (9), while *ADAP*$^{-/-}$ T cells show profound defects in β1 and β2 integrin clustering and adhesion (10, 27). Binding to SLP-76 is needed,

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▽ Published ahead of print on 23 July 2007.

as evidenced by the fact that mutation of the SLP-76 binding sites in ADAP impairs conjugation, supramolecular activation cluster (SMAC) formation, and cytokine production (39). The current model proposes that $p59^{fynT}$ phosphorylates YDDV sites in ADAP that interact with SLP-76 SH2 domains, followed by complex recruitment to LAT via binding of the adaptor GADS to a noncanonical motif in SLP-76 (31, 32, 44).

SKAP-55, another immune cell adaptor, is the main binding partner for ADAP in T cells (14, 23, 24). It is enriched in T cells and possesses a unique N-terminal region followed by a pleckstrin homology domain and a C-terminal SH3 domain (23, 24). SKAP-55 binds with high stoichiometry to ADAP (4, 25), an interaction that is mediated by binding of the SKAP-55 SH3 domain to a proline-rich region in ADAP (23, 25) and by weaker binding of the ADAP SH3c domain to a tyrosine-based RKXXYXXY motif in SKAP-55 (7, 14). SKAP-55 may also interact with the D1 domain of the phosphatase CD45 (43). We have previously shown that SKAP-55 is capable of enhancing TCR-mediated activation of β1 and β2 integrins and of forming conjugates between T cells and APCs (40) and that small interfering RNA-mediated knockdown of SKAP-55 impairs conjugate formation and LFA-1 clustering in mouse T cells (13). This effect occurred under conditions of normal expression of the homologue SKAP-55-related (SKAP-55R) or SKAP-55Hom, indicating a nonredundant function for SKAP-55 in T cells (13). An interaction between ADAP and SKAP-55 is needed for adhesion, since the loss of the SKAP-55 SH3 domain or the ADAP SH3c domain binding sites on SKAP-55 ablates adhesion and conjugate formation (7, 18, 40). ADAP–SKAP-55 may facilitate the translocation of Rap1 to membranes (18).

While ADAP–SKAP-55 comprises a module for integrin adhesion, it has been unclear whether ADAP and/or SKAP-55 is directly responsible for this function (10, 12, 18, 27). An important recent observation has been that ADAP modulates SKAP-55 protein turnover and degradation such that ADAP-deficient cells show a major loss of SKAP-55 expression (12). In this study, we report the phenotype of the SKAP-55-deficient mouse, where T cells lack SKAP-55 but retain ADAP expression. Despite this, SKAP-55-deficient T cells show major defects in integrin adhesion, cytokine production, and proliferation comparable to those for ADAP-deficient T cells. Dwell times of SKAP-55-deficient T cells in response to staphylococcal enterotoxin A (SEA) presented by dendritic cells (DCs) were shorter concurrent with reduced localization of the TCR/CD3 complex at the IS and reduced proliferation. However, the requirement for ADAP–SKAP-55 was not absolute. Adhesion assays and cell cycle analysis identified a subset of T cells that adhere without the need for the adaptors, a dependency that differs with the strength of the TCR signal. Our studies identify an effector function for SKAP-55 that operates independently of ADAP.

MATERIALS AND METHODS

SKAP-55 and ADAP KO mice. SKAP-55 knockout (KO) mice were generated in collaboration with Lexicon Genetics Ltd. (Texas), and the mutagenesis method was based on gene trapping, which allows the automated identification of sequence tags from the mutated genes, as described previously (45). Once germ line transmission had been validated, *SKAP-55*$^{+/-}$ animals were mated with animals of a C57JBL/6 background, resulting in a 129Sv/lex × C57JBL/6 hybrid background. The SKAP-55 KO line was backcrossed to C57JBL/6 for subsequent progeny. Age- and sex-matched mice from the same litters were used in our experiments. ADAP-deficient mice were kindly provided by Eric Peterson (University of Minnesota).

Cell culture and antibodies. Primary T cells from *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ mice were cultured in RPMI 1640 medium with 10% (vol/vol) fetal calf serum (FCS), 5% (vol/vol) glutamine, 5% (vol/vol) penicillin-streptomycin, and 5×10^{-5} M 2-mercaptoethanol. CD4-positive T cells were purified using anti-CD4 beads as previously described (40). For the generation of bone marrow DCs (BMDCs), bone marrow was flushed from femurs and passed through a 200-μm mesh to remove fibrous tissue, and red blood cells were lysed using ammonium chloride lysis buffer. Cells were cultured at 10^6/ml in RPMI medium supplemented with 10% FCS, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor, and 1,000 IU/ml IL-4. On day 5 of culture, floating cells were gently removed and fresh medium containing recombinant murine granulocyte-macrophage colony-stimulating factor was added. On day 7 of culture, BMDCs were induced to mature by addition of 1 μg/ml lipopolysaccharide to the cultures. After overnight incubation, nonadherent cells and loosely adherent proliferating BMDC aggregates were collected, washed, and replated for 1 h at 37°C to remove contaminating macrophages. DCs were then plated with various concentrations of SEA for 2 to 3 h prior to coculture with T cells for proliferation, cytokine, contact time, and imaging analysis. The contact time of individual T-cell–APC conjugates was followed over 1,800 s using MetaMorph imaging software.

Monoclonal antibodies against ADAP and SKAP-55 were purchased from Transduction Laboratories (San Diego, CA), while unconjugated or conjugated antibodies against CD11a (LFA-1, αL chain), CD18 (LFA-1, β2 chain), CD4, CD8, CD44, CD25, CD29 (anti-integrin β1 antibody), CD69, DX5, TCR, and B220 were from Pharmingen (Oxford, United Kingdom). Unconjugated antibodies against mouse CD3 (2C11; hamster anti-mouse CD3) or phosporylated tyrosine (4G10) were prepared and purified by Bioexpress. Alexa Fluor 546- or Alexa Fluor 633-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). Five-domain murine ICAM-1 Fc was purchased from R&D Systems. Antiactin was from Sigma, and an antibody against extracellular signal-regulated kinases 1 and 2 (anti-ERK1/2) was purchased from Cell Signaling [New England Biolabs (UK) Ltd., Hertfordshire, United Kingdom]. Anti-phospho-ERK1/2 T202/Y204 or T185/Y187 was purchased from BioSource United Kingdom (Nivelles, Belgium).

Intracellular staining and CFSE labeling. Intracellular staining and flow cytometry were used to measure the level of antibody against gamma interferon (anti-IFN-γ). After anti-CD3 stimulation, cells were fixed in 4% paraformaldehyde (Sigma) and permeabilized with 0.3% saponin (Sigma), followed by a second staining with allophycocyanin-tagged anti-IFN-γ in saponin containing phosphate-buffered saline (PBS)-bovine serum albumin (BSA). Cells were fixed and analyzed using a FACSCalibur flow cytometer. For carboxyfluorescein diacetate succinimide ester (CFSE) labeling, 5×10^6 cells were washed twice and resuspended in 250 μl of staining buffer (PBS supplemented with 0.1% BSA). A 250-μl volume of freshly diluted 2 μM CFSE (Molecular Probes) was added, and cells were incubated at room temperature for 10 min. An equal volume of FCS was added to stop labeling. After three washes, cells were activated with anti-CD3 for 72 h with a culture medium containing 10% FCS. Cells were harvested and stained with allophycocyanin-conjugated anti-CD4, followed by gating of CD4-positive cells for detection of CFSE fluorescence on a FACSCalibur system (BD Biosciences). Data were analyzed using CellQuest (BD Biosciences).

PCR. PCR amplification was performed in a 50-μl reaction mixture using 1 μl cDNA with 25 μl of 2× *Taq* master mix and reverse primer B with oligonucleotide primer LTR or primer A (0.5 μM each). The cycling parameters were as follows: 5 min at 95°C for 1 cycle; 1 min at 94°C, 1 min at 65°C, and 1 min and 30 s at 72°C for 10 cycles, with the annealing temperature decreasing 1°C in each cycle; 1 min at 94°C, 1 min at 55°C, and 1 min and 30 s at 72°C for 28 cycles; and a final extension at 72°C for 10 min. After PCR, the amplification reaction product was analyzed on a 2% agarose-ethidium bromide gel.

