LFA-1 Regulates CD8$^+$ T Cell Activation via T Cell Receptor-mediated and LFA-1-mediated Erk1/2 Signal Pathways$^*$

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LFA-1 regulates T cell activation and signal transduction through the immunological synapse. T cell receptor (TCR) stimulation rapidly activates LFA-1, which provides unique LFA-1-dependent signals to promote T cell activation. However, the detailed molecular pathways that regulate these processes and the precise mechanism by which LFA-1 contributes to TCR activation remain unclear. We found LFA-1 directly participates in Erk1/2 signaling upon TCR stimulation in CD8$^+$ T cells. The presence of LFA-1, not ligand binding, is required for the TCR-mediated Erk1/2 signal pathway. LFA-1-deficient T cells have defects in sustained Erk1/2 signaling, and TCR/CD3 clustering, which subsequently prevents MTOC reorientation, cell cycle progression, and mitosis.

LFA-1 regulates the TCR-mediated Erk1/2 signal pathway in the context of immunological synapse for recruitment and amplification of the Erk1/2 signal. In addition, LFA-1 ligation with ICAM-1 generates an additional Erk1/2 signal, which synergizes with the existing TCR-mediated Erk1/2 signal to enhance T cell activation. Thus, LFA-1 contributes to CD8$^+$ T cell activation through two distinct signal pathways. We demonstrated that the function of LFA-1 is to enhance TCR signaling through the immunological synapse and deliver distinct signals in CD8$^+$ T cell activation.

Leukocyte function-associated antigen 1 (LFA-1)$^2$ plays an important role in regulating leukocyte adhesion and T cell activation (1, 2). LFA-1 consists of the $\alpha$L (CD11a) and $\beta$2 (CD18) subunits. The ligands for LFA-1 include intercellular adhesion molecular 1 (ICAM-1), ICAM-2, and ICAM-3 (3).

LFA-1 participates in the formation of the immunological synapse, which regulates T cell activation synergistically with TCR engagement. The immunological synapse is a specialized structure that forms between the T cell and the APC or target cell (1, 2, 4). The function of the immunological synapse is to facilitate T cell activation and signal transduction. Mice deficient in LFA-1 (CD11a KO) have defects in leukocyte adhesion, lymphocyte proliferation, and tumor rejection (5–7).

Upon TCR stimulation, the nascent immunological synapse is initiated with surface receptor clustering and cytoskeleton rearrangement, then followed by mature synapse formation after prolonged stimulation (8, 9). In the mature immunological synapse, LFA-1 forms a ring-like pattern at the peripheral supramolecular activation cluster (pSMAC), which surrounds the central supramolecular activation cluster (cSMAC) containing TCR/CD3/lipid rafts (10, 11). The structure of the mature synapse is stable for hours and thought to be important for sustained TCR signaling (12–14). LFA-1 functions via pSMAC to stabilize the cSMAC and is associated with the induction of T cell proliferation, cytokine production, and lytic granule migration toward cSMAC (1, 15). Although LFA-1-containing pSMAC is self-evident in lipid bilayer systems and cell lines, whether it is required for T cell activation under physiological conditions remains controversial (15).

TCR stimulation rapidly induces the functional activity of LFA-1, which then provides unique LFA-1-dependent signals to promote T cell activation (16). The process can be divided into two steps. First, the intracellular signaling from TCR regulating LFA-1 activation is known as “inside-out” signaling; second, activated LFA-1, as a signaling receptor, can feedback to transduce the intracellular signal, the “outside-in” signaling (1, 17). It is widely accepted that TCR stimulation activates LFA-1 through affinity and/or avidity regulation, as supported by increased adhesion to ICAM-1 and pSMAC formation (16, 17). The “inside-out” signal process has been investigated extensively (18–21). The TCR proximal signal molecules, Lck, ZAP-70, and PI3K, are known to be important for TCR signaling to LFA-1 activation (22–26). The molecular mechanisms of LFA-1 “outside-in” signaling have been explored only recently. Perez et al. (27) have demonstrated that LFA-1 and ICAM-1 ligation activates the downstream Erk1/2 MAPK signaling pathway upon TCR stimulation, which ultimately leads to the qualitative modu-
LFA-1 Regulates Erk1/2 Signal Pathway in CD8+ Cells

Ligation of CD4+ T cell activation through distinct LFA-1-dependent signals. Another recent study provided compelling evidence that LFA-1 reshapes the Ras MAPK pathway downstream of TCR (28). However, the detailed molecular pathways that regulate these processes are poorly defined. Especially, the evidence in support of a distinctive role for LFA-1 in the T cell signaling pathway has lagged behind; whether the function of LFA-1 is to enhance TCR signaling through the immunological synapse and/or deliver distinct signal in T cell activation and whether LFA-1 is indispensable for or merely assists the existing TCR signal pathway. Furthermore, whether and how TCR proximal signal molecules regulate LFA-1 function remains unknown. Further studies are required to understand the LFA-1 and TCR signaling network.

