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Orai1 Function Is Essential for T Cell Homing to Lymph Nodes

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In T lymphocytes, Ca2+ release–activated Ca2+ (CRAC) channels composed of Orai1 subunits trigger Ag-induced gene expression and cell proliferation through the NFAT pathway. We evaluated the requirement of CRAC channel function for lymphocyte homing using expression of a dominant-negative Orai1-E106A mutant to suppress Ca2+ signaling. To investigate homing and motility of human lymphocytes in immunocompromised mouse hosts, we transferred human lymphocytes either acutely or after stable engraftment after a second transfer from the same blood donor. Human and mouse lymphocyte homing was assessed, and cells were tracked within lymph nodes (LN) by two-photon microscopy. Our results demonstrate that human T and B lymphocytes home into and migrate within the LN in immunocompromised NOD.Scid mice similar to murine lymphocytes. Human T and B cells localized in atrophied or reconstituted mouse LN, where T cells migrated in a random walk at velocities of 9–13 μm/min. Expression of Orai1-E106A inhibited CRAC channel function in human and mouse T cells, and prevented homing from high endothelial venules into murine LN. Ca2+ signals induced by CCL21 were also inhibited in T cells expressing Orai1-E106A. With CRAC channels inhibited, the high-affinity form of LFA-1 failed to become active, and T cells failed to migrate across endothelial cells in a transwell model. These results establish a requirement for CRAC channel–mediated Ca2+ influx for T cell homing to LN mediated by high-affinity integrin activation and chemokine-induced transendothelial migration. The Journal of Immunology, 2013, 190: 3197–3206.

Sustained Ca2+ influx through Ca2+ release–activated Ca2+ (CRAC) channels is essential for Ag-dependent T cell activation and proliferation via NFAT-driven gene expression (1, 2). In addition, after TCR engagement, CRAC channel activation elevates cytosolic calcium ([Ca2+]i) and acts to halt T cell migration and facilitate prolonged interactions with APCs (3). RNA interference screening has identified two proteins that activate and form the CRAC channel: STIM proteins (STIM1 and STIM2 in mammals) and mutagenesis have identified two proteins that activate and form the CRAC channel: STIM proteins (STIM1 and STIM2 in mammals) and mutagenesis have identified two proteins that activate and form the CRAC channel: STIM proteins (STIM1 and STIM2 in mammals) and mutagenesis have identified two proteins that activate and form the CRAC channel: STIM proteins (STIM1 and STIM2 in mammals).

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Abbreviations used in this article: B2, B2-microglobulin; [Ca2+]i, intracellular calcium concentration; CFPl, cyan fluorescent protein; CMF;HC, 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin; CMTMR, 5-(and-6)-[(4-chloromethyl)benzoyl] amino tetramethylrhodamine; CRAC, Ca2+ release–activated Ca2+; EC, endothelial cell; eGFP, enhanced GFP; ER, endoplasmic reticulum; HEV, high endothelial venule; LN, lymph node; SLO, secondary lymphoid organ; Tg, thapsigargin.

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as the endoplasmic reticulum (ER)–resident Ca2+ sensor, and Orai proteins (Orai1, Orai2, and Orai3 in humans) as the Ca2+-selective, pore-forming subunit (4–9). In T cells and other cells of the immune system, Ca2+ store depletion induces STIM1 to migrate as oligomers from the bulk ER to junctions immediately adjacent to the plasma membrane and mediates Ca2+ influx by opening Orai1 channels by direct STIM1–Orai1 interaction (10). Although the properties of CRAC channels and their role in T cell Ca2+ signaling have been described in detail (11, 12), the functional roles of CRAC channels in human T cell activation and immune defense remain incompletely characterized. In particular, CRAC channels may also be required for lymphocyte trafficking and migration. To address this, we developed methods to engraff and visualize human lymphocytes in the context of immunocompromised mouse models, and expressed a dominant-inhibitory Orai1 construct to evaluate the functional role of CRAC channel activity in lymphocyte homing to lymph nodes (LN) in vivo.