Immunoblotting. For immunobotting, 4×10^6 cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 (vol/vol) in 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_4VO_3, 10 mM NaF, and 1 mM $Na_4P_2O_7$ as described previously (29). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were then blocked with 5% skim milk in TBS (10 mM Tris-HCl [pH 7.6], 150 mM NaCl) and incubated for 1 h with the indicated antibodies. Levels of bound antibody were then measured using a horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody, fol-

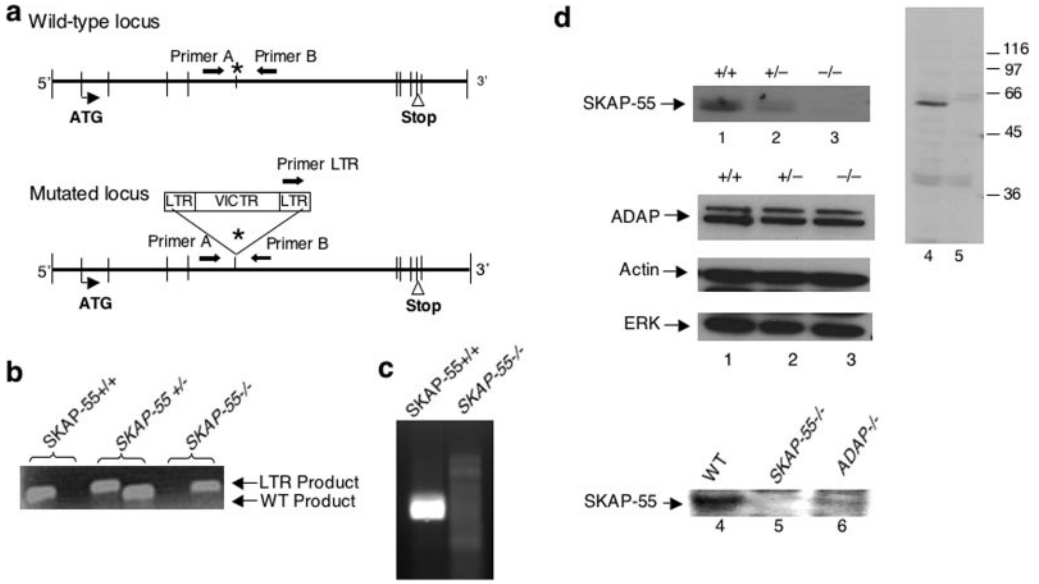

FIG. 1. SKAP-55-deficient mice lack SKAP-55 expression. (a) Site targeted vector used in the generation of *SKAP-55*$^{-/-}$ mice. SKAP-55 KO mice were generated as described previously (45). (b) PCR analysis of *SKAP-55*$^{-/-}$ mice. PCR analysis identified *SKAP-55*$^{+/+}$ mice with a wild-type (WT) band, *SKAP-55*$^{-/-}$ mice with an LTR band, and *SKAP-55*$^{+/-}$ mice with both WT and LTR bands. (c) The loss of SKAP-55 expression in SKAP-55 KO mice was confirmed by full-length SKAP-55 mRNA detection for the WT and KO mice by reverse transcription-PCR. (d) ADAP is expressed in *SKAP-55*$^{-/-}$ and *SKAP-55*$^{+/-}$ T cells. Cell lysates were blotted with various antibodies. (Top panels) Anti-SKAP-55. Lanes 1 and 4, WT (*SKAP-55*$^{+/+}$); lane 2, heterozygote (*SKAP-55*$^{+/-}$); lanes 3 and 5, homozygote (*SKAP-55*$^{-/-}$). Anti-N-terminal SKAP-55 antibodies were used in lanes 1 to 3, and anti-C-terminal SKAP-55 antibodies were used in lanes 4 and 5. (Center panels) Anti-ADAP, antiactin, and anti-ERK blots. Lanes 1, WT (*SKAP-55*$^{+/+}$); lanes 2, heterozygote (*SKAP-55*$^{+/-}$); lanes 3, homozygote (*SKAP-55*$^{-/-}$). (Bottom panel) Anti-SKAP-55 blot. Lane 4, WT T cells; lane 5, *SKAP-55*$^{-/-}$ T cells; lane 6, *ADAP*$^{-/-}$ T cells. *ADAP*$^{-/-}$ T cells show a loss of SKAP-55 expression.

lowed by detection with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

LFA-1 adhesion and clustering assays. Anti-CD3-stimulated or unstimulated T cells were added to plates coated with recombinant murine ICAM-1 (R&D Systems) and incubated for 30 min at 37°C. Nonadherent cells were gently washed off, and the remaining cells were counted as previously described (39). LFA-1 clustering/capping experiments were conducted as follows. Briefly, cells were incubated with 5 μg/ml anti-CD3 (clone 145-2C11) on ice for 30 min. Cells were then washed twice with cold medium and cross-linked by incubation with 10 μg/ml of a rabbit anti-hamster immunoglobulin G antibody (Jackson Immunoresearch) at 37°C for 30 min. Cells were fixed with 2% paraformaldehyde for 20 min, washed with PBS, and blocked with 3% BSA–5% FCS–PBS for 45 min. Samples were then stained for anti-CD11a and an isotype-specific Alexa Fluor 546-conjugated anti-rat antibody as described elsewhere (39). At least 50 T cells were counted for cap formation in each experiment. These experiments were conducted using four different SKAP-55 KO mice. Immunofluorescence microscopy was conducted as described previously (39).

IL-2 ELISA and T-cell proliferation assay. *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ primary mouse cells (2×10^5/well) were cultured in 2 μg/ml anti-CD3 (145-2C11) applied to plates for 48 h. The supernatants were collected, and IL-2 was measured by an enzyme-linked immunosorbent assay (ELISA) with a rat anti-mouse IL-2 monoclonal antibody (ELISA capture) and a biotinylated rat anti-mouse IL-2 monoclonal antibody (ELISA detection). For the T-cell proliferation assay, cells were stimulated with 1 μg/ml plated anti-CD3 for various times (e.g., 48 h). To assess proliferation, cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham Biosciences) for the last 12 h. Cells were collected on GSC filters (PerkinElmer, Wallace Dy, Finland). Radioactivity was measured in a microplate scintillation counter.

Statistical analysis. Results are given as means ± standard deviations. The difference between means was tested either by an unpaired Student *t* test (for two means) or by one-way analysis of variance with Dunnett's posttest (multiple test) using GraphPad Prism, version 3.02 for Windows (GraphPad Software, San Diego, CA). A P value of <0.05 was considered significant.

RESULTS

SKAP-55-deficient mice were generated using a gene-trapping approach that allows the identification of sequence tags from the mutated genes. *SKAP-55*$^{+/-}$ animals were then mated with C57JBL/6 mice to produce 129Sv/lex × C57JBL/6 hybrids. Mice have been backcrossed for more than six generations in this study. Age- and sex-matched mice from the same litters were used in our experiments. PCR was used to genotype SKAP-55 wild-type (*SKAP-55*$^{+/+}$), heterozygous (*SKAP-55*$^{+/-}$), and KO (*SKAP-55*$^{-/-}$) mice. As shown in Fig. 1a and b, upstream primer A, downstream primer B, and primer LTR were designed to amplify the wild-type sequence and the inserted trapping cassette. PCR analysis identified *SKAP-55*$^{+/+}$ mice with one wild-type band, *SKAP-55*$^{-/-}$ mice with one LTR band, and *SKAP-55*$^{+/-}$ mice with wild-type and LTR bands (Fig. 1b). The loss of SKAP-55 expression in SKAP-55 KO mice was confirmed by full-length SKAP-55 mRNA detection in wild-type and KO mice by reverse transcription-PCR (Fig. 1c) and by anti-SKAP-55 blotting using both C-terminal and N-terminal monoclonal antibodies (Fig. 1d, top panels, lanes 1 and 3 versus lanes 4 and 5). Significantly, SKAP-55-deficient T cells retained ADAP expression at wild-type levels (Fig. 1d, second panel from top, lanes 2 and 3 versus lane 1). Expression of ERK and actin served as positive controls (Fig. 1d). By contrast, and in agreement with a previous report (12), *ADAP*$^{-/-}$ T cells showed a loss of SKAP-55 expression, as shown by immunoblotting with anti-SKAP-55 (Fig. 1d, bottom panel, lane 6 versus lanes 4 and 5). Our finding that the loss of