In this study, we found that LFA-1 directly participates in CD8+ T cell activation. Upon TCR stimulation, LFA-1 regulates both TCR-mediated and LFA-1-mediated Erk1/2 signal pathways. First, the presence of LFA-1, not ligand binding, is required for the sustained Erk1/2 signaling and TCR/CD3 clustering on the surface of CD8+ T cells, subsequently leading to MT0C reorientation, cell cycle progression, and mitosis. Second, LFA-1 ligation with ICAM-1 enhances Erk1/2 signaling, which promotes T cell activation with increased IL-2 production and cell proliferation. This LFA-1-mediated Erk1/2 signal pathway integrates with the existing TCR-mediated Erk1/2 signal pathway to enhance T cell activation.

EXPERIMENTAL PROCEDURES

Animals—C57BL/6 mice were purchased from the Animal Production Area at NCI Frederick. CD11a KO mice (C57BL/6 background) were kindly provided by Dr. Christie Ballantyne (Baylor College of Medicine). The animal experiments were approved by the Institutional Animal Care and Use Committee at University of Texas M. D. Anderson Cancer Center.

Reagents—The monoclonal antibodies specific to mouse CD3 (145-2C11), CD4 (H129.19), CD8 (53-6.7), IL-2 (JES6-5H4), IFN-γ (XMG1.2), and Ki-67 (B56) were from BD Biosciences (San Diego, CA); TNF-α (MP6-XT22) was from eBioscience (San Diego, CA), and TCR-β was from Invitrogen Detection Technologies (Carlsbad, CA). The recombinant mouse ICAM-1/Fc chimera was from R&D Systems (Minneapolis, MN). The rabbit anti-mouse phospho-p44/22 MAPK (Thr-202/Tyr-204) antibody was obtained from Cell Signaling Technologies. The secondary Abs AlexaFluor 647 (1:50), and anti-mouse CD11a AlexaFluor 647 goat anti-rabbit IgG, AlexaFluor 647 goat anti-rat IgG, AlexaFluor 647 cholera toxin subunit B (CTB), and Prolong Anti-Fade™ mounting medium were purchased from Invitrogen Detection Technologies. The anti-β-tubulin (TUB2.1) was purchased from Sigma-Aldrich. The piceatannol, LY294002, cytochalasin D, and latrunculin were from EMD Biosciences (Darmstadt, Germany), and PP-1 was from Biomol (Plymouth Meeting, PA).

Cell Isolation and Stimulation—The single cell suspension was prepared from spleen and lymph nodes from C57BL/6 mice or CD11a KO mice by a standard method. CD8+ T cells were purified with mouse CD8α+ T Lymphocyte Enrichment Set-DM from BD Biosciences (San Diego, CA). In brief, 5 µl of biotin-antibody mixture including biotin-conjugated monoclonal antibodies against CD4 (GK1.5), CD11b (M1/70), CD45R/B220 (RA3-6B2), CD49b (HMo2), and TER-119/erythroid cells (TER-119) were mixed with 1 × 10^6 cells for 10 min on ice. Then, 5 µl of the BDTM Imag Streptavidin Particles Plus-DM were added to the single cell suspension, and CD8+ T cells were negatively selected with the BDTM IMagnet. The purified CD8+ T cells were diluted to 1 × 10^6 cells/ml in RPMI 1640 medium in 24-well microtiter plates and stimulated with coated anti-CD3 antibody in the presence or absence of coated ICAM-1 for indicated times.