Several mutations in Orai1 or STIM1 have been identified in human patients with SCID, leading to inhibition of CRAC channel function and lethal immune suppression (7, 13–16). In patients with the homozygous Orai1-R91W mutation, CRAC channels fail to open, and T cells cannot take up Ca2+ after stimulation (7). In contrast, Ca2+ influx in T cells isolated from Orai1-deficient mice was only partially inhibited (17, 18), perhaps because of compensation by other Orai isoforms. We therefore sought an alternative method to evaluate CRAC channel function. A charge-neutralization mutation of the critical glutamate residue to alanine in Drosophila Orai or human Orai1 has a dominant-negative effect on the CRAC channel, resulting in a nonconductive channel (4, 5). In this study, we use expression of the dominant-negative Orai1-E106A to silence the CRAC channel and inhibit Ca2+ influx to evaluate the functional role for Orai1 in homing.
adoptive transfer of human T cells. NOD.Cg-Prkdcscid B2mtm1Unc/J mice

Materials and Methods

Mice

NOD.CB17-Prkdcscid/J (NOD.SCID) mice obtained from Jackson Lab-
oratory (stock no. 001303; Bar Harbor, ME) were housed and monitored in
a specific pathogen-free environment with sterile food and water in our
animal facility. To inhibit NK cell activity, we injected NOD.SCID mice
t.v. with 20 μl anti-NK cell Ab (rabbit anti-Asialo GM1; Wako Biopro-
cuts, Richmond, VA) according to manufacturer’s instructions 3–4 d before
adoptive transfer of human T cells. NOD.Cg-Prkdcscid mice were
used to generate labeled (green), CMTMR-labeled (red), and CMF2HC-labeled (blue)

Purification of human monocytes and isolation of CD4+, CD8+, CD19+, and CD3+ cells

Human PBLs were isolated from blood of voluntary healthy donors by

Isolation of human CD4+ and CD8+ T cells was performed using human
CD4+ and CD8+ T cell isolation kits (Miltenyi Biotec). CD19+ B cells were isolated by positive selection according to manu-
facturer’s instructions (Miltenyi Biotec). In some experiments, human
CD3+ T cells were isolated using EasySep Human CD3+ Isolation kit
(Stem Cell Technologies, Vancouver, BC, Canada), according to manu-
facturer’s instructions. The purity of CD4+, CD8+, CD19+, or CD3+ cell
populations was confirmed to be >95% by flow cytometry.

Cell adoptive transfer in mice

For human T cells, purified CD4+ and CD8+ T cells and CD19+ B cells
were adoptively transferred into reconstituted NOD.SCID mice. For all adoptive
transfer experiments, 5 × 106 labeled cells of each cell type were trans-
ferred by tail-vein or retro-orbital injection.

Transfection and retroviral transduction

Primary human T cells were transfected by nucleofection (Amaxa,
Allendale, NJ), using the high-viability protocol with enhanced GFP (eGFP),
eGFP-tagged Orai1-E106A mutant, or mock transfected with no
plasmid as indicated. Human T cells were used for experiments 24 h after
transfection. Constructs were generated as previously (33). Retroviral transduction of purified mouse T cells was performed as de-
scribed previously (34). Thy1.1-cyan fluorescent protein (CFP) or Thy1.1-
eGFP-Orai1-E106A retroviral vectors were transfected into 293T cells
and retroviral supernatant was collected. Activated primary mouse T cells
were spin-fected using retroviral supernatant and 4 μg/ml Polybrene (Sigma-
Aldrich, St. Louis, MO) for 1 h at 1800 rpm and incubated for 24–48 h
at 37°C. Transduced mouse T cells expressing the retrovirus (Thy1.1+)
were magnetically isolated from nonexpressing cells (Thy1.1−) using a
biotinylated anti-Thy-1.2 Ab according to manufacturer’s instructions
(Miltenyi Biotec). After purification, Thy1.1+ CFP− cells were labeled
with CMTMR and Thy1.1+ eGFP-Orai1-E106A− cells were labeled
with CFSE. Also, Thy1.1+ CFP+ cells were labeled with CMTMR, and Thy1.1+ eGFP-
Orai1-E106A+ cells were labeled with CFSE. For determination of homing
efficiency, fluorescently labeled Thy1.1− or Thy1.1+ populations were
coadaptively transferred into wild-type C57.B16 recipient mice.

