A novel intracellular pool of LFA-1 is critical for asymmetric CD8+ T cell activation and differentiation

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The integrin lymphocyte function–associated antigen 1 (LFA-1; CD11a/CD18) is a key T cell adhesion receptor that mediates stable interactions with antigen-presenting cell (APC), as well as chemokine-mediated migration. Using our newly generated CD11a-mYFP knock-in mice, we discovered that naive CD8+ T cells reserve a significant intracellular pool of LFA-1 in the uropod during migration. Intracellular LFA-1 quickly translocated to the cell surface with antigenic stimulus. Importantly, the redistribution of intracellular LFA-1 at the contact with APC was maintained during cell division and led to an unequal inheritance of LFA-1 in divided T cells. The daughter CD8+ T cells with disparate LFA-1 expression showed different patterns of migration on ICAM-1, APC interactions, and tissue retention, as well as altered effector functions. In addition, we identified Rab27 as an important regulator of the intracellular LFA-1 translocation. Collectively, our data demonstrate that an intracellular pool of LFA-1 in naive CD8+ T cells plays a key role in T cell activation and differentiation.

Introduction

Naive T cells spend their lifespan circulating from the blood to lymphatic organs in search of cognate antigen presented by antigen-presenting cells (APCs) and then returning to the blood via the thoracic duct in a cyclical fashion. Successful expansion and differentiation of naive CD8+ T cells is dependent on the ability of cells to precisely localize with APCs in secondary lymphoid organs to form stable and prolonged interactions upon antigen recognition and T cell receptor (TCR) activation (Kaech et al., 2002; Cronin and Penninger, 2007; Chen and Flies, 2013). To undergo further T cell expansion and differentiation, T cells require additional stimuli from APCs and lymphatic cells that reside within niches in secondary lymphoid organs. Therefore, recirculation through lymph nodes, interactions with APCs, and localization to distinct immune niches are likely to impact CD8+ T cell division and differentiation. A key molecule regulating these processes is the integrin lymphocyte function–associated antigen 1 (LFA-1).

Adhesive force generated by LFA-1 ligation is essential for initial T cell entry into the lymph node through high endothelial venules (Weber et al., 2001) and subsequently T cell retention through interaction with the lymphatic stroma and APCs (Smith et al., 2003, 2007; Katakai et al., 2013). LFA-1 knockout (KO) T cells pass through the lymph node more rapidly and are three times more likely to exit (Reichardt et al., 2013). Enhanced LFA-1 adhesiveness is equally important for the maintenance of the immunological synapse and the signal integration necessary for complete T cell activation. Once a naive T cell encounters an antigen-bearing APC, LFA-1 engagement with ICAM-1 overcomes the glycocalyx repulsion of the T cell–APC contact and brings the two cells within a 40-nm proximity, allowing actin-mediated lamellipodia protrusion to sustain TCR signaling (Choudhuri et al., 2005). In addition to the physical adhesion, LFA-1 also provides important costimulation signals while excluding negative regulators of TCR signaling (Matsumoto et al., 2004; Graf et al., 2007).

Many signaling molecules have emerged as important players in regulating LFA-1 functions in T cells. Surface receptors, such as chemokine receptors or TCR, induce activation of downstream signaling molecules (Rap1 and talin) that leads to conformational changes in LFA-1 (Kim et al., 2003). Alternatively, outside-in signals occur when LFA-1 binds multivalent ICAM-1, stabilizing clusters of the active conformation and inducing downstream signals for cytokine production, proliferation, and survival (Salomon and Bluestone, 1998; Ni et al., 2001; Kandula and Abraham, 2004; Kim et al., 2004; Varga et al., 2010). In addition to receptor-induced activation, LFA-1 adhesiveness is also modulated by cell surface localization through lateral mobility (Cairo et al., 2010) and intracellular trafficking of important mediators of LFA-1 activation, including Rap1, Rap2, RapL, and Mst1, through Rab5, Rab11, Rab13, and EEA1 endosomes (Fabbri et al., 2005; Stanley et al., 2012; Svensson et al., 2012; Nishikimi et al., 2014). Although it has
been suggested that these vesicle cargos may contain LFA-1 (Hogg et al., 2011), dynamic regulation of LFA-1 redistribution during activation of naïve T cells has yet to be demonstrated.

Dynamic regulation of LFA-1 expression and functions in T cells is typically studied using cell lines and/or activated T cell blasts with transfection of recombinant genes or monoclonal antibodies that detect cell surface expression. Given the importance of the dynamic LFA-1 regulation during naïve T cell migration and activation, these approaches are not sufficient to completely understand LFA-1 biology. In this study, we generated CD11a-mYFP knock-in (KI) mice to study endogenous LFA-1 expression and distribution patterns. Using live imaging of fluorescence CD11a-mYFP in CD8⁺ T cells from the newly developed KI mice, we report a previously undescribed intracellular pool of LFA-1 that is critical for T cell activation and differentiation.

**Results**

**Naive CD8⁺ T cells possess an intracellular pool of LFA-1**

The integrin LFA-1 (CD11a/CD18) is expressed on most leukocytes and plays a key role in regulating leukocyte adhesion, migration, and activation. To study dynamic regulation of endogenous LFA-1 expression during T cell activation and differentiation, we generated a KI mouse in which the α subunit of LFA-1 (CD11a) was fused with monomeric YFP (CD11a-mYFP, Fig. 1, A–D). Extensive characterization revealed that immune development (Fig. S1 A), LFA-1 function (Fig. S1, B and C), T cell activation (Fig. S1 D), and T cell effector function (Fig. S1 E) in CD11a-mYFP and WT mice were comparable.