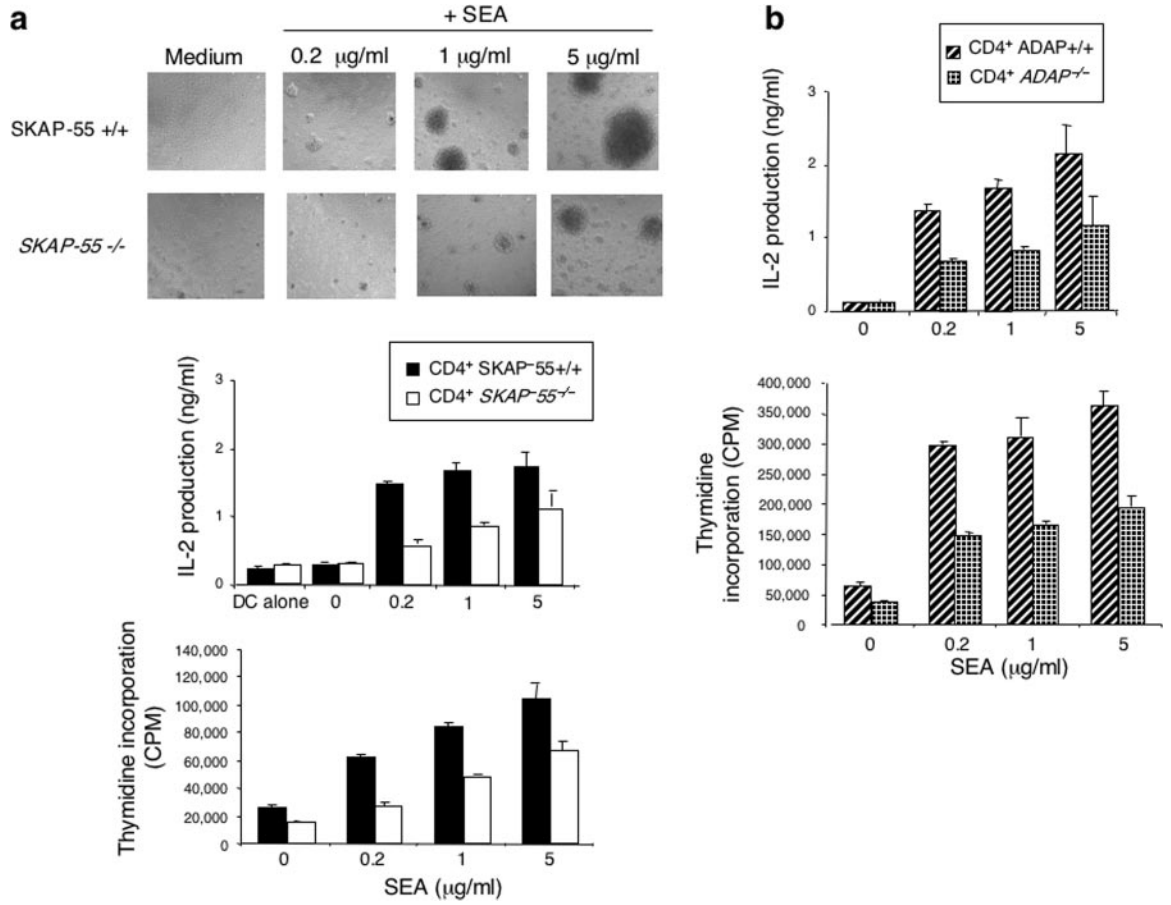

FIG. 2. SKAP-55-deficient T cells show defects in SEA-induced T-cell–DC aggregation. (a) Aggregates of SKAP-55-deficient T cells. Freshly isolated *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells were exposed to various concentrations of SEA (0 to 5 μg/ml) with DCs and were monitored by light microscopy for aggregation (upper panels), IL-2 production (upper graph), and proliferation (lower graph). (b) Freshly isolated *ADAP*$^{+/+}$ and *ADAP*$^{-/-}$ T cells were exposed to various concentrations of SEA (0 to 5 μg/ml) and monitored for IL-2 production (upper graph) and proliferation (lower graph) at 48 h. Data are means from three replicate wells. Error bars, standard errors of the means. A P value of <0.05 was considered significant.

SKAP-55 does not affect ADAP expression allowed an evaluation of SKAP-55 function in primary cells independent of ADAP expression.

We next assessed the responses of SKAP-55-deficient T cells to antigen as presented by APCs (Fig. 2). Purified CD4-positive T cells were cocultured with isolated mature DCs in the presence of the superantigen SEA, followed by assessment of aggregation. Aggregates were first observed at 12 to 24 h, with maximum levels at 45 to 60 h. As seen in Fig. 2a, the addition of SEA induced a concentration-dependent increase in the formation of aggregates at 24 h. Both the number and the size of the aggregates changed with increasing concentrations of SEA from 0.2 to 5 μg/ml (Fig. 2a, upper panels). By contrast, SKAP-55-deficient T cells showed smaller aggregates over the full range of SEA concentrations (Fig. 2a, lower panels). On average, the SKAP-55-deficient aggregates were 5- to 10-fold smaller than those for wild-type T cells. The addition of an anti-LFA-1 antibody prevented aggregate formation (data not shown). Consistent with reduced adhesion, IL-2 production and proliferation were impaired in SKAP-55-deficient T cells (Fig. 2a, upper and lower graphs, respectively). The response was impaired at similar levels in *ADAP*$^{-/-}$ T cells in terms of cell aggregation (data not shown), IL-2 production (Fig. 2b, upper graph), and proliferation (Fig. 2b, lower graph). These observations provided initial evidence that SKAP-55 is needed for the optimal ability of T cells to form aggregates and to respond to SEA.

To extend this, the interaction time of T cells with DCs was measured in the absence and presence of SEA (Fig. 3a). Contact times were monitored for 1,800 s, as outlined in Materials and Methods. SEA induced a shift from short-term to longer-term contacts of *SKAP-55*$^{+/+}$ T cells with DCs (Fig. 3a, left histogram), as reported previously (8, 21, 35). By comparison, CD4^{+} *SKAP-55*$^{-/-}$ T cells showed much shorter dwell times in the absence of SEA than wild-type cells. Although SEA caused a shift to longer contact times, these dwell times were still more transient than those observed for *SKAP-55*$^{+/+}$ T cells. This difference was especially evident when the number of conjugates at >1,800 s was measured (Fig. 3a, right histogram). In the absence of SEA, 60 to 65% of wild-type cells interacted with APCs longer than 1,800 s, while in the presence of SEA, more than 91% of cells formed longer-term contacts. By con-

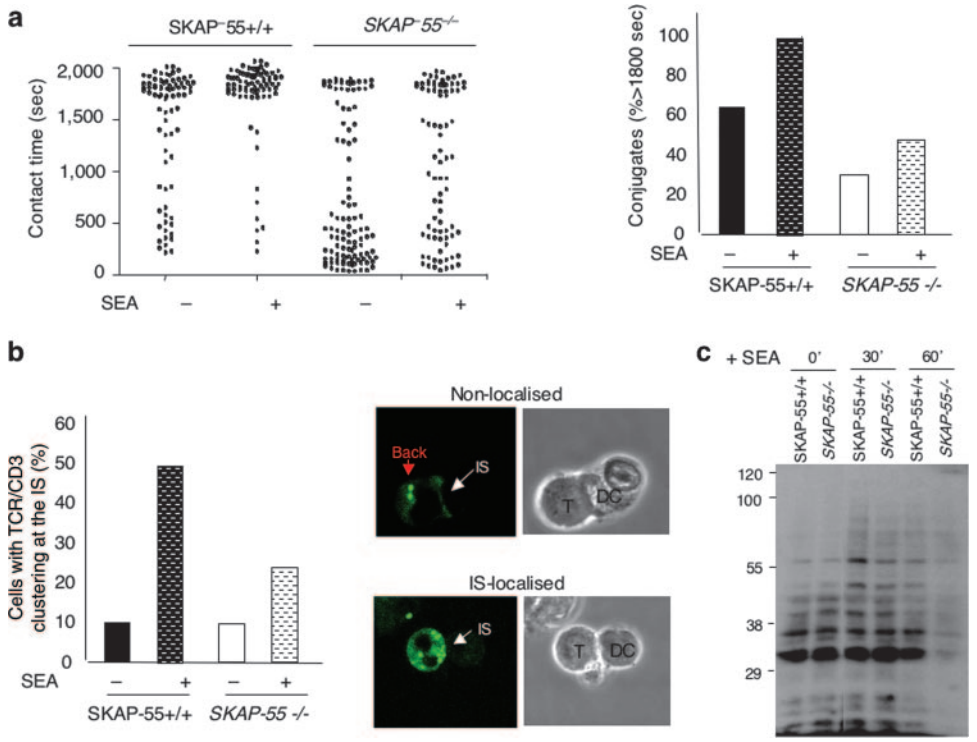

FIG. 3. SKAP-55-deficient T cells show defects in SEA-induced T-cell–DC conjugation and in the localization of TCR/CD3 at the IS. (a) $CD4^+$ *SKAP-55*$^{-/-}$ T cells show more-transient interactions between T cells and DCs. Purified $CD4^+$ T cells were incubated with mature DCs that had been preloaded with SEA. (Left) Dot plot analysis of T-cell interactions with DCs. (Right) Histogram showing the percentages of *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T-cell–DC conjugates of >1,800 s. (b) $CD4^+$ *SKAP-55*$^{-/-}$ T cells show defects in the movement of the TCR/CD3 complex to the IS. Purified $CD4^+$ T cells were incubated with mature DCs that had been preloaded with SEA. The localization of CD3 was imaged after staining with fluorescein isothiocyanate-conjugated anti-CD3. (Left) Histogram showing the percentages of T cells with CD3 localized at the IS. (Top right) Examples of nonlocalized CD3. (Bottom right) Examples of CD3 localized microclusters at the IS. (c) SKAP-55-deficient cells show a general reduction in tyrosine phosphorylation. Wild-type or SKAP-55-deficient splenocytes were incubated with 5 μg/ml SEA for 0, 30, or 60 min at 37°C. Cell lysates were prepared, followed by immunoblotting with antibody 4G10.

trast, only 35% of resting and 45 to 50% of SEA-exposed $CD4^+$ *SKAP-55*$^{-/-}$ T cells showed longer dwell periods. SKAP-55 therefore influences the duration of contact between T cells and APCs in antigen-dependent and -independent contexts. Further, these shorter contact times paralleled the reduced proliferation and reduced IL-2 production of *SKAP-55*$^{-/-}$ cells (Fig. 2a).