Two-photon imaging and analysis

Multidimensional (x, y, z, t, time, emission wavelength) two-photon mi-
croscopy was used to image fluorescently labeled lymphocytes in ex-
planted mouse LNs, using a 780-nm femtosecond pulsed laser as described
previously (35). Fluorescence emission was split by 510- and 560-nm
dichroic mirrors into three detector channels, used to visualize CSFE-
fluorescein (green), CMTMR-labeled (red), and CMF2HC-labeled (blue)

Immunohistochemistry

LN specimens were fixed in 4% neutrally buffered formaldehyde. Five- to 6-
μm-thick paraffin-embedded serial tissue sections were mounted on posi-
tively charged glass slides. Sections were taken for H&E staining or im-
munofluorescence staining. Sections were bathed in xylene, then treated
with a graded series of alcohol (100, 95, and 75% ethanol [v/v] H2O) and
rehydrated in PBS (pH 7.5). Sections were treated with Retrievagen A (pH
6.0), BD, and microwaved for 10 min for Ag retrieval. Sections were
then incubated with 3% H2O2 in PBS to block endogenous peroxidase activity
for 10 min followed by incubation with blocking solution containing 1%
normal goat serum for 20 min. Samples were then stained with anti-parallel

remaining cells by negative selection with a CD4+ T cell isolation mixture.
CD19+ B cells were isolated by positive selection according to manu-
facturer’s instructions (Miltenyi Biotec). In some experiments, human
CD3+ T cells were isolated using EasySep Human CD3+ Isolation kit
(Stem Cell Technologies, Vancouver, BC, Canada), according to manu-
facturer’s instructions. The purity of CD4+, CD8+, CD19+, or CD3+ cell
populations was confirmed to be >95% by flow cytometry.

Cell adoptive transfer in mice

For human T cells, purified CD4+ and CD8+ T cells and CD19+ B cells
were separately labeled with CellTracker dyes (Invitrogen, Grand Island,
NY) CFSE (4 μM), 5- (and-6)-[(4-chloromethylbenzoyloxy)tetra-

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node addressin mAb MECA-79 (1:20 dilution, rat IgM; BD) overnight at 4˚C. Secondary detection Abs were Cy5-conjugated goat anti-rat IgM+IgG (Jackson Laboratories). Slides were analyzed by fluorescence microscopy using a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY).

Flow cytometry

Cells were analyzed using FACSCalibur flow cytometer with Cell Quest software or a LSRII with FACSDiva software (Becton Dickinson, Mountain View, CA). Anti-human CD45, CD4, CD8, CCR7, and CD62L, and the corresponding isotype control Abs were obtained from eBioscience (San Diego, CA). Conformation-specific Abs against human LFA-1 (MEM-83) and VLA-4 (12G10) were purchased from Abcam (Cambridge, MA). TRITC-conjugated secondary Abs were purchased from KPL (Gaithersburg, MD). Annexin V–PE was purchased from BD. Cells were suspended in buffer containing 0.5% BSA and 2 mM EDTA in PBS (staining buffer), and stained for 20–30 min with specific PE-Cy5-conjugated mAbs. Unbound Abs were removed by two washes with staining buffer, and cells were resuspended in staining buffer and analyzed. For homing experiments, mouse LNs and spleen were collected 18 h after adoptive transfer and made into single-cell suspensions with 70-µm nylon cell strainers (BD Falcon, Mountain View, CA). Cells were gated for appropriate forward and side scatter. Data were saved as FCS 2.0 list mode files and analyzed with FCS Express 1.3 or FlowJo.

Single-cell Ca2+ imaging and intracellular Ca2+ measurement by flow cytometry

Ratiometric single-cell Ca2+ imaging was performed as described previously (36). Cells were loaded by incubation for 30 min with 3 µM fura-2 AM (Invitrogen) and analyzed after 2 µM thapsigargin (Tg; Sigma-Aldrich) treatment. Data were analyzed with MetaFluor software (Universal Imaging) and Origin software (OriginLab, Northampton, MA). For flow cytometry, cells were analyzed on a BD LSR II flow cytometer with FACSDiva software (BD). eGFP- or eGFP-Orai1-E106A–transfected CD3+ human T cells were loaded by incubation for 30 min with 8 µM fura-2 AM (Invitrogen) at 37˚C for 30 min, washed once, and resuspended in 2 mM Ca2+ RPMI 1640 supplemented with 1% FCS at 5 × 105 cells/ml. An 800-µl aliquot was warmed to 37˚C before stimulation. Cells were maintained at 37˚C and analyzed at 300 events/s. After establishment of the baseline, the tube containing the cells was removed, stimulant was added, and the tube was replaced. Recording was continued for up to 200 s. Data were saved as FCS 2.0 list mode files and analyzed with the FlowJo Kinetics tool. [Ca2+]i was determined as described previously (37).