Intracellular Staining—The intracellular staining was performed with the Fix & Perm cell permeabilization reagents according to the manufacturer’s protocol (Invitrogen) with a minor modification. For each sample, 1 × 10^6 cells were washed and resuspended in FACS buffer (phosphate-buffered saline, 0.1% NaN3, 1% fetal bovine serum). Cells were first incubated with various cell surface markers for 20 min, then washed and resuspended in reagent A (fixation medium). The rabbit anti-mouse phospho-p44/22 MAPK antibody was diluted in reagent B (permeabilization medium) and added onto cells. Then, cells were washed and incubated with secondary antibody AlexaFluor 647 goat anti-rabbit IgG. For intracellular IL-2, IFN-γ, and TNF-α staining, 10 µg/ml Brefeldin A was added for the last 8 h of stimulation. To detect intracellular cytokine production, cells were fixed (reagent A) and permeabilized (reagent B), then stained with AlexaFluor 647-conjugated IL-2, PE-Cy7-conjugated IFN-γ, and Pacific Blue-conjugated TNF-α antibody. Multicolor flow cytometry data were acquired with FACScalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Fluorescence Microscopy and Image Analysis—Fluorescence staining and image analysis of CD8+ T cells were prepared as described below. For cytoskeleton inhibition experiments, cells were pretreated with 100 µM cytochalasin D or 100 µM latrunculin A. For lipid raft experiments, cells were pretreated with 25 µg/ml AlexaFluor 647-conjugated CTB. After stimulation, cells were fixed with 4% paraformaldehyde, then stained with specific monoclonal antibodies conjugated with fluorescence dyes. The secondary antibody goat anti-hamster IgG AlexaFluor 594 (1:400) was used to observe the localization of CD3 on the cell surface. The anti-mouse CD8 AlexaFluor 488 (1:100), anti-mouse TCR-β AlexaFluor 647 (1:50), and anti-mouse CD11a AlexaFluor 488 (1:400) were used for cell surface staining. The rabbit anti-mouse phospho-p44/22 MAPK followed by secondary antibody AlexaFluor 647 goat anti-rabbit IgG in permeabilization medium were used to detect p-Erk1/2. The anti-β-tubulin Cy3 (1:200) was used to stain MT0C. After washing three times in phosphate-buffered saline, the cells were loaded onto poly-L-lysine-coated slides and mounted Prolong Anti-Fade™. Fluorescence images were acquired using a Leica TCS SE RS spectral laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) with the appropriate filters and a ×63 objective lens. Image analysis was performed using Leica ConfoScan Software (Leica Microsystems). Cells were examined with z stacks of 0.2 µm and at least 20 iterations.
RESULTS

LFA-1 Participates in Both TCR-mediated and LFA-1-mediated Erk1/2 Signaling—TCR stimulation and LFA-1 ligation can induce the activation of the Erk1/2 MAPK signal pathway in CD8$^+$ T cells and Jurkat cells (27, 29). Using the phospho-Erk1/2 flow cytometry assay and CD11a KO mice, we examined the role of LFA-1 and TCR stimulation in CD8$^+$ T cell activation. As shown in Fig. 1A (upper panel), purified CD8$^+$ T cells were stained with coated anti-CD3 antibody, the p-Erk1/2 activity was detected in 40.1% WT and 37.1% CD11a KO CD8$^+$ T cells after 5 min of stimulation. The mean fluorescence intensity (MFI) was 71.3 and 50.7, respectively. The p-Erk1/2 activity in WT (left panel) and CD11a KO (right panel) cells peaked at 5 min, and the signal subsided to background levels within 60 min. The percentage of p-Erk1/2-positive cells at each time point. Results are plotted as the mean of three independent experiments with S.D. The percentage and MFI of each population are displayed. B, phospho-Erk1/2 activity in CD8$^+$ T cells stimulated for 12 h. The panels from left to right are: WT cells (top) with various stimulation (unstimulated control, CD3 antibody, CD3 antibody and ICAM-1, ICAM-1) and CD11a KO cells (bottom) with various stimulation (unstimulated control, CD3 antibody, CD3 antibody and ICAM-1). The numbers in the quadrants represent the percentage of each population. The MFI of cell populations with relatively high p-Erk1/2 (p-Erk1/2$^{high}$) and low p-Erk1/2 (p-Erk1/2$^{low}$) activities are displayed in circles. The data are representative of at least three independent experiments.
LFA-1 Regulates Erk1/2 Signal Pathway in CD8+ Cells

A 30 min

WT

CD8

CD3

p-Erk1/2

Overlay

Membrane Intracellular

CD11a KO

12 hr

WT

CD8

CD3

p-Erk1/2

Overlay

Membrane Intracellular

CD11a KO

B

Cells with CD3 caps (%)

WT

unstimulated

WT +CD3

+Cyto. D

WT +CD3

+Latr. A

WT +CD3

CD11a KO +CD3

P < 0.001

Cells with CD3 caps (%)