Shorter interaction times between T cells and APCs were also reflected in a reduction in the number of cells with localized CD3 at the IS (Fig. 3b). T-cell–DC conjugates that formed after a 15-min exposure to SEA were stained with fluorescein isothiocyanate-conjugated anti-CD3 and examined for the localization of TCR/CD3 at the IS. While the number of cells with CD3 microclusters at the IS increased with the addition of SEA from 8 to 50% (Fig. 3b, left histogram), a lower number of SKAP-55-deficient T cells showed this pattern (i.e., the number increased from 8 to 22%). Examples of conjugate formation with nonlocalized and IS-localized CD3 are shown in the right panels of Fig. 3b. We also observed an overall reduction in the tyrosine phosphorylation of many proteins when splenocytes were incubated with SEA and DCs for either 30 min or 60 min (Fig. 3c). Overall, our observations showed that SKAP-55 can influence the conjugation time, the number of cells with IS-localized CD3, and the proliferation of T cells in response to SEA.

Given the effect on conjugation, it was important next to determine whether loss of SKAP-55 influenced anti-CD3-induced integrin adhesion of primary T cells (Fig. 4). Isolated mesenteric lymph cells were stimulated with anti-CD3, followed by an assessment of fibronectin (FN) and ICAM-1 binding, as previously described (40). First, CD29 (i.e., β1 FN receptor) expression was the same on *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells (Fig. 4a). Despite this, the SKAP-55-deficient cells showed low levels of adhesion to FN relative to those for wild-type cells (Fig. 4b). While anti-CD3 at 2 μg/ml increased wild-type cell adhesion from 4 to 32% of cells, only 15% of *SKAP-55*$^{-/-}$ T cells bound to FN (Fig. 4b, left). The loss of SKAP-55 therefore resulted in a >50% reduction in the number of cells capable of binding to FN. Examples of adherent cells on plates are shown in the right panels of Fig. 4b.

Defective adhesion was also noted in binding to immobilized ICAM-1 on plates (Fig. 5). CD18 (i.e., LFA-1 β2 chain) expression was the same on *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells (Fig. 5a). While anti-CD3 increased the level of adhesion of wild-type T cells to ICAM-1 from 2 to 30% of cells, only 19% of *SKAP-55*$^{-/-}$ T cells bound to plates (Fig. 5a, center). Images of adherent cells are shown in the right panels of Fig. 5a. Comparison of SKAP-55- and ADAP-deficient primary T cells showed similar levels of impaired binding to ICAM-1 (Fig. 5b). Occasionally, *ADAP*$^{-/-}$ cells showed a slightly greater impair-

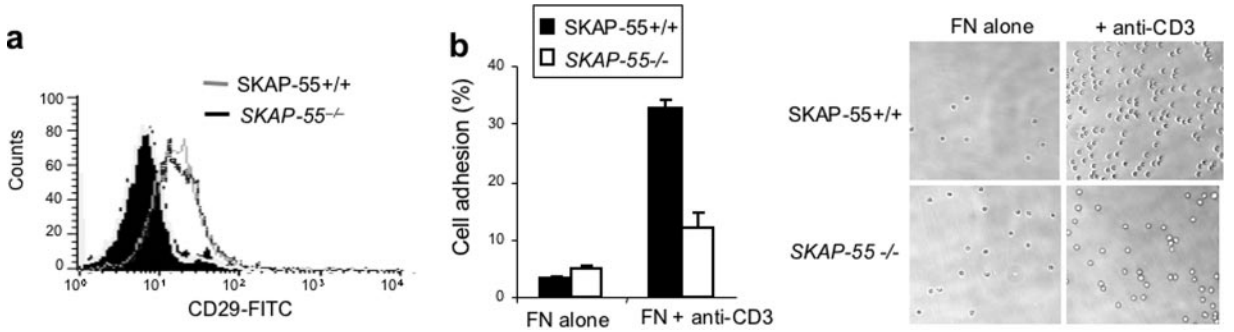

FIG. 4. Primary *SKAP-55*$^{-/-}$ T cells show impaired FN adhesion. Cells were stimulated with anti-CD3 (2C11), and binding to immobilized FN on plates was measured as described in Materials and Methods. (a) Fluorescence-activated cell sorter profile of CD29 surface staining on *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells. FITC, fluorescein isothiocyanate. (b) (Left) Histogram showing the levels of binding of *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells. Data are means ± standard errors of the means for three replicate wells. A P value of <0.05 was considered significant. (Right) Light microscopy images of cells on plates.

ment; however, this was not a reproducible finding. Last, the impaired adhesion of *SKAP-55*$^{-/-}$ T cells was reflected in a reduction of LFA-1 clustering on the surfaces of cells (Fig. 5c). For this experiment, freshly isolated lymph node cells were stimulated with anti-CD3 for 30 min, followed by staining with anti-CD11a, as previously described (39). While anti-CD3 induced an increase in the numbers of *SKAP-55*$^{+/-}$ cells with clustered LFA-1 (i.e., from 15 to 40%), fewer *SKAP-55*$^{-/-}$ cells showed LFA-1 clustering (i.e., an increase from 8 to 18%) (Fig. 5c, left). Examples of the LFA-1 clustered cells are shown in the right panels of Fig. 5c. Overall, these data show that the reduction in the ability of a significant portion of *SKAP-55*$^{-/-}$ T cells to bind to ICAM-1 was accompanied by a defect in LFA-1 clustering on the surface of T cells.

This defect in adhesion was reflected in reductions in cytokine production and proliferation by *SKAP-55*$^{-/-}$ T cells (Fig. 6).

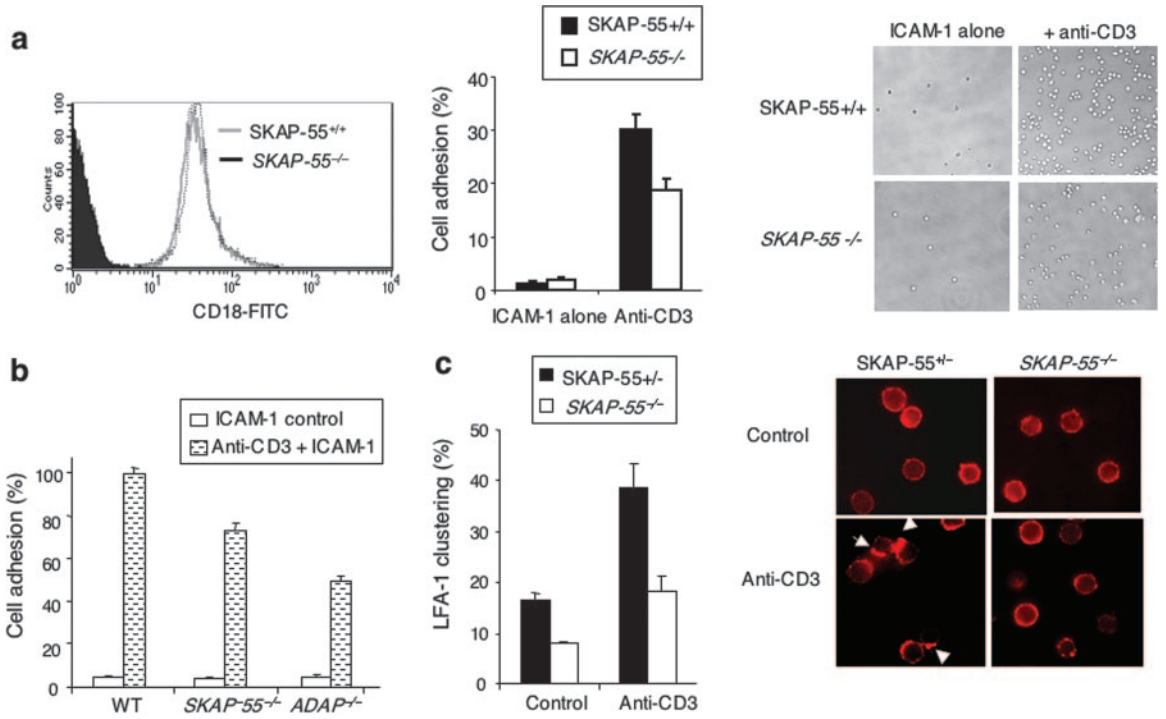

FIG. 5. Primary *SKAP-55*$^{-/-}$ T cells have impaired ICAM-1 adhesion and LFA-1 clustering. (a) Reduced ICAM-1 adhesion of SKAP-55-deficient T cells. (Left) Fluorescence-activated cell sorter profile showing CD18 surface expression on *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells. FITC, fluorescein isothiocyanate. (Center) Histogram showing difference in the induction of ICAM-1 adhesion. (Right) Light microscopy images of *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells. (b) Comparison of defective ICAM-1 binding between *ADAP*$^{-/-}$ and *SKAP-55*$^{-/-}$ T cells. Cells were stimulated with anti-CD3 (2C11), followed by measurement of cell binding to immobilized ICAM-1 on plates. Data are means ± standard errors of the means for three replicate wells. WT, wild type. (c) SKAP-55-deficient T cells have impaired LFA-1 clustering. Cells were incubated with anti-CD3 (2C11) and a rabbit anti-hamster secondary antibody, and LFA-1 surface expression was visualized as described previously (39). (Left) Histogram with the mean percentages (± standard deviations) of T cells that have formed polarized LFA-1 caps. At least 50 cells were counted for each sample by using fluorescence microscopy. (Right) Examples of LFA-1 clustering for *SKAP-55*$^{+/-}$ and *SKAP-55*$^{-/-}$ cells.