Chemokines and transwell assays

Chemokines were obtained from PeproTech (Rocky Hill, NJ) and prepared according to manufacturer’s instructions. Transwell assay plates with 5-µm pore size were obtained from Corning (Tewksbury, MA). HUVECs were isolated as described previously (38). HUVECs were plated in M199 (Invitrogen) supplemented with 10% FCS and endothelial cell supplement (EC; Invitrogen) and analyzed after 2 h before stimulation. Cells were maintained at 37˚C and analyzed at 500 events/s. After establishment of the baseline, the tube containing the cells was removed, stimulant was added, and the tube was replaced. Recording was continued for up to 200 s. Data were saved as FCS 2.0 list mode files and analyzed with the FlowJo Kinetics tool. [Ca2+]i was determined as described previously (37).

Soluble ICAM-1 binding assay

Transfected human CD3+ T cells were suspended in 2 mM Ca2+ RPMI 1640 supplemented with 1% FCS. Cells were treated with 100 ng/ml rmCCL21 in the presence of ICAM-1/Fc (20 µg/ml; R&D Systems) and allophycocyanin-conjugated anti-human IgG1 (Fc specific; Southern Biotechnology) for 10 min at 37˚C. Change in ICAM-1 binding determined by flow cytometry was measured as a fold increase over control.

Statistical analysis

Statistical signficance was determined using the Student t test. Two-way analysis of covariance was used where indicated and was calculated using R Statistical Computing Software (Vienna, Austria). A p value <0.05 was considered significant. Data are presented as mean ± SEM.

Results

Live-cell imaging of human lymphocytes in immunocompromised mice

To establish a system for in vivo imaging of human lymphocytes, we initially transferred human lymphocytes into NOD.SCID mice by i.v. injection (Supplemental Fig. 1A). Purified CD4+ and CD8+ T cells and CD19+ B cells distributed randomly throughout the atrophied LN, not localizing into any compartmentalized structure (Supplemental Fig. 2A, 2B, 2C), and actively migrated in a stop-and-go pattern of motility (Fig. 1A, Supplemental Video 1). Human CD4+ T cells migrated with a mean velocity of 9.3 ± 0.4 µm min−1 (Fig. 1B). For comparison, adoptively transferred wild-type mouse CD4+ T cells also distributed randomly throughout the LN of NOD.SCID mice (Supplemental Fig. 2D) and migrated with slightly higher mean velocities averaging 10.9 ± 0.2 µm min−1 (Supplemental Fig. 2E). Migrating human CD4+ T cells traversed a wide area, and their displacement from the origin increased in proportion to the square root of time (Fig. 1C, 1D). These properties are consistent with a default random-walk pattern of motility of human T cells in the immunocompromised mouse host. Analysis of turning angles between consecutive imaging frames revealed a mean turning angle of 69 ± 4.5 degrees (Fig. 1E). Human CD8+ T cells performed similarly to human CD4+ cells, migrating with a mean velocity of 9.7 ± 0.4 µm min−1 (Fig. 1F), with displacement increasing as a square root function of time (Fig. 1G, 1H) and with a similar mean turning angle (Fig. 1I). Human CD19+ B cells colocalized with and migrated more slowly than CD4+ or CD8+ T cells, with a mean velocity of 5.8 ± 0.3 µm min−1 (Fig. 1J), displacing about half the distance of human T cells (Fig. 1K, 1L), and along more tortuous paths with a mean turning angle of 94 ± 4.8 degrees (Fig. 1M). This analysis of human lymphocyte motility is in agreement with previous two-photon imaging, which showed that murine T cells migrate faster and along straighter paths than B cells (39). We conclude that human T and B cells migrate similarly to mouse T and B cells in the host LN environment, with velocities that are only slightly slower than mouse cells. Despite the lack of organization into separate T zones and follicles, both human and mouse T cells migrated faster than colocalized B cells, implying an intrinsically faster mechanism for crawling.