WT

unstimulated

WT +CD3

CD11a KO +CD3

P < 0.001
LFA-1 Regulates Erk1/2 Signal Pathway in CD8\(^+\) Cells

activation (2, 4, 8–9). To further investigate whether LFA-1 plays a role in sustained Erk1/2 signaling in CD8\(^+\) T cells, we measured p-Erk1/2 activity after prolonged TCR cross-linking with CD3 antibody for 6–18 h. As shown in Fig. 1B, there were two distinct subsets of cells with intracellular p-Erk1/2 activity in the presence of ICAM-1: one with relatively high amounts of p-Erk1/2 (p-Erk1/2\(^{2\text{High}}\)) and one with relatively low amounts of p-Erk1/2 (p-Erk1/2\(^{2\text{Low}}\)). The p-Erk1/2 activity measured by the MFI in the p-Erk1/2\(^{2\text{High}}\) subset was 17 times that of the p-Erk1/2\(^{2\text{Low}}\) subset (815 versus 50). However, the CD3 antibody stimulation alone only induced the p-Erk1/2\(^{2\text{Low}}\) subset. The p-Erk1/2 activity of cells stimulated with ICAM-1 in the absence of TCR cross-linking was similar to that of the unstimulated control without any detectable p-Erk1/2 activity, which indicated that signal from LFA-1 ligation with ICAM-1 alone could not activate the Erk1/2 signal pathway. In addition, only the p-Erk1/2\(^{2\text{High}}\) but not the p-Erk1/2\(^{2\text{Low}}\) could be inhibited with an LFA-1-blocking antibody M17/4, which abolished LFA-1 binding to ICAM-1 (data not shown). Thus, LFA-1 ligation with ICAM-1 provided a unique LFA-1-dependent Erk1/2 signal upon TCR stimulation. Furthermore, there was only a minimal amount of detectable p-Erk1/2 activity in CD11a KO cells upon continuous TCR stimulation for 12 h (Fig. 1B), although a significant amount of p-Erk1/2 was detected 5 min after CD3 antibody stimulation (Fig. 1A). Therefore, the p-Erk1/2\(^{2\text{Low}}\) subset is generated with continuous TCR stimulation in the presence of LFA-1.

In summary, we found that LFA-1 contributes to CD8\(^+\) T cell activation in two ways: first, the presence of LFA-1 is required for the p-Erk1/2 activity induced by TCR stimulation, which is represented by the p-Erk1/2\(^{2\text{Low}}\) subset (TCR-mediated Erk1/2 signaling); second, the ligation of LFA-1 with ICAM-1 significantly enhances intracellular Erk1/2 signaling, which is represented by the p-Erk1/2\(^{2\text{High}}\) subset (LFA-1-mediated Erk1/2 signaling). Although TCR stimulation and LFA-1 ligation can activate Erk1/2 signaling in CD8\(^+\) T cells, these signal pathways depend on the presence of LFA-1 on the cell surface.

The Presence of LFA-1 Is Essential for TCR-mediated Erk1/2 Signaling and TCR/CD3 Clustering—To further investigate the mechanism of LFA-1 in regulating TCR-induced Erk1/2 activity, we used fluorescence microscopy to examine the subcellular location and dynamics of p-Erk1/2 activity. As shown in Fig. 2A (upper panel), p-Erk1/2 was enriched proximal to the membrane and associated with the prominent CD3 macro-cluster in the WT CD8\(^+\) T cells after 30 min of TCR stimulation. The relative location and quantity of p-Erk1/2 (blue line) and CD3 (red line) were plotted on the right side of the overlay image, with two peaks of p-Erk1/2 and CD3 co-localized to the cell membrane. In the CD11a KO cells, the predominant pattern was different with multiple CD3/p-Erk1/2 micro-clusters distributed on the cell membrane. However, the p-Erk1/2 activity was evident and co-localized with CD3 proximal to the cell membrane. This is consistent with the data in Fig. 1A showing that p-Erk1/2 activity in WT and CD11a KO cells was comparable. After 8–12 h of stimulation with CD3 antibody, p-Erk1/2 remained co-localized with CD3 macro-clusters in the WT CD8\(^+\) T cells (Fig. 2A, middle panel). Furthermore, p-Erk1/2 was translocated inside of the cell, and a significant amount of p-Erk1/2 activity was detected in the cytosol, which indicated the continuous recruitment and amplification of p-Erk1/2 upon TCR stimulation. However, the p-Erk1/2 activity was barely detectable proximal to the membrane in CD11a KO cells, although CD3 micro-clusters remained evenly distributed across the cell surface. In addition, the p-Erk1/2 signal inside of the cells was minimal and at background levels. Although the p-Erk1/2 activity can be transiently initiated upon TCR stimulation in CD11a KO cells, the sustained Erk1/2 signaling requires the presence of LFA-1.