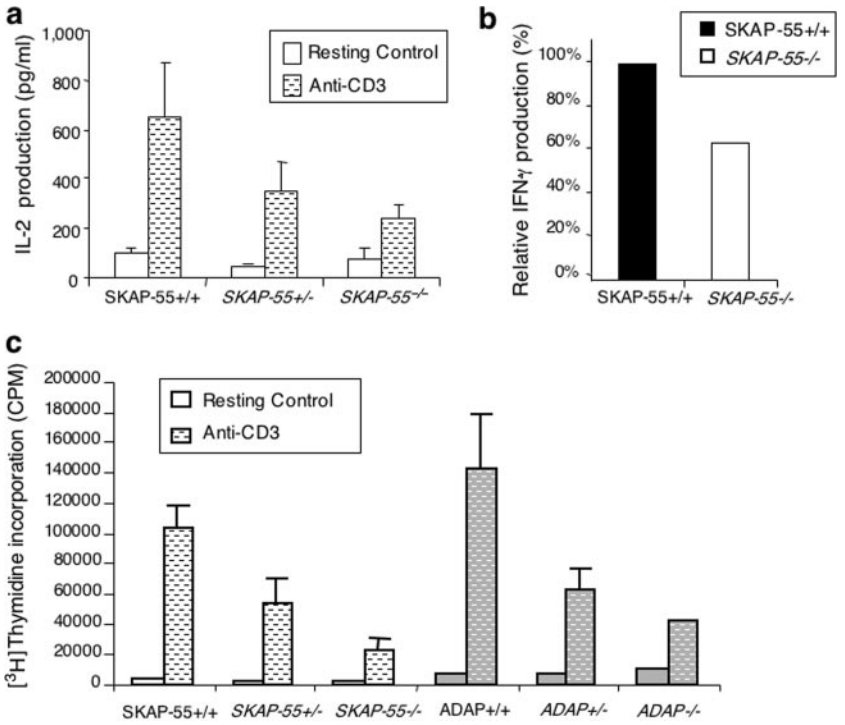

FIG. 6. SKAP-55-deficient T cells show defects in cytokine production and proliferation. Cells were stimulated with anti-CD3 (2C11), followed by measurement of IL-2 and IFN-γ production. (a) *SKAP-55*$^{-/-}$ T cells showed lower IL-2 production levels than *SKAP-55*$^{+/+}$ T cells. *SKAP-55*$^{+/-}$ IL-2 production was 50% lower, and *SKAP-55*$^{-/-}$ IL-2-production was 60 to 70% lower. (b) *SKAP-55*$^{-/-}$ T cells showed reduced IFN-γ production. (c) SKAP-55-and ADAP-deficient T cells showed similar impairment of proliferation. Cells were stimulated with anti-CD3, followed by [^{3}H]thymidine incorporation. Data are means from three replicate wells. Error bars, standard errors of the means. A P value of <0.05 was considered significant.

Anti-CD3 was used to activate T cells, followed by assessments of IL-2 and IFN-γ production. While anti-CD3 increased IL-2 production from 70 to 630 pg/ml in *SKAP-55*$^{+/+}$ cells at 48 h of culture, IL-2 production at this time was only 350 pg/ml for *SKAP-55*$^{+/-}$ and 280 pg/ml for *SKAP-55*$^{-/-}$ T cells (Fig. 6a). Similar decreases were observed at other time points (i.e., 36 and 60 h) (data not shown). IFN-γ production was also reduced in SKAP-55-deficient T cells (Fig. 6b). Intracellular staining was used to measure IFN-γ as described in Materials and Methods. On average, there was a 40 to 50% reduction in the percentage of T cells expressing IFN-γ. This reduction in adhesion and cytokine production was mirrored by reduced *SKAP-55*$^{-/-}$ T-cell proliferation (Fig. 6c). Proliferation was 50 to 75% lower for *SKAP-55*$^{-/-}$ T cells than for wild-type T cells. The reduced proliferation of *SKAP-55*$^{+/-}$ and *SKAP-55*$^{-/-}$ cells closely resembled that of *ADAP*$^{+/-}$ and *ADAP*$^{-/-}$ T cells. These observations indicate that SKAP-55 expression in primary cells is needed for optimal IL-2 and IFN-γ production and proliferation.

The partial reduction in responses was unexpected given previous reports of a more complete blockade in cells lacking the ADAP–SKAP-55 module (10, 27). The reduction in cytokine production by, and proliferation of, SKAP-55-deficient T cells could be related to a general impairment of function in the overall population or could reflect the presence of subsets of SKAP-55-dependent and -independent T cells. The binding of a portion of *SKAP-55*$^{-/-}$ T cells to ICAM-1 plates (Fig. 5) and their ability to form conjugates with TCR/CD3 localization at the IS in response to SEA (Fig. 3) supported the notion of a subset of SKAP-55-independent cells. To assess this in more depth in the context of proliferation, T cells were labeled with CSFE and monitored for the number of cell divisions in response to anti-CD3 (2 μg/ml) by using flow cytometry (Fig. 7A). While resting *SKAP-55*$^{+/+}$ T cells showed a single peak (Fig. 7a, top left), anti-CD3 induced several cell divisions from M2 to M4 (top right). A decreasing number of wild-type cells was observed in each successive division: 35% in M2, 15% in M3, and 2.6% in M4 (Fig. 7a). SKAP-55-deficient T cells differed from wild-type cells in that fewer cells entered the first round of division (Fig. 7a, bottom panels). In this case, 69% of cells remained in M1 compared to 46% of *SKAP-55*$^{+/+}$ cells. The *SKAP-55*$^{-/-}$ T cells that entered the first round of division continued to M3 and M4 with the same decreasing frequency as the *SKAP-55*$^{+/+}$ cells (Fig. 7b). Overall, these data indicate that the peripheral T-cell compartment is heterogeneous, with some T cells requiring and other cells not requiring SKAP-55 to undergo cell division. The principal defect in the *SKAP-55*$^{-/-}$ response was an inability of a subset of T cells to enter the first phase of the cell cycle. Once committed, the other proliferating subset of *SKAP-55*$^{-/-}$ cells cycled in the same way as wild-type cells.

Given the presence of these two populations, it was of interest next to assess whether the dependency on SKAP-55 could be influenced by the strength of the TCR/CD3 signal. For this purpose, anti-CD3 antibody concentrations were varied from 1 to 10 μg/ml. Our studies so far had used 2 μg/ml of