Functional CRAC channels are required for human T cell homing in NOD.SCID mice

The human xenograft model provides an opportunity to observe human lymphocytes that have been manipulated by transfection before adoptive transfer. Purified human CD4+ T cells were transfected with control eGFP or with the dominant-negative eGFP-Orai1-E106A CRAC channel mutant and rested overnight in culture. Expression of eGFP-Orai1-E106A had no effect on cell viability before transfer as determined by Annexin V staining, an indicator of apoptosis (Supplemental Fig. 3A). Aliquots of cells were evaluated by flow cytometry, revealing a transfection efficiency of >50% (Fig. 2A). Ca2+ imaging confirmed that, as expected, human T cells expressing eGFP-Orai1-E106A showed impaired Ca2+ influx after readdition of external Ca2+ after Tg treatment to deplete ER Ca2+ stores (Fig. 2B). T cells were transferred into an NOD.SCID recipient and analyzed by flow cytometry (Supplemental Fig. 1B) to quantify human T cell homing to the SLO in the xenograft model. Control eGFP human T cells efficiently homed to both the LNs and spleen. In contrast, eGFP-Orai1-E106A human T cells were almost entirely deficient in homing to the LN and, to a lesser extent, the spleen (Fig. 2C). To confirm that the homing defect is not due to differential CD62L or CCR7 expression, we quantified the surface phenotype of control eGFP and eGFP-Orai1-E106A–transfected human T cells, eGFP-Orai1-E106A human T cells maintained CD62L expression at marginally lower levels than the control population (Fig. 2D). CCR7 expression was unchanged in both populations (Fig. 2E).

We evaluated whether suppression of Ca2+ signaling through Orai1 channels could lead to an accumulation of Orai1-E106A.
T cells in the HEV after adoptive transfer. To determine the localization of T cells relative to the HEV, we isolated LNs from NOD.SCID mice after adoptive transfer of equal numbers of control CMTMR-labeled and eGFP-Orai1-E106A–transfected human T cells (Supplemental Fig. 3B). Correspondingly colored cell tracks depict cell movements over the indicated time periods. Both control and eGFP-Orai1-E106A+ human T cells were observed in regions that contained HEVs (Fig. 3A). Control human T cells (red) were positioned both within and outside of HEVs (blue) that perfuse the LN cortex, whereas T cells expressing eGFP-Orai1-E106A (green) were observed predominantly within the HEVs (Fig. 3B). Fewer total eGFP-Orai1-E016A+ T cells were observed in selected areas containing HEVs, compared with control T cells (Supplemental Fig. 3C). Collectively, these findings indicate that human T cells require CRAC channel function for LN homing across the HEVs in our xenograft model.

**Migration and homing in reconstituted immunocompromised mice**

To extend these results on the requirement of CRAC channel function for homing, we performed additional validation studies using NOD.SCID.B2 mice that had been previously reconstituted with human PBLs. Reconstitution of immunocompromised mice was achieved by i.p. injection of purified human PBLs. Three weeks after PBL injection, LNs and spleens from NOD.SCID.B2 mice increased in size. Mean LN perimeter grew from 0.39 to 0.82 cm; mean spleen perimeter increased from 2.0 to 3.5 cm (Supplemental Fig. 4A). H&E staining of nonreconstituted NOD.SCID.B2 LN slices showed a lack of structure and nuclear...
density. Cellular density increased after 3 wk of reconstitution by human PBL, but cellular density was lower and follicular structure undefined compared with wild-type LNs (Supplemental Fig. 4B).

To confirm that human T cells could migrate with characteristic motility behavior in the reconstituted NOD.SCID.B2 LN, CD3+ T cells from the original donor were transferred into the host animal after reconstitution (Supplemental Fig. 1C). Time-lapse two-photon imaging revealed robust T cell migration in the reconstituted LN (Fig. 4A, Supplemental Video 2), where T cells migrated with a mean velocity of 12.8 ± 0.4 μm min⁻¹ (Fig. 4B). Human CD3+ T cells migrated away from their starting coordinates, with mean displacement increasing as a square root function of time, typical of a random walk (Fig. 4C, 4D). The average CD3+ step-wise turning angle was 110.9 ± 2.5 degrees (Fig. 4E). Normal representative T cell migration and increased LN cellularity indicated that reconstituted NOD.SCID.B2 mice can also be used for analysis of transfected human immune cells.

To validate our finding that human T cells expressing the dominant-negative Orai1 mutant are unable to home to the LN and spleen in vivo, we performed a homing study in reconstituted NOD.SCID.B2 mice. Equal numbers of mock-transfected, CMTMR-labeled control and Orai1-E106A–transfected human T cells were adoptively transferred into NOD.SCID.B2 mice that had previously been reconstituted with blood from the same donor.