To further confirm that the cellular expression of endogenous LFA-1 in CD11a-mYFP mice was comparable to that of WT mice, we investigated the distribution pattern of LFA-1 in naïve CD8⁺ T cells. First, CD11a-mYFP mice showed normal cell surface expression of LFA-1 on naïve CD8⁺ T cells compared with WT mice (Fig. S2 A). To our surprise, however, optical scanning (Fig. 1 E and Video 1) and flow cytometry analysis using two different cell-permeabilization methods (Fig. S2, B and C) of naïve CD8⁺ T cells from CD11a-mYFP/OT-I mice revealed a previously unrecognized intracellular pool of LFA-1. Intracellular LFA-1 was primarily concentrated to the uropod of migrating cells (Fig. 1 E, migration; and Video 2). Strikingly, time-lapse imaging of live naïve CD8⁺ T cells showed that the majority of intracellular LFA-1 in the uropod rapidly localized to the T cell and ovalbumin (OVA) (N4)-loaded APC contact site (Fig. 1 E, conjugation; and Video 3).

**Intracellular LFA-1 redistributes to the cell membrane during T cell activation**

To determine whether this rapid redistribution of intracellular LFA-1 was dependent on antigen affinity to the TCR, we stimulated naïve CD8⁺ T cells with N4 or a low-affinity altered peptide ligand (D7; Koniaras et al., 1999) that showed reduced peptide ligand (D7; Koniaras et al., 1999) that showed reduced

**Figure 1. Naive CD8⁺ T cells possess an intracellular pool of LFA-1.** (A) Schematic of CD11a-mYFP mouse generation. The mYFP sequence was knocked into the C terminus of the mouse integrin CD11a subunit. (B) CD11a PCR depicting the increased size of CD11a corresponding with the mYFP tag. (C) Corresponding size increase was also detected in a silver stain at the protein level. mAb M17/4 was used for immunoprecipitation (IP) and total cell lysate from splenocytes of CD11a-mYFP mice. (D) Western blot analysis of YFP expression in YFP immunoprecipitate (IP) and total cell lysate from splenocytes of CD11a-mYFP mice showing the intact YFP conjugation to CD11a. No evidence of proteolytic cleavage of YFP was detected. (E) Representative images of permeabilized naïve CD8⁺ T cells stained with CD11a (LFA-1) or α-tubulin antibodies on noncoated glass surface (unstimulated/round), after 30 min of migration on ICAM-1 and CCL21 coating (migration) or after 60 min of conjugation with N4-pulsed BMDCs (conjugation) showing the intracellular pool of LFA-1. Bars, 2 µm. Graph shows colocalization of YFP signal versus anti–CD11a antibody (LFA-1) signal in naïve CD11a-mYFP CD8⁺ T cells. Pearson coefficient was generated as YFP [CD11a-mYFP]/red (anti-CD11a). Note that the YFP signal and the anti-CD11a antibody (LFA-1) signal are highly colocalized in naïve CD11a-mYFP CD8⁺ T cells. Data are presented as mean ± SEM; n = 4 mice/group (10–20 cells per mouse). Note that there are equivalent levels of total LFA-1 [intracellular staining and surface LFA-1] detected in both saponin and Triton X-100 permeabilized samples (Fig. S2 C).

to induce the rapid translocation of LFA-1 to the cell surface (Fig. 2, B–E; and Video 4).

We further confirmed the dynamic redistribution of intracellular LFA-1 to the cell surface during early T cell activation.
using flow cytometry analysis of naive CD8+ T cell isolated from WT mice (Fig. S3 D). Furthermore, the integrin VLA-4 and TCR did not exhibit the same dynamic redistribution pattern as LFA-1, suggesting the presence of a specific redistribution pathway for LFA-1 (Fig. 2 E). Total CD11a-mYFP protein expression levels measured by YFP intensity remained constant during the T cell activation (Figs. 2 E and S3 E), and Exo1, an inhibitor of exocytosis of newly synthesized proteins, did not alter redistribution of LFA-1 (Fig. S3 F). Therefore, the results suggest that LFA-1 redistribution is not a consequence of de novo protein production. In addition, cells treated with Dynasore, a dynamin inhibitor that blocks a majority of endocytosis (Macia et al., 2006), exhibited similar intracellular and cell surface levels of LFA-1 as detected by flow cytometry, suggesting that endocytic recycling has minimum impact on intracellular LFA-1 in naive T cells (Fig. S3 G). Finally, we confirmed that LFA-1