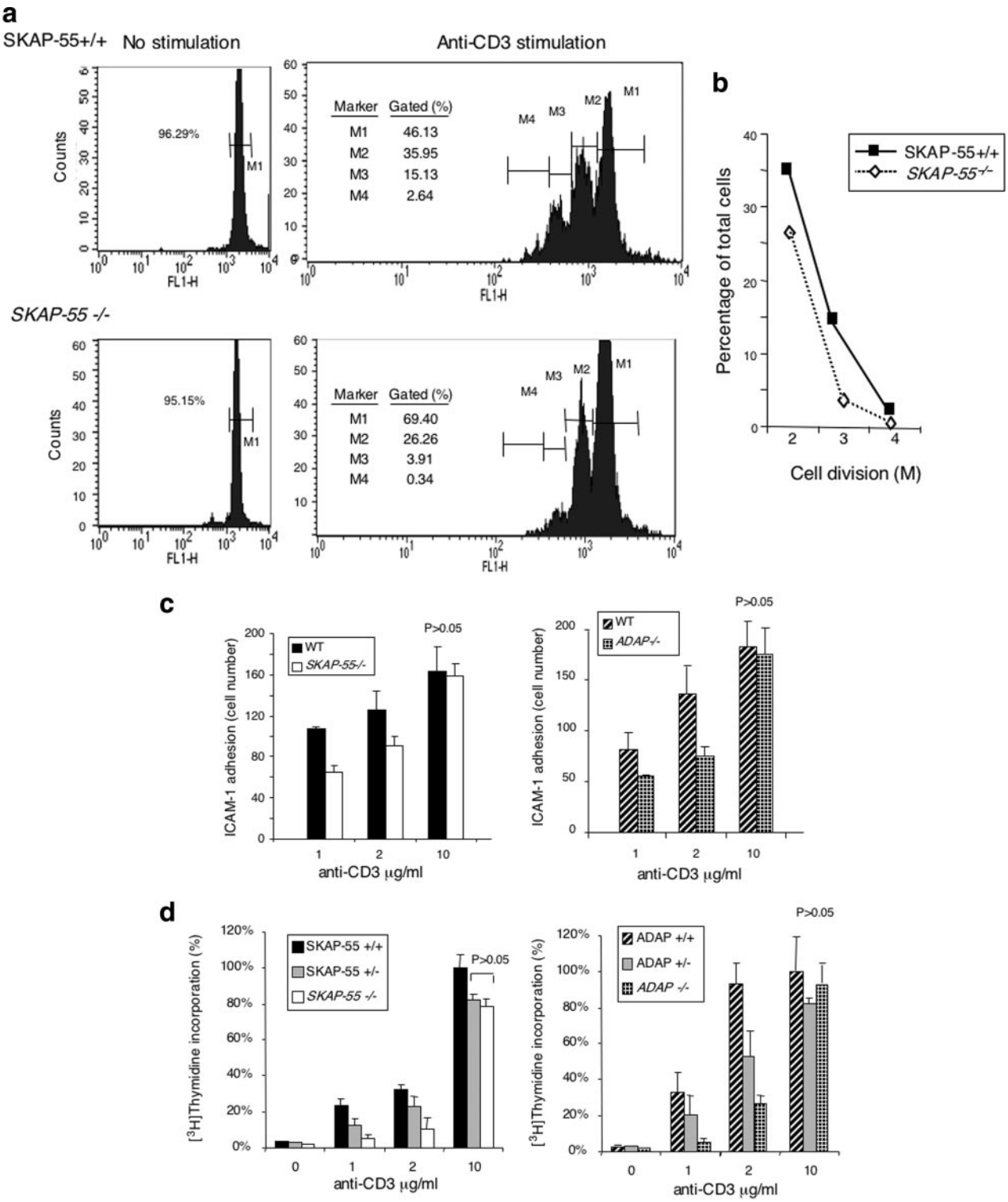

FIG. 7. Identification of SKAP- and ADAP-dependent and -independent T-cell subsets. Dependency differs with the strength of the TCR signal. (a) Subsets of primary *SKAP-55*$^{-/-}$ T cells were dependent on or independent of SKAP-55 for entry into the cell cycle as detected by CFSE labeling. Freshly isolated T cells were labeled with CFSE and either left in a resting state or stimulated with anti-CD3. Then they were monitored for cell division by fluorescence-activated cell sorting. (Top) Wild-type *SKAP-55*$^{+/+}$ cells. (Left) Resting cells; (right) anti-CD3-activated cells. (Bottom) *SKAP-55*$^{-/-}$ cells. (Left) Resting cells; (right) anti-CD3-activated cells. (b) Percentages of cells undergoing cell division (M2, first division; M3, second division; M4, third division). A decreasing number of cells undergo multiple divisions. The decreases in the abilities of activated *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells to undergo subsequent divisions are similar. (c) The requirements for SKAP-55 and ADAP for ICAM-1 adhesion differ with the potency of the TCR signal. T cells were stimulated with anti-CD3 (2C11), and the binding of cells to ICAM-1 immobilized on plates was then measured. (Left) *SKAP-55*$^{+/+}$ and *SKAP-5*$^{-/-}$ T cells; (right) *ADAP*$^{+/+}$ and *ADAP*$^{-/-}$ T cells. (d) The requirements for SKAP-55 and ADAP for proliferation differ with the potency of the TCR signal. Cells were stimulated with anti-CD3, followed by [^{3}H]thymidine incorporation at 60 h. (Left) *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ T cells; (right) *ADAP*$^{+/+}$, *ADAP*$^{+/-}$, and *ADAP*$^{-/-}$ T cells. Data are means from three replicate wells. Error bars, standard errors of the means.

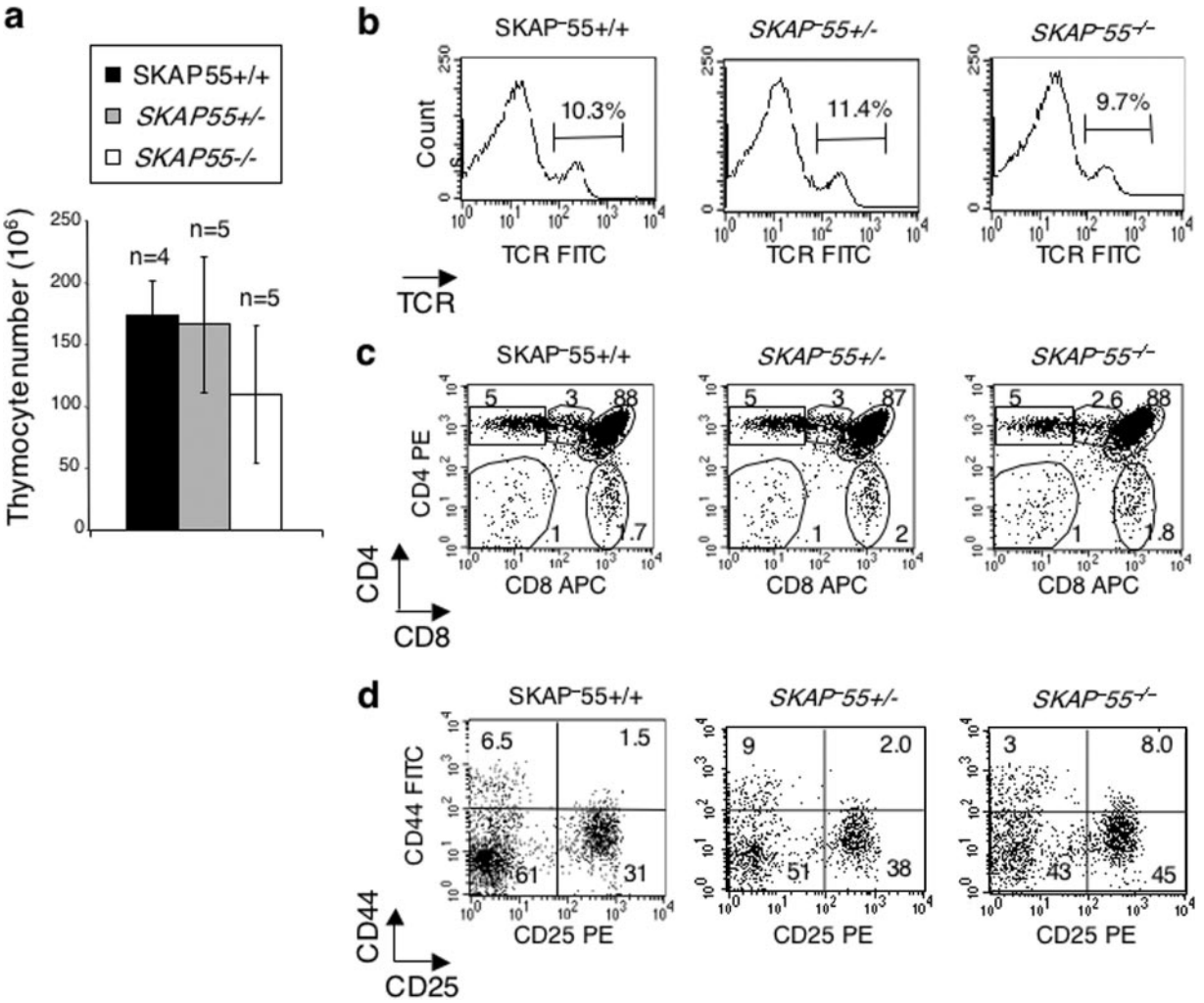

FIG. 8. SKAP-55-deficient mice show normal thymic cellularity and normal stages of thymic differentiation. (a) Normal numbers of thymocytes in *SKAP-55*$^{-/-}$ mice. (b) Anti-TCR staining of thymocytes of *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ mice. TCR expression on thymocytes from *SKAP-55*$^{-/-}$ and *SKAP-55*$^{+/-}$ mice was normal. FITC, fluorescein isothiocyanate. (c) Anti-CD4 and anti-CD8 staining of thymocytes of *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ mice. Normal numbers of CD4 or CD8 SP, DP, and DN thymocytes were observed in *SKAP-55*$^{-/-}$ and *SKAP-55*$^{+/-}$ mice. (d) Anti-CD44 and anti-CD25 staining of thymocytes from *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ mice. Thymic development involves the progression from stage DN1 (CD25$^-$ CD44$^+$) to DN2 (CD25$^+$ CD44$^+$), DN3 (CD25$^+$ CD44$^-$), and DN4 (CD25$^-$ CD44$^-$). Five independent experiments were conducted, and representative results from one set of experiments are shown.

anti-CD3 to activate cells. As seen in Fig. 7c (left), 2 μg/ml of anti-CD3 increased the numbers of *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ T cells binding to ICAM-1 relative to those exposed to 1 μg/ml (from 105 to 123 wild-type cells and from 60 to 90 KO cells). The use of 10 μg/ml (or 5 μg/ml) essentially induced similar levels of binding to ICAM-1 (160 cells in both cases). This indicated that an increase in the concentration of the antibody (i.e., the strength of the TCR/CD3 signal) can override the dependency of T cells on SKAP-55. A comparable situation was observed for *ADAP*$^{-/-}$ T cells: dependency on ADAP was reduced with increasing levels of anti-CD3 antibody (Fig. 7c, right). Again, the use of 10 μg/ml obviated the need for ADAP expression in the induction of ICAM-1 adhesion. These results were matched by those for the proliferation of T cells, where the use of 10 μg/ml increased the similar levels of proliferation of *SKAP-55*$^{+/+}$ versus *SKAP-55*$^{-/-}$ and *ADAP*$^{+/+}$ versus *ADAP*$^{-/-}$ T cells (Fig. 7d). Therefore, while these concentrations of anti-CD3 and the potency of the resultant signal are likely to be much greater than those that would be encountered in response to a foreign peptide, our observations make the important point that the dependency on SKAP-55 and ADAP is relative and differs with the potency of the TCR signal. They might also indicate that TCR signaling can use an alternate pathway in the regulation of adhesion.

