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The Late Endosomal Transporter CD222 Directs the Spatial Distribution and Activity of Lck

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The spatial and temporal organization of T cell signaling molecules is increasingly accepted as a crucial step in controlling T cell activation. CD222, also known as the cation-independent mannose 6-phosphate/insulin-like growth factor 2 receptor, is the central component of endosomal transport pathways. In this study, we show that CD222 is a key regulator of the early T cell signaling cascade. Knockdown of CD222 hampers the effective progression of TCR-induced signaling and subsequent effector functions, which can be rescued via reconstitution of CD222 expression. We decipher that Lck is retained in the cytosol of CD222-deficient cells, which obstructs the recruitment of Lck to CD45 at the cell surface, resulting in an abundant inhibitory phosphorylation signature on Lck at the steady state. Hence, CD222 specifically controls the balance between active and inactive Lck in resting T cells, which guarantees operative T cell effector functions. The Journal of Immunology, 2014, 193: 2718–2732.

Tyrosine 394 (pY394); 3) an inactive “closed” form phosphorylated at the COOH-terminal tyrosine 505 (pY505); and 4) a double phosphorylated form (pY394 + pY505) that is thought to possess an equal kinase activity as single phosphorylated Lck at Y394 (3). Hence, active Lck is constitutively present in resting T cells, and it is its coordinated intracellular distribution that is crucial to balance T cell activation and nonreactivity (5–7). The antagonistic activities of the phosphatase CD45 and the kinase Csk control Lck’s mode of action (2, 8, 9). CD45 plays a pivotal role in the activation of Lck via dephosphorylation of Y505 (10, 11), but it is not clear where the interaction takes place and how it is regulated in space and time.

The late endosomal transmembrane molecule CD222, also known as the cation-independent mannose 6-phosphate/insulin-like growth factor 2 receptor, is a multifunctional broadly expressed regulator of protein trafficking (12). It binds mannose 6-phosphate–bearing proteins including acid hydrolases and TGF-β (13), the insulin-like growth factor 2 (14) and plasminogen (15). On delivering its cargo, CD222 traffics between the trans-Golgi network (TGN), endosomes, and the plasma membrane (16). CD222 is barely expressed on the surface of resting T cell but has been reported to be upregulated at the plasma membrane upon T cell activation in rodents (17). In this study, we show that CD222 is also upregulated on the surface of human T cells upon stimulation. Remarkably, we found that CD222 transports Lck intracellularly and directs its distribution to CD45 at the plasma membrane. The loss of CD222 causes a decrease in signal transduction and subsequently a profound blockade of T cell effector functions.

Materials and Methods

Abs

The mAb to CD222, unlabeled and conjugated with AF647 (MEM-238), CD45, both unlabeled (MEM-28) and conjugated with Pacific Orange (HI30), and CD4, unlabeled (MEM-241), were purchased from EXBIO (Prague, Czech Republic); the mAb to CD3 (MEM-57), CD39 (MEM-43/5), pTag (H902), and α-fetoprotein (AFP-12) were provided by Dr. V. Horejsi (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). The anti–TCRβ-chain mAb C305 was a gift from Dr. A. Weiss (University of California, San Francisco, San Francisco, CA). The stimulatory CD3 mAb OKT3 was from Ortho...
Pharmaceuticals (Raritan, NJ). The CD28 mAb Leu-28, anti-phospho-
CD3/CD234 (Y142) mAb K25-407,69 and anti-phospho- linker of activated T cells (LAT; Y171) were from BD Biosciences (San Jose, CA). The HRP-conjugated anti-phosphotyrosine mAb 4G10 was from Merck Millipore (Billerica, MA). The anti-GPDP mAb l4C10, anti-phospho-
ZAP70 Ab (Y319/Syk) (Y352), anti-ZAP70 mAb 99F2 and LIES, anti-
phospho-p44/42 MAPK Ab ERK1/2 (Y202/204), anti-p44/42 MAPK Ab ERK1/2, anti–phospho-Src mAb (Y416), anti–non-phospho-Src mAb (Y416), anti–phospho-Lck Ab (Y505), anti-Lck mAb 73A, and the rabbit mAb to early endosome Ag I (EEA1) C45B10 were from Cell Signaling Technology (Danvers, MA). The anti-Lck mAb H-95 was from Santa Cruz Biotechnology (Santa Cruz, CA). Biotinylation of unconjugated mAb was performed in our laboratory. As secondary reagents we used HRP-con-
jugated goat anti-mouse and anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO), streptavidin-HRP conjugate (GE Healthcare, Buckinghamshire, U.K.) and goat anti-mouse IgG+IgM (H+L)-FITC conjugate (An der Gruh, Kaumberg, Austria).

Cell culture

The T cell line Jurkat E6.1 and the B cell line Raji were from the American Type Culture Collection. The Lck-deficient Jurkat T cell line JCaM 1.6 (18) was from the European Collection of Cell Culture (Salisbury, U.K.). The immortalized CD222 negative mouse fibroblasts were provided by E. Wagner (Spanish National Cancer Research Centre, Madrid, Spain). The Jurkat E6.2–Luc and E6.1 cells were transfected with a luciferase expressing IL-2 promoter reporter construct as described previously (19). The Jurkat NFAF Luc cells containing an artificial promoter region with four NFAT binding sites were created in our laboratory. Human PBMC were isolated from purchased buffy coats of healthy adult volunteers (Rotex Kreuz, Vienna, Austria) by density gradient centrifugation on Lymphoprep (Nycodemed). All primary human T cells as well as mouse and human T cell lines were maintained in RPMI 1640 medium, the HEK-293 cells in DMEM, all supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 2 mmol/l L-glutamine (Life Technologies, Carlsbad, CA), 100 μg/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies). Cells were grown in a humidified atmosphere at 37°C and 5% CO2 and passed every 2–3 d.