Figure 2. Redistribution of intracellular LFA-1 during T cell activation. (A) Representative flow cytometry analysis of T cell activation (CD69 and CD25) and proliferation (CFSE dilution) after stimulation of naive T cells with cognate ligand (N4) or altered peptide ligand (D7)–loaded irradiated splenocytes; n = 4 mice. (B) Representative images from real-time T cell contacts with APCs loaded with N4 or D7 peptide on plates coated with ICAM-1 and CCL21. Bars, 5 µm. In the graph, each bar represents the percentage of total cells scored after 45 min of co-culture. The gray portion of each bar is the fraction of cells exhibiting dominant YFP signal at the immunological synapse (IS) region, and the white portion is the fraction of the cells that showed YFP signal at the posterior region. Data represent mean ± SEM; n = 3 mice/group (30–42 cells per mouse). *, P < 0.05. (C) Representative fluorescence intensity of CD11a-mYFP cell surface from B. YFP fluorescence intensity is shown in a pseudocolor scale (from low [black] to high [red]). +/− 180°, rear of cell; 0°, leading edge; white lines depict the T cell–APC interface; arrowheads indicate the beginning of the T cell–APC contact. (D) Representative Western blot analysis of YFP expression in cell cytosol and plasma membrane (PM) fractions from naive CD11a-mYFP CD8+ cells or cells stimulated with CD3/CD28 antibodies for 30 min. Note that CD11a-mYFP protein level was increased in PM after T cell activation. n = 3 mice. (E) Flow cytometry analysis of surface LFA-1, VLA-4, and TCR levels after indicated times of naive CD11a-mYFP/OT-I CD8+ T cell and peptide-pulsed or PBS-treated BMDC co-culture. YFP+ T cells were fixed at indicated times and stained for surface expression. Total LFA-1 levels measured by mYFP intensity (yellow line). Data normalized to PBS control. Data are expressed as mean ± SEM of six separate experiments. *, P < 0.001. (F) Representative image of naive CD11a-mYFP CD8+ T cells stained with ER Tracker (MTOC; red) during N4-loaded APC interaction on the ICAM-1+CCL21–coated surface. Note that CD11a-mYFP and the MTOC are not colocalized during the LFA-1 redistribution. Bar, 5 µm. (G) The MTOC and CD11a-mYFP are colocalized during migration and mature APC contact, but not during early LFA-1 translocation to the APC contact (“early contact”) when stimulated by N4. The Pearson’s correlation coefficient was generated as YFP (LFA-1)/red (MTOC). Circles represent individual cells from three independent experiments with mean shown as a line. Data represent mean ± SEM. *, P < 0.01.
The frequency of APC conjugation of LFA-1 high (YFP high) versus LFA-1 low (YFP low) CD8+ T cells was measured. Among cells imaged, 75% of LFA-1 high T cells spent more than 70% of imaging time in contact with an APC demonstrated a pronounced polarized redistribution of LFA-1 into the two daughter cells (YFP high vs. YFP low; Figs. 3 C and S4). In addition to LFA-1, naive CD8 T cells express integrin VLA-4 (CD49d/CD29), which plays a key role in extravasation through high endothelial venules and intranodal migration, during which APC scanning occurs (Hyun et al., 2009). Furthermore, chemotactic molecules such as CCL21, CCL19, CXCL12, and S1P presented in the lymph node and their receptors, including CCR7, CXCR4, and S1PR, are essential for T cell migration and the signal integration necessary for complete T cell activation (von Andrian and Mackay, 2000; Pham et al., 2008). Although flow cytometry analysis clearly showed asymmetric expression of LFA-1 in first-division T cells, we did not observe disparate expression of other T cell surface molecules known to mediate T cell migration (Fig. 3 D).

Differential LFA-1 levels lead to changes in T cell migration and conjugation

To determine whether disparate LFA-1 expression in first-division CD8+ T cells regulates the dynamic patterns of T cell migration and APC interactions during early T cell activation, we isolated YFP high (LFA-1 high) and YFP low (LFA-1 low) first-division CD8+ T cells from influenza-infected mice (Fig. 3 C). In vitro migration assays on plates coated with ICAM-1 and CCL21 revealed two distinct cell migration patterns in the LFA-1 high and LFA-1 low T cells (Fig. 4 A). To evaluate T cell--APC conjugation patterns, OVA-pulsed BMDCs were first adhered to a chamber coated with ICAM-1 and CCL21. T cells were then placed in the chamber, and cell–cell interactions were imaged. The frequency of APC conjugation of LFA-1 high (YFP high) versus LFA-1 low (YFP low) CD8+ T cells was measured. Among cells imaged, 75% of LFA-1 high T cells spent more than 70% of imaging time forming stable contacts with OVA-loaded APCs, whereas the majority (over 80%) of LFA-1 low T cells never or only transiently (less than 30% of imaging time) contacted APCs. Importantly, the majority of stopped T cells (>80%) productively engaged with APCs during their conjugation time as measured by calcium flux (Fig. S3 B). Thus, migration and APC interaction patterns in T cells with differential LFA-1 expression demonstrate that higher LFA-1 expression allows formation of stable and prolonged T cell–APC conjugates over time, whereas reduced LFA-1 expression permits a highly migratory state. Our data suggest unequal partitioning of LFA-1 during cell division generates daughter cells with differential behavior patterns, guiding T cell migration, interactions, and localization.