We next assessed the numbers of cells in the thymi of *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ mice (Fig. 8a). Although an occasional *SKAP-55*$^{-/-}$ mouse showed a reduction in thymic cellularity, this was not found to be statistically significant over five independent experiments. The expression of the antigen receptor (TCR) was normal for *SKAP-55*$^{+/-}$ and *SKAP-55*$^{-/-}$ thymocytes (Fig. 8b), as was the presence of CD4 and CD8 double-negative (DN), double-positive (DP), and CD4 or CD8 single-positive (SP) cells (Fig. 8c). Using CD44 and CD25 as markers, the DN1-to-DN4 transition appeared normal in

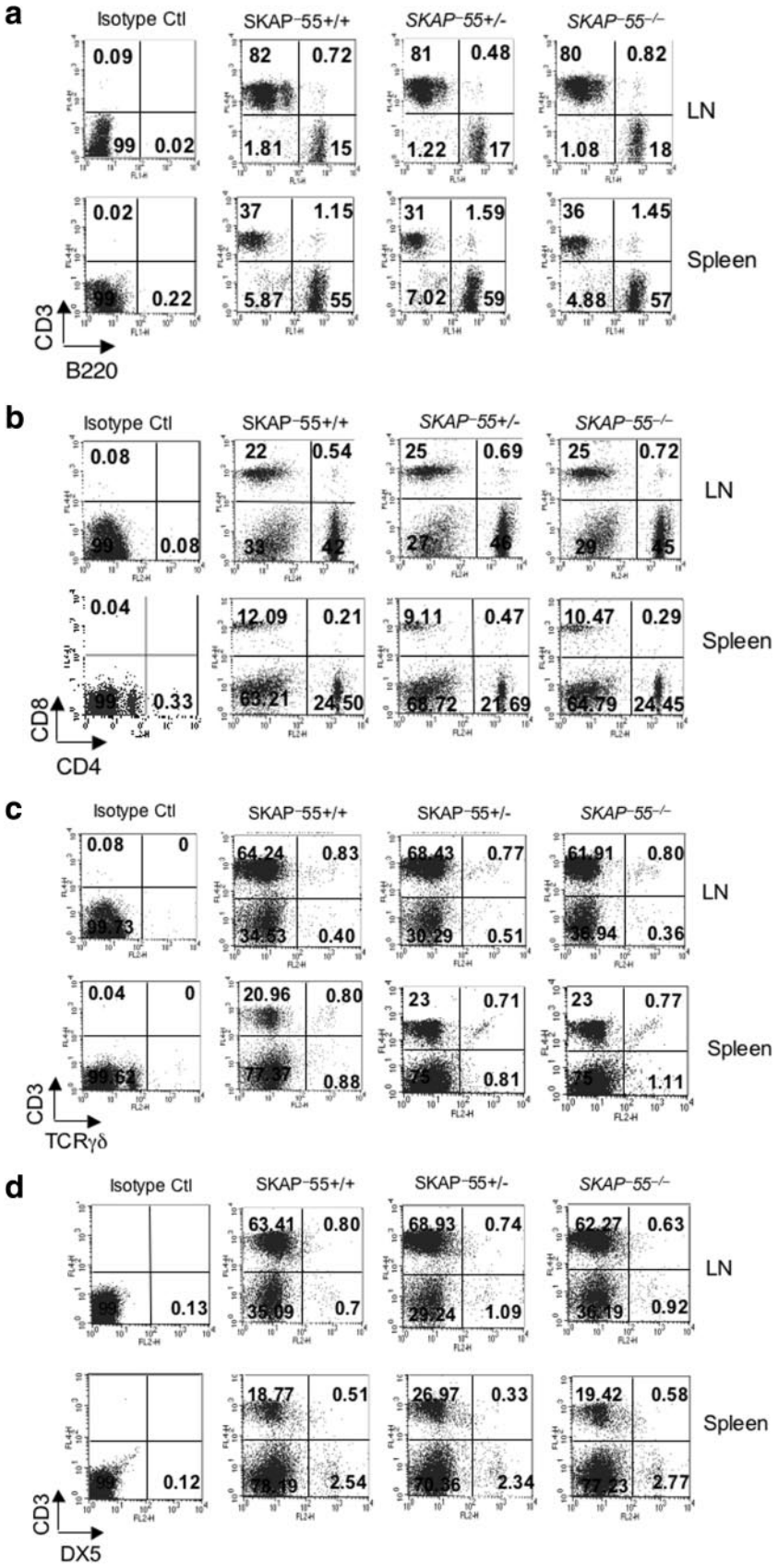

FIG. 9. SKAP-55-deficient mice show normal composition of T-cell and B-cell subsets and NK cells. Cells from the lymph nodes (LN) and spleens of *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ mice were stained with either anti-CD3 versus anti-B220 (a), anti-CD4 versus anti-CD8 (b),

SKAP-55$^{+/-}$ and *SKAP-55*$^{-/-}$ mice (Fig. 8d). A slight reduction in the CD44- and CD25-negative compartment was occasionally observed; but this was not a consistent observation. We also confirmed that normal ratios of T and B cells were observed in the lymph nodes and spleen when anti-CD3 and anti-B220 were used to stain cells (Fig. 9a), and normal ratios of peripheral mature CD4 SP and CD8 SP T-cell subsets were observed (Fig. 9b). Occasionally, *SKAP-55*$^{-/-}$ and *SKAP-55*$^{+/-}$ mice showed slight increases in the numbers of CD4$^+$ CD8$^+$ T cells; however, this was not reproducible. In addition, the compartments of γ/δ T cells in the lymph nodes and spleen were equivalent in *SKAP-55*$^{+/+}$, *SKAP-55*$^{+/-}$, and *SKAP-55*$^{-/-}$ mice (Fig. 9c), as were those of NK cells as defined by CD3$^-$ DX5$^+$ staining (Fig. 9d). These data confirm a normal composition of cells in the peripheral T-cell compartments of SKAP-55-deficient mice.

DISCUSSION

Although current data support a central role for the ADAP–SKAP-55 module in the regulation of TCR-induced integrin clustering and adhesion (9, 10, 12, 13, 14, 18, 27), it has been unclear whether ADAP and/or SKAP-55 acts as an effector of the response. Since ADAP controls SKAP-55 degradation and ADAP-deficient T cells lack SKAP-55 expression (i.e., ADAP SKAP-55 double deficient), it had not been possible to determine whether the immunodeficient phenotype was due to ADAP and/or to SKAP-55. In this study, we have reported the phenotype of the SKAP-55-deficient mouse where *SKAP-55*$^{-/-}$ T cells retain ADAP expression yet show defects in integrin clustering and adhesion, IL-2 and IFN-γ cytokine production, and proliferation. In fact, *SKAP-55*$^{-/-}$ T cells showed the same general impairment of function as *ADAP*$^{-/-}$ T cells, indicating that SKAP-55 is an effector of the ADAP–SKAP-55 module. At the same time, the requirement for ADAP and SKAP-55 was not absolute, since a subset of peripheral T cells adhere without the need for either adaptor. Further, dependency on SKAP-55 or ADAP differed with the strength of the TCR signal. Higher concentrations of anti-CD3 enabled greater numbers of cells to bind ICAM-1 and proliferate in the absence of SKAP-55 or ADAP. Our findings identify a clear effector role for SKAP-55 in the up-regulation of LFA-1 adhesion in peripheral T cells and demonstrate that higher levels of TCR engagement can bypass the need for SKAP-55 and ADAP in the up-regulation of T-cell adhesion.

ADAP and SKAP-55 form a module where they bind to each other with high stoichiometry, and immunoprecipitation with an anti-ADAP antibody depletes most SKAP-55 from cell lysates and vice versa (4, 25). Further, ADAP controls the degradation of SKAP-55 such that ADAP-deficient T cells have little if any SKAP-55 protein expression (12) (Fig. 1d). The question of whether SKAP-55 might also influence ADAP expression was addressed previously; small interfering RNA-mediated reduction of SKAP-55 levels in a mouse antigen-specific T-cell line markedly reduced integrin adhesion without altering ADAP expression (13). Similar findings have recently been reported with Jurkat cells (18). Our study has now extended this finding by demonstrating that SKAP-55 has no obvious role in regulating ADAP expression in normal primary peripheral T cells. ADAP therefore serves a unidirectional chaperone function for its binding partner within the module.