The cytoskeleton remodeling and lipid rafts have been demonstrated to be involved in TCR clustering and immunological synapse formation (1, 30, 31). To examine the role of LFA-1 in regulating CD3 macro-cluster formation, CD8\(^+\) T cells were preincubated with CTB before CD3 stimulation. As shown in Fig. 2A (bottom panel), a CD3 macro-cluster was associated with the lipid microdomain enriched with GM1 gangliosides in WT cells upon TCR stimulation for 30 min. The CD3 macro-clusters were detected in about 50% of the WT cells after 30 min of TCR stimulation (left panel in Fig. 2B). In the CD11a KO cells, there was a significant reduction of cells containing CD3 macro-clusters (18.75%), which was similar to WT control (13.77%), WT cells pretreated with actin polymerization inhibitor cytochalasin D (11.64%), or latrunculin A (18.61%). After 12 h of TCR stimulation (right panel in Fig. 2B), CD3 macro-clusters presented on 80.72% of the wild-type cells compared with 10.69% CD11a KO cells. Thus, LFA-1 plays an essential role in CD3 macro-cluster formation, which maintains the sustained Erk1/2 signaling. The presence of LFA-1 on the cell sur-

FIGURE 2. The presence of LFA-1 is required for sustained Erk1/2 signaling and CD3 macro-cluster formation induced by TCR stimulation. CD8\(^+\) T cells from WT or CD11a KO mice were stimulated with coated anti-CD3 antibody for the indicated times. A, LFA-1 is required for sustained Erk1/2 signaling induced by TCR stimulation. Cells were harvested and stained with CD6 (green), CD3 (red), and phospho-p44/22 MAPK antibodies (blue). For lipid raft experiments, cells were pretreated with CTB (blue) before the stimulation, and then stained with CD3 (red) and CD8 antibodies (green). Images were acquired using Leica TCS SE RS spectral laser-scanning confocal microscopy with the appropriate filters and a 63 oil objective lens. Images in the middle sections of the cells were captured, and each set of images was representative of at least 50 cells examined in three independent experiments. In the merged images, the arrowheads indicate the enrichment and colocalization of CD3 and p-Erk1/2 on the cell membrane. In the fluorescence intensity profiles of p-Erk1/2 (blue line) and CD3 (red line), cells were quantified using the Quantity Package of the Leica Confocal Software (version 2.61 of LCS Lite), with sampling at every 1.16E-08 µm distance and averaged for the mean total fluorescence intensity in arbitrary fluorescence units. The vertical lines represent the boundaries of the cell membrane and intracellular fluorescence signals. B, LFA-1 is required for CD3 macro-cluster formation induced by TCR stimulation. CD8\(^+\) T cells from WT and CD11a KO mice were quantified after stimulation for 30 min (left panel) or 12 h (right panel). In addition, WT cells were stimulated for 30 min in the presence or absence of cytochalasin D (100 µM) or latrunculin A (100 µM). AlexaFluor 594-conjugated secondary antibody to CD3 and AlexaFluor 488-conjugated CD8 antibody were used to detect CD3 macro-clustering (capping) on CD8\(^+\) T cells under a confocal microscope. The plot represents the percentages of cells with CD3 capping with three independent experiments analyzed. The samples are the WT unstimulated (n = 208), WT with CD3 antibody in the presence of cytochalasin D (n = 567) or latrunculin A (n = 274), WT with CD3 antibody (n = 875), CD11a KO with CD3 antibody (n = 271), respectively. A significance level of p < 0.001 was determined using the WT CD3 antibody stimulation sample as the reference.

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LFA-1 Regulates Erk1/2 Signal Pathway in CD8$^+$ Cells

**A**

12 hr

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**B**

Polar (%)

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**C**

Polar (%)

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**D**

Ki-67

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Cells (%)

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face is required for TCR-mediated Erk1/2 activity and TCR/CD3 clustering.