Different patterns of T cell behavior may lead to increased integration of signals required for differentiation versus further APC scanning, migration to other regions of the lymph node, or access to sites of egress. Thus, the exposure of first-division T cells to diverse immune niches could alter T cell differentiation programs by reinforcing or redefining existing environmental cues. To determine whether unequal LFA-1 inheritance affected T cell retention in the lymph node, we conducted a competitive egress assay. To evaluate the rate at which cells exit the lymph node, mice were treated with an antibody against CD62L to block entry of additional T cells 12 h before the first division. The number of T cells retained in the draining lymph node was compared with the number of T cells measured in mice treated with both the CD62L antibody and FTY720, an inhibitor of T cell egress (Matloubian et al., 2004). Flow cytometry analysis of T cell number in the draining lymph node and spleen revealed that when additional T cell entry was blocked by CD62L antibody, the first division LFA-1 low T cell population was quickly egressed from the draining lymph node, whereas a larger number of LFA-1 high T cells remained in the inflamed lymph node for a longer period (Fig. 4 B). To determine whether the migration and retention of T cells in the draining lymph node resulted in cells receiving differential effector functions, we first measured the mRNA levels of the transcription factor T-bet and effector molecules interferon-γ and granzyme B (Kelso et al., 2002). LFA-1 high T cells isolated from influenza-infected mice exhibited higher expression of effector gene products than LFA-1 low T cells (Fig. 4 C). Second, we tested the ability of LFA-1 high and LFA-1 low CD8+ T cells to generate memory cells after primary influenza infection. Upon infection of recipients with influenza virus X31-OVA, we found that both LFA-1 high and LFA-1 low cells expanded equally well in the lymph node and lung during the primary response (8 d post infection [dpi]; Fig. 4 D). However, the number of CD11a-mYFP+ T cells in the lung 60 dpi was significantly reduced when LFA-1 low T cells were transferred before primary infection (Fig. 4 E). Furthermore, LFA-1 high T cells were unable to form the central memory, effector memory, and tissue-resident memory compartments 60 dpi (Fig. 4 F). This result is in agreement with earlier studies demonstrating that CD8 low T cells, but not CD8 high T cells, clear secondary infection (Chang et al., 2007; Ciocca et al., 2012). Collectively, these data demonstrate that LFA-1 high and LFA-1 low CD8+ T cells exhibit distinct migration, T cell–APC interaction, and lymph node retention patterns that generate unique differentiation programs.

Rab27 mediates redistribution of intracellular LFA-1 during naive CD8+ T cell activation

Several molecules are known to asymmetrically partition into first-division T cells (Arsenio et al., 2015). To determine the contribution of asymmetric inheritance of LFA-1 on the behavioral and differentiation phenotypes observed in LFA-1 high and LFA-1 low CD8+ T cells, we sought to identify cytoplasmic molecules...
required for the redistribution of intracellular LFA-1 to the cell surface. Based on the molecular model of YFP expression in our CD11a-mYFP mice (Fig. 5 A), we predicted that an anti-YFP antibody would selectively isolate LFA-1–containing endosomes from CD8+ T cells. To confirm this hypothesis, we first isolated the total endosomes from homogenized CD11a-mYFP CD8+ T cells using flotation ultracentrifugation (Fig. 5 B, left) and selectively immunoprecipitated CD11a-mYFP+ endosomes using beads coated with a monoclonal GFP (E36) antibody that cross-reacts with YFP. Western blot analysis with a polyclonal YFP antibody was used to distinguish CD11a-mYFP+ and CD11a-mYFP− endosome fractions (Fig. 5 B, right). To confirm that the majority of the intracellular CD11a is paired with CD18 and thus forms intact LFA-1 heterodimers in the CD11a-mYFP+ endosomal compartments, YFP endosomes from naive CD11a-mYFP/CD8+ T cells were analyzed on a native-PAGE together with total cell lysate. Immunoblotting with anti-GFP (to detect CD11a-mYFP) or anti-CD18 antibody revealed a single band presumably corresponding to an intact heterodimeric LFA-1 (CD11a-mYFP/CD18) with both antibodies and no evidence of unpaired single subunit of CD11a or CD18 was detected (Fig. 5 C). We then compared these immunoblots with those of denatured samples and observed a band at ~180 kD with anti-GFP corresponding to the CD11a-mYFP single chain and a band at ~95 kD with anti-CD18 corresponding to single-chain CD18 of LFA-1 (Fig. 5 C). Therefore, this approach allowed us to fractionate a highly purified LFA-1+ endosome population free of cytosolic and cell membrane contaminants.

Using this highly pure endosome fraction, we first confirmed that LFA-1+ endosomes are not associated with CD3ζ+ endosomes required for TCR signals (Fig. 5 B, right). To further investigate a potential cell-recycling pathway associated with LFA-1+ endosomes in naive T cells, we screened purified LFA-1+ endosomes isolated from naive CD8+ T cells generated from OT-I mice 56 h after infection with influenza virus x31-OVA [as described in C]. Transferred cells were sorted on proliferation dye expression and stained for cell surface receptors, Con, isotype control. Results are representative of three independent experiments (one mouse per experiment).