Whole-organ flow cytometry revealed that control CMTMR-labeled human T cells homed to the SLO, representing 0.8 and 0.9% of human cells expressing eGFP or eGFP-Orai1-E106A in SLO determined by flow cytometry (Fig. 4F). Surface expression of CD62L on eGFP+ (left) and eGFP-Orai1-E106A+ human T cells (right), 18 h posttransfection. Isotype control staining represented in dotted gray lines. Surface expression of CCR7 on eGFP+ (left) and eGFP-Orai1-E106A+ human T cells (right), 18 h posttransfection. FACS plots are representative of three separate experiments.

FIGURE 2. Human T cells expressing Orai1-E106A cannot home into the SLO of NOD.SCID mice. (A) Expression of eGFP and eGFP-Orai1-E106A in human T cells 24 h after transfection. (B) Averaged Tg-dependent influx of Ca²⁺ in human T cells expressing eGFP or eGFP-Orai1-E106A. Data are representative of three different experiments. (C) Human lymphocyte homing capacity in NOD.SCID mouse LN and spleen 18 h after adoptive transfer. Percentage of human cells expressing eGFP or eGFP-Orai1-E106A in SLO determined by flow cytometry. (D) Surface expression of CD62L on eGFP+ (left) and eGFP-Orai1-E106A+ human T cells (right), 18 h posttransfection. Isotype control staining represented in dotted gray lines. (E) Surface expression of CCR7 on eGFP+ (left) and eGFP-Orai1-E106A+ human T cells (right), 18 h posttransfection. FACS plots are representative of three separate experiments.

FIGURE 3. Human T cells expressing Orai1-E106A remain localized to the HEV. (A) Localization of human CD3+ T cells in the LN of a NOD.SCID mouse. This image shows CMTMR-labeled control (red) or eGFP-Orai1-E106A–transfected (green) human CD3+ T cells in a LN slice from a NOD.SCID mouse. HEVs were labeled with anti–periipheral node addressin Ab MECA-79. Inset shows labeled human cells localized in the HEV and cortex. (B) The percentage of human T cells found within the HEV.
0.2% of the total cells in the LN and spleen, respectively (Fig. 4F). Human T cells expressing the Orai1-E106A mutant lost their homing capacity to the SLO, representing 0.1% of the total cellularity in both organs (Fig. 4F). The majority of the cells recovered by flow cytometry were CMTMR+ controls (Fig. 4G), demonstrating that eGFP-Orai1-E106A+ human cells cannot home to the SLO in the human-reconstituted mouse model.

**Homing block in mouse T cells with disrupted CRAC channel function**

The results so far point to a requirement for CRAC channel function in the homing of human T cells in acutely and stably engrafted immunocompromised mice. To determine whether functional CRAC channels are also required for the homing of mouse T cells into the LN, we engineered MSCV-IRES retroviral vectors containing the variant Thy1.1 gene as a marker for expression, together with either eGFP-Orai1-E106A or a control CFP construct. We then transduced wild-type mouse T cells and examined their Ca\(^{2+}\) responses after store depletion by single-cell Ca\(^{2+}\) imaging. As expected, Ca\(^{2+}\) influx was suppressed in mouse T cells expressing eGFP-Orai1-E106A, relative to control CFP+ T cells (Fig. 5A). Using Thy1.1 coexpression and magnetic bead separation to purify cells, we labeled control and eGFP-Orai1-E106A–expressing T cells with CMTMR or CFSE, respectively, and evaluated homing after cotransfer into a wild-type recipient (Supplemental Fig. 1D). By flow cytometric analysis of recovered cells, Thy1.1− cells that did not express retroviral constructs were effectively recovered from LN and spleen (Fig. 5B). In addition, Thy1.1+CFP+ T cells, representing control cells that were successfully transduced, also were able to home to the SLO, representing >8% of the total cellularity (Fig. 5C, top gate). In contrast, adoptively transferred Thy1.1+eGFP-Orai1-E106A+ mouse T cells represented <0.2% of the total cellularity of the LN and spleen (Fig. 5C, bottom gate). We conclude that mouse T cells deficient in CRAC channel function are unable to home to the SLO of syngeneic mice, in agreement with our results using human cells in the xenograft models.