This observation, combined with the finding of impaired β1- and β2-mediated adhesion of *SKAP-55*$^{-/-}$ T cells, has, for the first time, identified an effector role for SKAP-55 within the ADAP–SKAP-55 module in primary T cells. SKAP-55-deficient T cells showed multiple defects, including defects in cell aggregation and subsequent cytokine production and proliferation in response to SEA (Fig. 2) and defects in anti-CD3-induced ICAM-1 adhesion, cytokine production, and proliferation (Fig. 6). Occasionally, *ADAP*$^{-/-}$ cells showed greater impairment of ICAM-1 binding; however, this was not a consistent observation. Instead, the level of defective function for SKAP-55-deficient T cells was remarkably similar to that for ADAP-deficient T cells. This was observed in multiple assays including assessment of cell aggregation, cytokine production, and proliferation in response to SEA (Fig. 2), ICAM-1 binding (Fig. 5b), anti-CD3-induced proliferation (Fig. 6c), and the abilities of high anti-CD3 concentrations to bypass the need for the adaptor (Fig. 7c and d). These findings introduce the intriguing question of whether the impaired function of ADAP-deficient mice is due primarily to the loss of SKAP-55 or whether ADAP can play an additional, independent role in adhesion. In the former scenario, SKAP-55 would be the sole effector of the ADAP–SKAP-55 module. ADAP would indirectly influence adhesion by altering SKAP-55 expression, or possibly its conformation or localization. In the more likely scenario, ADAP provides overlapping or alternate signals for adhesion. The presence of an EVH1 domain and G-actin binding motifs in ADAP would support this alternate role (19). The modulation of the cytoskeleton at the IS could facilitate more effective adhesion without directly affecting the status of LFA-1 on the surface of the cell. However, it is interesting that multiple mutations in the EVH1 motif had no obvious effect on the ability of T cells to form conjugates with APCs (39). In either case, ADAP acts to bridge SLP-76 with downstream SKAP-55. p59^{fynT} phosphorylates YDDV sites in ADAP, leading to SLP-76 SH2 domain binding and possibly to recruitment of the ADAP–SLP-76–GADS complex to LAT (31, 32, 41). Mutation of YDDV sites in ADAP impairs conjugation, SMAC formation, and cytokine production (39). Further, loss of the SKAP-55 SH3 domain, loss of the ADAP SH3c domain, or over expression of a peptide which binds to the proline-rich region of ADAP ablates adhesion (7, 18, 39). These observations are consistent with the existence of a cascade linking

anti-CD3 versus anti-TCRγδ (c), or anti-CD3 versus anti-DX5 (d). Ctl, control. (a) Normal levels of T and B cells were observed in *SKAP-55*$^{-/-}$ LN (top) and splenocytes (bottom). (b) Normal levels of CD4 SP and CD8 SP T cells were observed in *SKAP-55*$^{-/-}$ LN (top) and splenocytes (bottom). (c) Normal levels of γ/δ T cells were observed in *SKAP-55*$^{-/-}$ and *SKAP-55*$^{-/+}$ LN (top) and splenocytes (bottom). (d) Normal levels of one subset of NK cells (CD3$^-$ DX5$^+$) were observed in *SKAP-55*$^{-/-}$ and *SKAP-55*$^{-/+}$ LN (top) and splenocytes (bottom). Four independent experiments were conducted, and representative results from one set of experiments are shown.

SLP-76 to ADAP and ADAP to SKAP-55 in the control of adhesion. The data from this study now underscore the central role of SKAP-55 as a downstream effector in the cascade.

The loss of SKAP-55 impaired anti-CD3-induced adhesion to FN (Fig. 4) and ICAM-1 (Fig. 5). Generally β1-mediated binding was impaired more than β2-mediated binding, introducing the possibility that SKAP-55 may differentially regulate the different classes of integrins. In terms of response to antigens, the loss of SKAP-55 reduced SEA-induced T-cell aggregation (Fig. 2) and the dwell times of T cells with APCs (Fig. 3). The length of time that a T-cell spends attached to a DC will influence the level of antigen-receptor clustering and the intracellular signaling needed for proliferation. In this regard, while the difference in general cell aggregation induced by SEA did not correlate strictly with reduced proliferation, a correspondence between the reduction in the percentage of long-term contacts (i.e., >1,800 s), the reduction in the number of cells with the TCR/CD3 complex localized at the IS, and the level of IL-2 production and proliferation was observed (i.e., 40 to 50%) (Fig. 2 and 3). A small reduction in number of TCR/CD3 microclusters in *SKAP-55*$^{-/-}$ T cells was also observed (data not shown). TCR/CD3 and intracellular signaling proteins form microclusters at the IS, where signals are generated for proliferation (5, 8, 35). The reduction in conjugation time and TCR microcluster formation in turn correlated with an overall reduction in the tyrosine phosphorylation of many proteins in T cells (Fig. 3c). To date, a selective reduction in the phosphorylation of one protein relative to another has not been observed.

Interestingly, SKAP-55 also played a role in antigen-independent conjugate formation. Even in the absence of SEA, *SKAP-55*$^{-/-}$ T cells interacted with APCs for shorter periods than wild-type cells (Fig. 3a). The addition of SEA caused a shift to longer adhesion periods for both *SKAP-55*$^{+/+}$ and *SKAP-55*$^{-/-}$ cells. The inability of *SKAP-55*$^{-/-}$ cells to achieve longer contact times appeared to be influenced by the fact that they already exhibited shorter contacts in an antigen-independent context. Only 40% of *SKAP-55*$^{-/-}$ T cells achieved the longer conjugation periods (i.e., >1,800 s) in response to SEA. Many *SKAP-55*$^{-/-}$cells failed to respond to TCR ligation, while others formed conjugates of intermediate duration. Overall, our findings are consistent with a general role for SKAP-55 in antigen-dependent and antigen-independent conjugate formation.

One surprising observation was the existence of heterogeneity in the peripheral T-cell population with regard to their dependence on SKAP-55 and ADAP. A percentage of *SKAP-55*$^{-/-}$ T cells underwent normal adhesion, conjugation, and proliferation (Fig. 2 to 6). In fact, some 30 to 50% of SKAP-55- and ADAP-deficient T cells proliferated normally in response to SEA presented by DCs (Fig. 2), bound to ICAM-1 on plates (Fig. 5), produced IL-2 and IFN-γ, and proliferated (Fig. 6). Cell cycle analysis extended this observation by showing that a significant portion of SKAP-55-deficient T cells underwent multiple rounds of cell division (Fig. 7). Once committed to proliferation, these cells underwent the same number of subsequent cell divisions as wild-type cells. The defect was in the ability of a subset of T cells to enter the first cycle of division. This indicates that the "inside-out" pathway is more complex than previously appreciated and that SKAP-55 is not essential for the function of all peripheral T cells. Similar findings were made with ADAP-deficient T cells. A subset of T cells appears to have an alternate mechanism that can substitute for SKAP-55.

Further, it is noteworthy that the relative proportions of SKAP-55- and ADAP-dependent and -independent cells in the peripheral compartment are not fixed. Rather, they appear to reflect a heterogeneity in the population of T cells that can be altered by the strength of the TCR signal. An increase in the anti-CD3 concentration from 1 to 2 μg/ml (otherwise, 2 μg/ml was used throughout the study) and 10 μg/ml induced increasing numbers of cells to adhere to ICAM-1 and to proliferate without the need for SKAP-55 or ADAP (Fig. 7c and d). In fact, 10 μg of antibody/ml activated the entire population of T cells without the need for SKAP-55 or ADAP. This level of receptor cross-linking and this signal strength are probably higher than those that would normally be encountered under physiological conditions. Nevertheless, the observation is informative in that it indicates that SKAP-55 and ADAP are not obligatory components in the TCR signaling leading to adhesion. The strength of the signal can override the need for SKAP-55. In this context, the ADAP/SKAP-55 pathway may preferentially support low- to intermediate-affinity ligand stimulation of T cells. The degree to which the ADAP/SKAP-55 pathway supports physiological responses to peptides of different affinities and the nature of the alternate pathway remain to be determined. Another protein(s) may constitute a pathway that is distinct from SKAP-55, or the TCR complex may directly activate another mediator downstream of SKAP-55 in the same pathway. Possible candidates include the GTPase Rap1 and/or its binding partners RapL and RIAM (6, 15, 16, 22, 28, 34).

Last, a similarity between the phenotypes of the SKAP-55- and ADAP-deficient mice was also observed in the lack of a profound effect on thymic differentiation and the composition of the peripheral T-cell compartment. Both ADAP- and SKAP-55-deficient mice expressed a normal array of thymocytes (i.e., CD4, CD8, DN1 to DN4) and peripheral cell subsets (i.e., CD4 SP, CD8 SP, and TCRγδ T cells; B-cells; and NK cells) (Fig. 8 and 9). These findings do not exclude the possibility that more-subtle alterations will eventually be observed in TCR-transgenic animals, as reported for *ADAP*$^{-/-}$ mice (42). We occasionally observed a decrease in *SKAP-55*$^{-/-}$ thymic cellularity; however, when averaged over six animals, this difference was not found to be statistically significant. This differs from the observations for *ADAP*$^{-/-}$ mice, which showed a moderate decrease in thymocyte numbers (10, 27, 43). In instances of mice with decreased numbers of thymocytes, the cells showed an increased propensity to undergo cell death in media (data not shown). Although occasionally *SKAP-55*$^{-/-}$ thymocytes showed slight increases in the percentages of DN2 and DN3 thymocytes with a reduction in the percentage of the DN4 subset, this also was not reproducibly different from findings for non-TCR-transgenic mice. Consistent with this, LFA-1-deficient mice show no apparent defects in thymic differentiation (36). Further studies will be needed to uncover whether ADAP- and SKAP-55-deficient T cells influence the activation of other pathways linked to the regulation of T-cell adhesion.

ACKNOWLEDGMENT

This work was supported from a grant from the Wellcome Trust, London, United Kingdom (C.E.R. is the recipient of a Principal Research Fellow Award).

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