Figure 3. Intracellular LFA-1 redistribution leads to unequal partitioning during division. (A) Representative image of real-time cell division on ICAM-1–coated plates 30 h after co-culture of naive CD11a-mYFP/OT-I CD8+ T cells (yellow) with N4-pulsed BMDCs. H, LFA-1high; L, LFA-1low. Bar, 5 µm. (B) Quantification of relative LFA-1 expression levels (mYFP intensity) of daughter T cells with and without APC contact during division. Data were analyzed based on real-time imaging under N4-conditions. Each circle represents the ratio of total YFP intensity in each daughter cell by proximity to APC (proximity vs. distal). For divisions occurring outside of APC contact (no APC contact), proximity was assigned arbitrarily and both proximal and distal cells from the same parent cell showed similar YFP intensity (fold change = 1). Circles represent individual cells from five independent experiments with mean shown as a red line. Data represent mean ± SEM; n = 5. * P < 0.0003. (C) Naive CD11a-mYFP/OT-I CD8+ T cells (1–3 × 10^6) were labeled with Cell Proliferation Dye eFluor670 and i.v. transferred 24 h before infection with influenza virus x31-OVA. 56 h after infection, transferred cells were sorted and identified as the first division (Div1) and undivided (Undiv) cells based on proliferation dye dilution. Cell surface CD8 and CD11a-mYFP showed asymmetric expression on the first-divide cells (Div1), but not the cell activation marker (CD69). Results are representative of 10 independent experiments (one mouse per experiment). (D) Flow cytometry analysis of cell surface receptors of the first division (Div1) and undivided (Undiv) CD8+ T cells generated from OT-I mice 56 h after infection with influenza virus x31-OVA [as described in C]. Transferred cells were sorted on proliferation dye expression and stained for cell surface receptors, Con, isotype control. Results are representative of three independent experiments (one mouse per experiment).
CD8+ T cells from Rab27 KO mice were comparable to naive CD8+ T cells from WT mice (Fig. 6A and Fig. S5, A–I). However, the redistribution of intracellular LFA-1 to the cell surface (Fig. 6C) and the contact site with APCs (Fig. 6B) was completely abolished in CD11a-mYFP/OT-I/Rab27 KO CD8+ T cells. Importantly, CD11a-mYFP/OT-I/Rab27 KO CD8+ T cells failed to induce asymmetric inheritance of both LFA-1 and CD8 after the first division and their retention time in the draining lymph node was comparable to LFA-1high CD8+ T cells (Fig. 6D). Additionally, the first-division CD8+ T cells from CD11a-mYFP/OT-I/Rab27 KO mice (KO Div 1) failed to exhibit similar bimodal patterns of cell migration as WT divided cells (WT Div 1; Fig. 6E). The frequency of APC conjugation of KO Div 1 versus WT Div 1 CD8+ T cells was measured. Among the cells imaged, 45% of WT Div 1 T cells spent more than 70% of imaging time forming stable contacts with OVA-loaded APCs, whereas the rest of the cells only made transient contacts. In contrast, the majority of KO Div 1 T cells (65% of total) never or only transiently (less than 30% of imaging time) contacted APCs. Therefore, we concluded that Rab27-mediated LFA-1 redistribution is a key regulator of the unequal partitioning of LFA-1 during the T cell division, which is critical for the distinct patterns of migration and APC conjugation during T cell differentiation (Fig. 4, A and B).

To corroborate our hypothesis that the differential expression of LFA-1 is the key functional determinant that dictates the distinct migration patterns of first-division LFA-1high proximal and LFA-1low distal daughter T cells, we evaluated OT-I/CD11a heterozygous KO mice (LFA-1 Het). Reduction of LFA-1 surface expression by 49–57% in LFA-1 Het CD8+ T cells was not altered early T cell activation, such as APC conjugation, CD69 and CD25 expression, and redistribution of intracellular LFA-1 expression, in naive LFA-1 Het CD8+ T cells (not depicted). However, reduced LFA-1 expression levels abolished the polarized localization of intracellular LFA-1 to the contact site between T cells and APCs and subsequent
differential expression of LFA-1 in the first-division CD8^+ T cells (Fig. 7, B and C). Importantly, the lack of asymmetric expression of LFA-1 in first-division T cells completely abolished the distinct patterns of T cell migration and APC interactions observed in WT first-division T cells (Fig. 7 D). Although our studies with LFA-1 Het T cells do not define the role of LFA-1 under normal physiology, these data suggest that asymmetric expression levels of LFA-1 in the first-divided CD8^+ T cells are essential for the distinct motility pattern, which may lead to the differential fates of daughter CD8^+ T cells. To corroborate our hypothesis that differential expression (high vs. low) of LFA-1 has an immunological consequence, we performed T cell memory experiments using Rab27 KO and LFA-1 Het mice. Because asymmetric cell division does not occur in these cells and there are no YFP high/low cells in the first division, we sorted total division 1 cells and transferred them into a naive WT recipient. We then infected the recipient mice with influenza virus and assessed the ability of Rab27 KO and LFA-1 Het cells to form T cell memory. Unlike WT T cells, Rab27 KO and LFA-1 Het T cells were unable to form the central memory, effector memory, and tissue-resident memory compartments 60 dpi (Fig. 7 E), suggesting that cell surface LFA-1 expression plays a key role in immunological memory formation.

Discussion

In this study, we generated CD11a-mYFP KI mice that allowed us to identify novel intracellular LFA-1 endosomes, which actively redistribute to the cell surface upon antigen stimulation. Redistribution and sequestration of LFA-1 to the immunological synapse during early T cell activation was required for unequal partitioning of LFA-1 into first-division daughter cells. Subsequent isolation of daughter T cells with unequal LFA-1 expression further revealed different functional phenotypes with distinct patterns of migration, APC conjugation, lymph node retention, and T cell effector programs. Interestingly, our in vivo egress assay demonstrated LFA-1^low^ T cells egress from the lymph node faster than LFA-1^high^ cells, suggesting these cells may home to other tissues. It is possible that LFA-1^low^ T cells migrate to other lymphoid organs to create additional inflammatory niches or that they reenter the same lymph node at a later time point after the tissue established a favorable microenvironment that facilitates altered differentiation. Alternatively, a highly migratory phenotype of LFA-1^low^ T cells may enable them to rapidly egress from the lymph node and home in to target peripheral tissues. These events might promote long-term developmental plasticity to enable both self-renewal and terminal differentiation at the target tissue sites. Therefore, we conclude that dynamic redistribution of intracellular LFA-1 during early antigen stimulation controls T cell differentiation and effector functions of daughter cells.