**Orai1-E106A expression disrupts CCL21-dependent Ca\(^{2+}\) signaling and LFA-1 activation**

Because treatment with CCL21 (the chemokine implicated in T cell homing) evokes Ca\(^{2+}\) signals in human T cells (25, 26), we hypothesized that CRAC channels mediate this response. To test for CRAC channel activity during the chemokine response, we used time-lapse flow cytometry to measure Ca\(^{2+}\) influx after Tg or CCL21 treatment in untransfected control human T cells and in cells transfected with either eGFP or eGFP-Orai1-E106A (Supplemental Fig. 1E). To achieve this, we used the Ca\(^{2+}\) indicator dye fura red, which reports increases in [Ca\(^{2+}\)]\(_i\), by a decrease in...
fluorescence signal. As expected, Tg treatment resulted in a sustained \([\text{Ca}^{2+}]_i\) increase in both untransfected and eGFP+ control T cell populations (Fig. 6A). In contrast, cytosolic \([\text{Ca}^{2+}]_i\) did not change in eGFP-Orai1-E106A T cells. Recombinant mouse CCL21 stimulation resulted in a rapid and transient increase in \([\text{Ca}^{2+}]_i\), in both control untransfected and eGFP+ human T cells. However, human T cells expressing the dominant-negative eGFP-Orai1-E106A construct did not respond to CCL21 (Fig. 6B). Measurements of peak \([\text{Ca}^{2+}]_i\) levels demonstrate that human T cells deficient in CRAC channel function are unable to mobilize \([\text{Ca}^{2+}]_i\) after Tg or CCL21 treatment (Fig. 6C).

Because CCL21 treatment has been previously shown to induce T cell binding to ICAM-1 (22), we tested whether suppression of CCL21-dependent \([\text{Ca}^{2+}]_i\) signaling could lead to a defect in migration across a confluent EC monolayer in vitro. Using a standard chemotaxis assay, we measured migration of control eGFP+ or eGFP-Orai1-E106A human T cells in response to CCL21 across an uncoated polycarbonate transwell. Both T cell populations migrated robustly in response to the chemokine gradient (Fig. 7A), revealing that Orai1 function is not required for chemotaxis in the uncoated transwell. However, eGFP-Orai1-E106A human T cells were deficient in migrating across transwells coated with ECs independent of the CCL21 concentration (Fig. 7B), suggesting a role for Orai1 in facilitating T cell–EC interactions independent of CCR7. Because chemokine-induced activation of integrins is required for transendothelial migration and LN entry (22, 23), we investigated the role of Orai1 in integrin activation after CCL21 treatment (Supplemental Fig. 1F). Flow cytometry revealed that CCL21-induced activation of LFA-1 integrin was abolished in eGFP-Orai1-E106A human T cells (Fig. 7C), although VLA-4 \(\beta_1\) integrin was unaffected (Fig. 7D). Chemokine treatment has been shown to enhance binding of soluble ICAM-1 to leukocytes, demonstrating LFA-1 activation (41). CCL21 treatment enhanced ICAM-1 binding to control T cells, and binding was reduced in eGFP-Orai1-

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FIGURE 5. Mouse T cells expressing Orai1-E106A cannot take up \([\text{Ca}^{2+}]_i\) or home to the SLO of wild-type syngeneic mice. (A) Fura-2 fluorescence ratios in transduced mouse CD3+ T cells loaded with fura-2. (B) Homing of control mouse T cells after transduction. Successfully transduced cells (Thy1.1+) were purified via positive selection. Unbound mouse T cells (Thy1.1−) transduced with the CFP vector were labeled with CMTMR, and Thy1.1− cells transduced with the eGFP-Orai1-E106A vector were labeled with CFSE and adoptively transferred. Whole organs were analyzed with T cell gates and are drawn as indicated. (C) Homing of positively isolated (Thy1.1+) mouse T cells. Cells were labeled: CFP+ with CMTMR and eGFP-Orai1-E106A+ with CFSE.

FIGURE 6. Inhibition of \([\text{Ca}^{2+}]_i\) signals evoked by Tg or CCL21 treatment in human T cells expressing Orai1-E106A. (A) Flow cytometry analysis, using the FlowJo Kinetics tool, of human T cells loaded with fura red. After 90 s to establish a baseline, cells were treated with 2 \(\mu\text{M} \text{Tg}\). Mean fluorescence changes (lower fluorescence = higher \([\text{Ca}^{2+}]_i\)) are shown in untransfected human T cells (top), eGFP+ human T cells (middle), and eGFP-Orai1-E106A+ human T cells (bottom). (B) Fluorescence changes in untransfected human T cells (top), eGFP+ human T cells (middle), and eGFP-Orai1-E106A+ human T cells (bottom) after addition of 100 ng/ml recombinant mouse CCL21. Data are from one donor and are representative of three donors. (C) Mean peak \([\text{Ca}^{2+}]_i\) signals evoked by Tg and CCL21 in control and eGFP-Orai1-E106A+ human T cells.
E106A+ T cells (Fig. 7E). We conclude that Orai1 is required for CCL21-induced Ca2+ signaling in human T cells and subsequent LFA-1 activation, transendothelial migration, and LN homing.