Requirement of LFA-1 for MTOC Reorientation, Cell Cycle Progression, and Mitosis Induced by TCR Stimulation—Upon TCR stimulation, MTOC is reoriented to the immunological synapse through cytoskeleton reorganization, and it has been proposed that microtubules anchor in the pSMAC (32–34). In addition, Erk1/2 signaling is required for the polarization of MTOC in NK cells (35). Because the presence of LFA-1 is required for the Erk1/2 signaling in CD8+ T cells, we examined MTOC localization in WT and CD11a KO cells. Upon TCR stimulation for 30 min, there were slightly increased MTOCs reoriented to the proximal cell membrane in WT cells compared with CD11a KO (data not shown). After TCR stimulation for 12 h, the MTOC was reoriented to the CD3 macro-cluster in WT cells, whereas the MTOC remained inside of the CD11a KO cells (upper panel in Fig. 3A). As shown in Fig. 3B, there were 77.7% WT cells with polarized MTOC in comparison to only 17.24% CD11a KO cells. After 48 h of stimulation, the WT CD8+ T cells were mostly premitotic blasts with a single polarized MTOC (74.78%) and about 10.81% mitotic cells with two polarized MTOCs (lower panel in Fig. 3A). We observed the dissolution of cytoskeleton structure including MTOC and significant apoptosis in CD11a KO cells after 48 h of stimulation (data not shown). Thus, in the absence of LFA-1, the MTOC polarization induced by TCR stimulation is defective.

We further investigated whether the absence of LFA-1 and Erk1/2 signaling plays a role in cell cycle entry and mitosis. The percentage of viable cells in the G0, G1, S, and G2/M phases were determined by double staining of Ki-67 and PI (36, 37). As shown in Fig. 3D, there were 5.02, 3.03, and 2.63% wild-type cells in G1, S, and G2/M phases respectively in WT cells after 48 h of stimulation. Although CD11a KO cells could progress through the cell cycle, there were reduced cells in G1, S, and G2/M phases (3.33, 1.05, and 1.08%). In addition, there were increased dying cells in CD11a KO cells in comparison to WT (16.7% versus 42.1%). Collectively, CD11a KO CD8+ T cells can enter the cell cycle upon TCR stimulation but at a much slower rate than WT.

LFA-1-mediated Erk1/2 Signaling Enhances the Activation and Proliferation of CD8+ T Cells—We further investigated the functional consequences of LFA-1 ligation with ICAM-1 in CD8+ T cell activation. Intracellular IL-2, TNF-α, and IFN-γ were measured simultaneously 12 h after stimulation with CD3 antibody or CD3 plus ICAM-1. As shown in Fig. 4A (upper panel), there were two subsets of activated CD8+ T cells with relatively high and low levels of intracellular IL-2 in the presence of ICAM-1. The total amount of intracellular IL-2 was significantly increased in the presence of ICAM-1 compared with CD3 antibody stimulation alone (MFI: 133 versus 18), as shown in the lower panel of Fig. 4A. Thus, the activation of the Erk1/2 signal pathway by LFA-1 ligation enhances IL-2 production in CD8+ T cells. In addition, the cells positive for intracellular IL-2 also coexpressed TNF-α and IFN-γ. Whereas the amount of TNF-α remained the same in the presence of ICAM-1, the production of IFN-γ was significantly increased as shown in Fig. 4A (lower panel).

Subsequently, we examined the effect of LFA-1 ligation on CD8+ T cell proliferation. As shown in Fig. 4B (upper panel), there were similar amounts of cells entering G1 phase after 72 h of stimulation with CD3 antibody in the absence or presence of ICAM-1 (13.6 versus 13.9). However, LFA-1 ligation with ICAM-1 produced a higher percentage of cells in the S phase than did CD3 antibody stimulation alone (7.4 versus 6.43), and a similar effect was seen in cells entering G2-M phase (3.6 versus 2.22). Overall, there were significantly increased cells in cycle (S+G2-M) in the presence of ICAM-1 (Fig. 4B, lower panel). Thus, LFA-1 ligation with ICAM-1 promotes cell cycle entry of CD8+ T cells.

DISCUSSION

We have investigated the molecular mechanisms of LFA-1 in CD8+ T cell activation. We found that LFA-1 regulates CD8+ T cell activation through two distinct signal pathways. Upon CD3 stimulation, the presence of LFA-1 on the cell surface is required for TCR-mediated Erk1/2 signaling. In addition, LFA-1 ligation with ICAM-1 produces a unique LFA-1-mediated Erk1/2 signal, which integrates with the TCR-mediated Erk1/2 signal for optimal T cell activation. Our data demonstrated the differential contribution of TCR stimulation and LFA-1 ligation in the activation of the Erk1/2 pathway.