Recent studies have shown that important mediators of LFA-1 functions, including Rap1, Rap2, RapL, and Mst1, are contained in Rab5, Rab11, Rab13, and EEA1 vesicles (Fabbrì et al., 2005; Stanley et al., 2012; Svensson et al., 2012; Nishikimi et al., 2014) and that LFA-1 is endocytosed and recycles through a cholesterol- and Rab11-dependent manner through a YXXXΦ motif in the cytoplasmic region of the β2 subunit (Fabbrì et al., 2005). However, the presence of LFA-1 in these endosomal cargo and functions of the intracellular LFA-1 in naive T cell activation remains unknown. In addition to detection of endogenous LFA-1 redistribution in live T cells, our CD11a-mYFP KI mice allowed us to isolated highly pure LFA-1^+ endosomes from naive T cells using anti-YFP immunoprecipitation. Our Western blot analysis with a polyclonal YFP antibody to isolate CD11a^+ endosome fractions revealed that Rab27 localized exclusively to LFA-1^+ endosomes and was not observed in
LFA-1+ endosomes (Fig. 5 D). The results from in vitro and in vivo experiments with Rab27 KO CD8+ T cells (Fig. 6) strongly support our conclusion that Rab27 is a key regulator for trafficking of intracellular LFA-1 to the cell surface, an essential step for asymmetric segregation of LFA-1 into first-division daughter T cells and subsequent distinct patterns of migration and APC interactions during T cell activation.

Surprisingly, we observed an extreme enrichment of LFA-1 at the point of contact between T cells and APCs during the early phase (<10 min) of immunological synapse formation, followed by prolonged accumulation of LFA-1 at the contact zone for 60 min (an ~5-fold increase until 30 min and an ~2.5-fold increase between 30 min to 60 min; Fig. S3 C). TCR activation during the synapse formation triggers a cascade of signaling events, which may differentially regulate LFA-1 adhesiveness and distribution. In addition to the intracellular signals that directly regulate LFA-1 functions, centripetal flow of T cell actin cytoskeleton recruits LFA-1 to the synapse and induces mechanical maturations of distinct affinity status (Comrie et al., 2015). Interestingly, analysis of T cell–APC interaction
CD11a-mYFP+/-CD8+ T cells found in the draining lymph node and lung 60 dpi (CD11a+/-CD44high), and effector memory (TEM; CD62L neg, CD44high) CD11a-mYFP+ [integrin αEβ7]+T cells; Figure 7. LFA-1 expression and disparate migration patterns in first-division CD8+ T cells. 

Data represent mean ± SEM; mYFP/OT-I T cells: Het, CD11a-mYFP/OT-I/Rab27 KO T cells: Rab27KO). Het Div1 (*, P < 0.01). (E) Number of tissue-resident memory (TRM; CD103+) CD8+ T cells. Data are fit to nonlinear regression and multimodality was assessed with the Kolmogorov–Smirnov test. Asterisk indicates significance between WT Div1 and Het Undiv, n = 4 mice/group. *, P < 0.05.

**Figure 7.** CD11a heterozygous knockout T cells fail to induce asymmetric LFA-1 expression and disparate migration patterns in first-division CD8+ T cells. (A) Representative flow cytometry of surface (CD11a Ab) and total [mYFP] LFA-1; expression levels from CD11a-mYFP/OT-I Het and WT OT-I naive CD8+ T cells; n = 3 mice. (B) Quantification of relative fluorescence intensity of mYFP from CD11a-mYFP/OT-I (WT) versus CD11a-mYFP/OT-I Het CD8+ T cells at the contact site with Ag-bearing APCs (N4 or D7). Data are expressed as mean ± SEM of total 25–40 cells. (C) Representative flow cytometry analysis of asymmetric expression of CD11a-mYFP in WT (CD11a-mYFP/OT-I) versus LFA-1 Het (CD11α−/−mYFP/OT-I) CD8+ T cell division from x31-OVA–infected mice 56 hpi; n = 6 mice. (D) Frequency distribution of migration indices measured from CD11α-mYFP (WT Div1) and CD11α-mYFP/−/−Het Div1) divided or undivided CD8+ T cells. Data collected from three independent experiments (one mouse per experiment; 45–60 cells/mouse) were fit to nonlinear regression and multimodality was assessed with the Kolmogorov–Smirnov test. Asterisk indicates significance between WT Div1 and Het Div1 (*, P < 0.01). (E) Number of tissue-resident memory (TRM); CD103−[integrin αEβ7]+, CD62L-, CD44high, central memory (CM); CD62L+, CD44low, and effector memory (TEM); CD62L-CD44high CD11a-mYFP CD8+ T cells found in the draining lymph node and lung 60 dpi [CD11α−/−mYFP/OT-I; T cells: Het, CD11α-mYFP/OT-I/Rab27 KO T cells: Rab27KO]. Data represent mean ± SEM; n = 4 mice/group. *, P < 0.05.
CD8+ T cells primarily use LFA-1 (Van Seventer et al., 1990; Berlin-Rufenach et al., 1999; Wang et al., 2009; Contenko et al., 2010; Varga et al., 2010; King et al., 2012). Although previous studies highlighted the importance of LFA-1 interactions with its ligand ICAM-1 in CD8+ T cell memory development (Parameswaran et al., 2005; Ghosh et al., 2006; Scholer et al., 2008; Bose et al., 2013; Cox et al., 2013; Zumwalde et al., 2013), a direct link between LFA-1 and memory formation has yet to be identified. Our study provides insight into how the differential migration patterns mediated by asymmetric expression of LFA-1 control the fate decisions of daughter T cells.