**Discussion**

We show that CRAC channel function is required for T cell homing into SLO. Our results are demonstrated in a combined in vivo and in vitro assessment of human T cells in mouse xenograft models, as well as in retrovirally transduced mouse T cells in syngeneic recipient mice. By inhibiting native CRAC channels with a dominant-negative Orai1 mutant (E106A), our approach avoids potential compensation in Orai1 knockout mouse models that retain residual CRAC channel function. Moreover, these results establish a system for live two-photon imaging of labeled or transfected human lymphocytes in an in vivo human xenografted mouse environment.

**Homing and motility of human lymphocytes in xenografted mice**

Using human lymphocytes transferred acutely into immunocompromised mice or into reconstituted human xenografted mice, we describe two-photon imaging of human T and B lymphocytes within intact LNs in a preparation that does not require human fetal tissue. Our methods differ from a recent study that used two-photon microscopy to image human T cell migration in mice reconstituted using fetal human blood, liver, and thymus (42). Both NOD.SCID and NOD.SCID.B2 mice retain peripheral LNs that support engraftment of human lymphocytes. These two strains were chosen because LNs from other immunocompromised mouse strains, including Rag2−/− and NSG, are even more atrophied (43, 44), rendering them unsuitable for imaging. NOD.SCID mice require depletion of resident NK cells before transfer of human lymphocytes, and LNs remain small after engraftment, although imaging was possible. Conversely, NOD.SCID.B2 mice readily support human xenografts, allowing for expansion of lymphocyte populations and stable engraftment of human PBLs. After reconstitution, LNs are enlarged and easily used for tracking individual labeled cells from the same blood donor by two-photon microscopy.

Our results demonstrate that adoptive transfer, both acutely and into reconstituted SCID mouse models, enables quantitative imaging and analysis of human cells. Human T cells homed into and actively migrated through the atrophied mouse host LNs of NOD.SCID mice with velocities that were somewhat slower than mouse T cells in the same environment (9.3 ± 0.4 versus 10.9 ± 0.2 μm min−1, re-
spectively), similar to velocities reported for the fetal human blood, liver, and thymus mouse model (42). Compared with the acutely transferred model, human T cells transferred into reconstituted mice migrated with faster mean velocity (12.8 ± 0.4 μm min⁻¹), closely similar to wild-type mouse T cells in the LN (27). Human T and B cell relative velocities mirrored the migration of mouse lymphocytes; both human and mouse T cells migrate more rapidly than CD19⁺ B cells in the xenograft environment (Fig. 1), as originally observed for mouse T and B cells in mouse LN (39). Displacement analysis confirmed that, like mouse B cells, human B cells migrate with lower velocities and motility coefficients than T cells, reflecting slower migration. Our results suggest that more rapid migration is an intrinsic characteristic of T cells.

**CRAC channel activity required for homing**

Our results used the dominant-negative Orai1-E106A mutant to suppress CRAC channel function in mouse and human T cells, and thereby elucidate mechanisms by which these ion channels regulate T cell function. Orai1-E106A has been used previously to determine the requirements for Orai1 clustering at the immunological synapse (33), to identify STIM1–Orai1 interacting domains (45), to determine regulation of voltage-gated Ca²⁺ channels by STIM1 (46), to reveal mechanisms for protection against apoptotic signaling through CD95 (47), and to demonstrate Ca²⁺ release from secretory granules (48). In Orai1-deficient mice, Ca²⁺ entry is not fully abolished in T cells (17, 18). Moreover, whereas human patients with the homozygous Orai1-R91W mutation do not develop a syndrome of immunodeficiency and autoimmunity (12), disruption of VLA-4 (1220–1223), which we demonstrate to result in a severe homing defect in both the xenograft or wild-type mouse environment (Figs. 2, 4, 5).

We further show that transendothelial migration and homing of lymphocytes to LNs requires Ca²⁺ influx through Orai1 channels. During homing, T cells must adhere to ECs and then extravasate from the HEV to the LN paracortex. Consistent with a role of Ca²⁺ influx, our results demonstrate that Orai1 assists in LFA-1 and CRAC channel activity.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