We have shown that LFA-1 plays an essential role in the sustained Erk1/2 signaling induced by TCR stimulation. LFA-1 is not required to initiate the TCR-mediated Erk1/2 signal pathway because Erk1/2 signaling occurs in the absence of LFA-1. However, the presence of LFA-1 is required for TCR/CD3 macro-cluster formation and sustained Erk1/2 signaling. Subsequently, the CD8+ T cells commit to the activation program such as cytokine production and cell cycle progression. We demonstrated that the TCR/CD3 macro-cluster is co-localized with p-Erk1/2 activity. Newly formed TCR/CD3 micro-clusters are actively transported and reorganized into the macro-cluster upon TCR stimulation (1, 4, 38). The absence of LFA-1 results in defective TCR/CD3 macro-cluster formation, which has a structural and functional similarity to the cSMAC. The mature immunological synapse is a long-lived structure, and the
LFA-1 Regulates Erk1/2 Signal Pathway in CD8+ Cells

A

Unstimulated
CD3
CD3+ICAM-1

IL-2
CD8

B

Unstimulated
CD3
CD3+ICAM-1

Ki-67
PI

Cells (%)

G1
S+G2-M

Mean fluorescence intensity (MFI)

P < 0.05

CD3
CD3+ICAM-1
cSMAC is required for the maintenance of sustained signaling (1–2, 4). In the absence of LFA-1 and the TCR/CD3 macro-cluster as an anchor, the local concentration of p-Erk1/2 is not maintained and amplified over time, and the MTÖC does not polarize toward the immunological synapse. This results in incomplete CD8+ T cell activation.

The presence of LFA-1 is essential for sustained TCR-mediated Erk1/2 signaling (p-Erk1/2low), and LFA-1-mediated Erk1/2 signaling (p-Erk1/2High) promotes the optimal activation of CD8+ T cells. Both signal pathways depend on TCR and LFA-1. To investigate whether the LFA-1-mediated Erk1/2 signal pathway is directly regulated by the proximal signals from TCR stimulation, we used the pharmacological inhibitors Lck (PP1), PI3K (LY294002), and ZAP70 (piceatannol). As shown in supplemental Fig. S1A, preincubation of CD8+ T cells with PP1, piceatannol, or LY294002 blocked TCR-mediated Erk1/2 activity (p-Erk1/2low), regardless of the absence (upper panel) or presence (lower panel) of ICAM-1. However, the p-Erk1/2High subset resulting from LFA-1 ligation with ICAM-1 was not affected by LY294002 and piceatannol, but partially impaired by PP1 with 66% reduction of cells with p-Erk1/2High activity (supplemental Fig. S1B). These data indicated the existence of two signal pathways: the TCR-mediated Erk1/2 signal pathway (p-Erk1/2Low) depends on Lck, ZAP-70, and PI3K, whereas the LFA-1-mediated Erk1/2 signal pathway (p-Erk1/2High) is dependent on Lck but independent of ZAP-70 and PI3K. Thus, TCR-mediated and LFA-1-mediated Erk1/2 signal pathways are differentially regulated by proximal signals from TCR activation.

Lck, ZAP-70, and PI3K function sequentially to initiate TCR signal transduction and activate the downstream Erk1/2 MAPK pathway (15, 23). In addition, the TCR-dependent Lck/Zap70/PI3K pathway can trigger actin cytoskeleton rearrangement and recruit LFA-1 to the peripheral zone of the pSMAC (17, 21). We have demonstrated that the TCR-mediated Erk1/2 signal pathway depends on Lck, ZAP-70, and PI3K. Furthermore, cytochalasin D and latrunculin A, inhibitors that disrupt the cytoskeleton, prevent sustained Erk1/2 activity and TCR/CD3 clustering on the cell surface. We propose that the function of LFA-1 in the TCR-mediated Erk1/2 signal pathway as follows: TCR signaling recruits and activates LFA-1 via cytoskeleton rearrangement; subsequently, LFA-1 functions via pSMAC to stabilize the cSMAC and sustain Erk1/2 signaling in T cell activation. Thus, LFA-1 regulates the TCR-mediated Erk1/2 signal pathway in the context of the immunological synapse.

The LFA-1-mediated Erk1/2 signal pathway, which is Zap70- and PI3K-independent but partially Lck-dependent, requires TCR stimulation and LFA-1 ligation for optimal CD8+ T cell activation. The p-Erk1/2High population indicates that LFA-1 ligation with ICAM-1 can significantly enhance intracellular Erk1/2 MAPK signaling in the presence of TCR stimulation. This is consistent with the previous observation that LFA-1 signaling cooperates with TCR stimulation to augment phosphorylation of Erk1/2 and lower the activation threshold in CD4+ T cells (27). Here, we demonstrated that LFA-1 influences CD8+ T cell activation by providing a distinct signal through ligation with ICAM-1, which integrates with the TCR-mediated Erk1/2 signal to enhance T cell activation.