The selectivity of naive CD8+ T cells to redistribute intracellular LFA-1 in recognition of strong, but not weak, antigenic stimulation suggests that cell surface LFA-1 expression may serve as an evolutionarily conserved mechanism in T cells. Such selectivity may ensure that the immune system uses the Rab27-mediated mechanism during appropriate immune activation against highly pathogenic infections. It would be interesting to investigate whether diversity in T cell memory is due, at least in part, to distinct LFA-1 redistribution patterns and thus changes in the occurrence of unequal LFA-1 partitioning during cell division by pathogens with different antigenicity. However, further studies to investigate direct roles of Rab27 in T cell memory formation in our system are infeasible, as examining memory T cell development in Rab27 KO is limited by the inability of these mice to clear primary infection. Both LFA-1 and Rab27 play critical roles in cytotoxic T cell functions, and although the host immune system can clear viral infection, the transferred KO T cells would subsequently differentiate in altered conditions. Nevertheless, further elucidating the contribution of LFA-1 in memory generation at later stages of T cell activation may be critical for future therapeutic developments.

Materials and methods

**Antibodies and reagents**

CCR7-PE (4B12), CXCR4-PE (2B11), CD11a-eFluor450 (M17/4), CD25-APC (PC61.5), purified CD62L (MEL-14), CD4-PE (GK1.5), MHCI-APC (H2Kb; AF6-88.5.5.3), and OneComp eBeads were purchased from eBioscience. CD69-BV605 (H1.2F3), CXCR5-BV421 (L138D7), CD8+ T cells primarily use LFA-1 (Van Seventer et al., 1990; Berlin-Rufenach et al., 1999; Wang et al., 2009; Contenko et al., 2010; Varga et al., 2010; King et al., 2012). Although previous studies highlighted the importance of LFA-1 interactions with its ligand ICAM-1 in CD8+ T cell memory development (Parameswaran et al., 2005; Ghosh et al., 2006; Scholer et al., 2008; Bose et al., 2013; Cox et al., 2013; Zumwalde et al., 2013), a direct link between LFA-1 and memory formation has yet to be identified. Our study provides insight into how the differential migration patterns mediated by asymmetric expression of LFA-1 control the fate decisions of daughter T cells.

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25 µg lipopolysaccharide (Sigma). Where noted, altered peptide ligands were used at the same concentration (D7: SHINFEDL; BioPeptide).

**Competitive egress assay**
For the competitive egress assay, equal numbers of T cells (10^6 each) were differentially labeled and i.v. injected to WT recipients. Lymph nodes were harvested at 24 h, and single-cell suspensions were analyzed by flow cytometry. The homing index was calculated as the ratio between the number of each differentially-labeled cell population present. For the first-division egress assay, 100 µg anti-CD62L (BD) and 1 µg/g FTY720 (Cayman Chemicals) were injected i.v. and i.p., respectively. For the first-division egress assay, 100 µg anti-CD62L (BD) and 1 µg/g FTY720 (Cayman Chemicals) were injected i.v. and i.p., respectively, 12 h before harvest. The homing index of Div1 YFPhigh or Div1 YFP<sub>low</sub> cells was calculated as the ratio between CD62L-treated mice and (CD62L + FTY720)–treated mice.

**T cell memory**
For memory assays, LFA-1<sup>high</sup> and LFA-1<sup>low</sup> cells were harvested at 56 hpi from influenza-infected mice and sorted based on YFP expression. 2,000 LFA-1<sup>high</sup> or LFA-1<sup>low</sup> cells were transferred into a naive WT recipient. Mice were then inoculated with X31-OVA. To distinguish cells in the vasculature versus tissue, mice were treated with CD8β-APC (BD) i.v. 3 min before harvest. Draining lymph node, spleen, and lung were harvested 8 or 60 dpi. Macerated lung tissue was digested with 5 mg/ml collagenase/dispace (Roche) for 1 h at 37°C. To distinguish memory phenotypes, single-cell suspensions were stained with CD44-BV421 (BioLegend), CD62L-PE/Cy7 (BD), CD103-BV711 (BD), and TCRβ-BV605 (BD).

**In vitro imaging**
Cell migration chambers (Millipore; Bioptech) were prepared by coating their glass bottom with 5 µg recombinant mouse ICAM-1 (Sino Biological) in PBS with or without indicated chemokines. For in vitro migration imaging, leukocytes were placed in L15 medium (Invitrogen) in the chamber at 37°C and video microscopy was conducted using a TE2000-U microscope (Nikon) coupled to a CoolSNAP HQ CCD camera with a 20x objective (CFI Plan Fluor ELWD DM; Nikon) and 0.45 numerical aperture. For conjugation studies, 10 µm peptide-pulsed BMDCs adhered to a Delta T dish coated with ICAM-1 and 2 µg CCL21 (R&D) for 1 h before imaging. For in vitro imaging studies, BMDCs and naive CD8 T cells were cultured on ICAM-1–coated Delta T dish in complete media with 20% FCS and 5 µg/ml rhIL-2 (PeproTech) for 24 h. BMDCs were fixed, and stained for LFA-1 surface levels. Experiments were normalised to control groups (PBS-pulsed BMDCs). For image-based conjugation studies, 10 µM peptide-pulsed BMDCs adhered to a Delta T dish coated with ICAM-1 and 2 µg CCL21 (R&D) for 1 h before imaging. T cells were added at a 1:1 ratio for the indicated times, and conjugate frequencies were determined from live imaging. All conjugation studies were normalized to no antigen controls (PBS-pulsed BMDCs).

**Flow cytometry**
For flow cytometry–based LFA-1 surface studies, naive CD8<sup>+</sup> T cells were co-cultured with antigen-pulsed BMDCs for the indicated times, fixed, and stained for LFA-1 surface levels. Experiments were normalized to no-antigen controls (PBS-pulsed BMDCs), and fold change was determined from time 0 (T cells only, no BMDCs). We used a BD LSR II flow cytometer with a solid-state Coherent Sapphire blue laser (20-100 mW at 488 nm), and the emission signal was detected by an associated photomultiplier tube.

**Endosome isolation and Western blot analysis**
Purified CD8<sup>+</sup> T cells were homogenized as previously described (Graham, 2002) in Diluent (50 mM Hepes-NaOH, 500 mM KOAc, and 5 mM MgOAc) and Halt protease and phosphatase inhibitor with a 27G needle 25 times and mixed with 50% solution (Diluent, 0.25 mM sucrose; Optiprep) for a 30% density. The gradient was loaded from bottom to top with the following densities: 2 ml of 30% homogentane, 8 ml of 25%, and 2 ml of 5%. Gradients were subjected to 250,000 g for 3 h at 4°C. Endosomes were collected from the 25–5% interface and processed for immunoprecipitation with anti-GFP (mouse monoclonal 3E6; Molecular Probes) covalently linked to CrossLink IP beads (Pierce). YFP<sup>−</sup> endosomes were diluted in PBS and subjected to 100,000 g for 10 h at 4°C. Both YFP<sup>+</sup> beads and YFP<sup>−</sup> pellet were re-suspended in Laemmli sample buffer (Bio-Rad) and boiled. For Western blotting, the membrane was blocked with 5% nonfat milk or 5% BSA in PBS plus 0.1% Tween 20 for 30 min after proteins were transferred from PAGE Gold Precast 4–20% Tris-Glycine gel (Lonza). Blots were incubated overnight at 4°C with 1:1,000 of anti-GFP (ab290; Abcam), CD3z (H146-968; Thermo) EEA1 (CST), Rab4 (BD), Rab7 (CST), Rab8 (CST), Rab11 (Abcam), Rab13 (Abcam), Rab21 (Santa Cruz), and Rab27a (Santa Cruz). The membrane was then incubated with 1:5,000 horseradish peroxidase–conjugated anti-rabbit or anti-mouse Fc-specific IgG antibody (Jackson ImmunoResearch) for 1 h at room temperature. Protein was detected using Super Signal chemiluminescent reagent (Thermo). Where described, native PAGE Bis-Tris Gels (nonreducing) were used (Thermo).

**Statistical analysis**
All statistical tests were done with GraphPad Prism and Jmp Software (SAS). For frequency distribution analyses, nonlinear regression was performed and multimodality was assessed with the Kolmogorov–Smirnov test. Analyses of multiple variances were analyzed with two-way ANOVA with a Bonferroni post-test. Other analyses used one-way ANOVA with a Bonferroni post-test, unpaired t test, and Mann–Whitney when appropriate.

**Online supplemental material**
Fig. S1 shows LFA-1 expression and function in CD11a-mYFP mice. Fig. S2 shows expression of intracellular LFA-1 in naive CD8<sup>+</sup> T cells from WT and CD11a-mYFP mice. Fig. S3 shows LFA-1 redistribution.
to the T cell surface. Fig. S4 shows correlation of CD11a-mYFP high and low populations with CD8 expression. Fig. S5 shows that LFA-1 expression and functions are comparable in naive CD8+ T cells from WT and Rab27 KO mice. Video 1 shows localization of LFA-1 in naive CD8+ T cells. Video 2 shows localization of LFA-1 during naive CD8+ T cell migration. Video 3 shows intracellular LFA-1 redistribution to the contact site with Ag-bearing APCs. Video 4 shows that intracellular LFA-1 fails to redistribute to the contact site upon encountering APCs bearing altered peptide ligands with reduced TCR affinity. Video 5 shows that CD8+ T cell asymmetrically divides in real time.

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The authors declare no competing financial interests.

Author contributions: T. Capece conducted most of the experiments and performed the statistical analysis of the data; B.L. Walling helped with virus infection, mouse injections, and in vitro imaging; S. Boe performed cytotoxic T cell killing assay. K.-D. Kim and K. Lim helped with western blot analysis. H.-L. Chung designed MATLAB algorithm. D.J. Topham assisted with experimental design. M. Kim conceived and directed the study. T. Capece and M. Kim wrote the manuscript with suggestions from all authors.

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