LFA-1 is constitutively expressed on the surface of leukocytes in an inactive state (39–41). It is controversial whether the affinity or avidity state of LFA-1 is involved in the inside-out and outside-in signals. A recent publication demonstrated that cytoskeletal regulation couples LFA-1 conformational changes to receptor lateral mobility and clustering; thus, both affinity and avidity states of LFA-1 provide regulation of receptor function on lymphocytes (42). Previous studies reported that LFA-1 ligation can co-stimulate T cells for IL-2 production, cell proliferation, and Th1 polarization (27, 43–44). However, the detailed molecular pathways that regulate these processes are not defined. In particular, evidence in support of a distinctive role for LFA-1 activation in T cell signaling pathway is lacking. Based on our data, a plausible model is derived as follows: the TCR signaling to LFA-1 (inside-out) activates both the avidity and affinity of LFA-1; the avidity regulation of LFA-1 in the absence of ICAM-1 ligand is mediated through pSMAC formation, which leads to the maintenance of the stable immunological synapse for the TCR-mediated Erk1/2 signal pathway. This is consistent with a previous report that TCR stimulation activates Rap1, which leads LFA-1 to rapidly accumulate at immunological synapses through spatial redistribution (45). Furthermore, integrin-activating agonists have been shown to cause LFA-1 to move into lipid rafts (30). In addition, the polarization and clustering of LFA-1 can be observed when T cells are stimulated with CD3 antibody (2, 4). The TCR signaling to LFA-1 also changes the affinity state of LFA-1 simultaneously. Subsequently, the ligation of high affinity LFA-1 with ICAM-1 transduces the outside-in signal. The ligation between activated LFA-1 and ICAM-1 provides extra strength to stabilize the immunological synapse and accessory signals to accelerate T cell activation. Thus, our results suggest that TCR controls the avidity and affinity of LFA-1, which are coupled to provide regulation of CD8+ T cell activation.

In summary, we found that LFA-1 contributes to CD8+ T cell activation through two distinct Erk1/2 signal pathways.

FIGURE 4. LFA-1 ligation with ICAM-1 promotes T cell activation and proliferation. A, intracellular cytokine production in CD8+ T cells with LFA-1 ligation. CD8+ T cells were stimulated with coated anti-CD3 antibody with or without coated ICAM-1 for 12 h. Intracellular IL-2, TNF-α, and IFN-γ were measured simultaneously. The upper panel shows the representative dot plots of intracellular IL-2 production. The IL-2+ cells are displayed in circles. The lower panel shows the MFI of each cytokine quantified. Results are plotted as the mean of three independent experiments with S.D.; a significance level of p < 0.05 was determined using CD3 antibody stimulation sample as the reference. B, cell cycle analysis of CD8+ T cells with LFA-1 ligation. CD8+ T cells were stimulated with coated anti-CD3 antibody with or without coated ICAM-1 for 72 h. The percentage of viable cells in the G0, G1, S, and G2/M phases of the cell cycle were determined by double staining of Ki-67 and PI. Values of PI lower than 500 were considered as cells in G0/G1 (1n DNA); values from 500–700 were considered as cells in S (1n–2n DNA); values higher than 700 were considered as cells in G2/M (2n DNA). Low Ki-67 signals were considered as cells in G0; Ki-67 signals with 2n DNA were considered as cells in G1. The percentages of cells in G1 and S + G2/M were quantified. Results are representative data and plotted as the mean of three independent experiments with S.D.; a significance level of p < 0.05 was determined using CD3 antibody stimulation sample as the reference.
LFA-1 Regulates Erk1/2 Signal Pathway in CD8⁺ Cells

The first Erk1/2 signal is delivered through the TCR by recruiting LFA-1 to the immunological synapse. This TCR-mediated Erk1/2 signal pathway is required for CD8⁺ T cell activation. The second Erk1/2 signal is delivered through LFA-1/ICAM-1 binding. This LFA-1-mediated Erk1/2 signal pathway induces optimal activation of CD8⁺ T cells.

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REFERENCES

LFA-1 Regulates CD8+ T Cell Activation via T Cell Receptor-mediated and LFA-1-mediated Erk1/2 Signal Pathways

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