T cell activation assay

Primary human T cells isolated by CD14-depletion from PBMC were cultured for 2 d to separate non-adherent cells. T cell lines and primary human T cells (5 × 10⁴) were seeded in 96-well plates and kept unstimu-
lated or stimulated by: 1) plate-bound CD3 mAb OKT3 (1 μg/ml) alone or together with soluble CD28 Ab Leu-28 (0.5 μg/ml); 2) CD3 Ab-coupled beads; 3) staphylococcal enterotoxin E (SEE)–loaded Raji B cells; or 4) staphylococcal enterotoxin A (SEA)–loaded Raji B cells or 5) SEA-loaded Raji B cells supplemented with 10 ng/ml IL-2. The cells were harvested and analyzed via flow cytometry, immunoblotting, and samples were prepared, according to the filter aided sample preparation procedure following analysis via liquid chromatography–mass spectrometry (LC-MS). Lck immunoprecipitates were separated on 4–12% gradient Bis-Tris NuPAGE gels (Invitrogen Life Technologies), the gels were washed in distilled water, lightly stained with Colloidal Blue (Invitrogen Life Technologies), and subjected to Gel-MS as described previously (21).

Gene knockdown via RNA interference and retroviral transduction

Stable knockdown cell lines were produced by lentiviral introduction of short hairpin (sh)RNA specific for the CD222 mRNA. The following CD222 silencing sequences were used for cloning into the vector plKOPuro1 (a gift from S. Steward, Washington University School of Medicine, St. Louis, MO): shCD222P1 at position 6588 with the sequence 5′-GCCCAACGATCAG-
CACCCTCtcgaagAGATGCTGATCGTTGGGC-3′ and shCD222P2 at position 4525 with the sequence 5′-GAGCAGGACGACATGCTcagaga-
gTCATACGTcgtGTTGCTC-3′. The MISSION nontarget shRNA control vector (pKlKOpurol; Sigma-Aldrich) was used as a negative control (shCTR).

Virus particles were generated via transfection of HEK-293 cells with the silencing and the lentiviral envelope coding plasmid pMD2.G and the packaging vectorsPAX2 and psPAX2 by using viraMax Transfection Reagent (Ecole Polytechnique Federal de Lausanne, and obtained from Addgene, Cambridge, MA) as described previously (22). After 48 h, the virus particles were harvested, filtered, and used for the target cell line infections in the presence of 5 μg/ml polybrene (Sigma-Aldrich). The next day, the cells were washed with and maintained in medium containing 1 μg/ml of puromycin (Sigma-Aldrich) to select transduced cells. CD222 knockdown cells were used from 10 d to 2 mo postinfection for all experiments.

Lck constructs were prepared in the retroviral expression vector pBMM-Z for transfection of the Phoenix amphotropic virus producer cell line (both provided by G. Nolan, Stanford University School of Medicine, Stanford, CA) (15). The target cells were transduced one to three times with the viral supernatants depending on the efficacy of transduction and maintained afterward in normal culture medium.

Gene knockout via zinc finger nucleases

Cys2His2-based zinc fingers were created via the Context Dependent Assembly method provided by the free software tool ZiFiT to identify and target the genomic sequence of CD222 specifically (23). The zinc finger constructs targeting exons 18 and 22 were cloned into pMML290/pMLM292 and pMSM800/pMML802 (both from Addgene) to generate zinc finger nuclease expression constructs. These constructs were used for the transfection of Jurkat T cells via electroporation. For this, 2 × 10⁵ cells were suspended in 100 μl Cytoxins (120 mM KCl, 0.15 mM CaCl₂, 5 mM MgCl₂, 10 mM K₂HPO₄, 25 mM HEPES [pH 7.6], 2 mM EGTA, 5 mM Glutathione, 1.25% DMSO [v/v], and 100 mM sucrose), mixed with the plasmids (each 0.5 μg), transferred in electroporation cuvettes, and transfected with the Amaxa System program S-18 (Amaxa, Lonza, Basel, Switzerland). Cells were immediately removed from the cuvettes, shortly spun down, and cultured in 12-well plates containing prewarmed medium. At 6 h post transfection, CD222-negative cells were collected, sorted with a FACSAria (BD Biosciences), expanded in Jurkat T cell–conditioned medium, and cultured in RPMI 1640 medium until analysis.

Immunofluorescence staining and confocal laser scanning microscopy

Primary T cells or T cell lines were let to adhere on adhesion slides (Superior-Marienfeld Laboratory Glassware, Lauda-Könighofen, Germany).
fixed, and permeabilized with 4% formaldehyde and 0.1% saponin, respectively, and stained with the following Ab: the rabbit anti-Lck Ab H-95 and the rabbit anti-human mAb EEAI (C45B10), followed by a goat anti-rabbit AF488 Ab, the anti-Lck mouse Ab (73A5), followed by anti-mouse AF488 Ab, the AF647-conjugated CD222 mAb MEM-238, and the Pacific Orange-conjugated CD45 mAb H130 (all diluted 1:50) in PBS containing 2% BSA. For stimulation of T cells before staining, cells were incubated with SEE-loaded Raji B cells at 37°C for the indicated time points. Mouse fibroblasts were either cultured in 8-well chamber slides (Nunc, Thermo Scientific Fisher) until confluency or trypsinized and cytopsins onto glass slides prior to fixation and staining as described above. For each cell, one vertical and one horizontal cut was made through the cell, and the Lck distribution was analyzed using the ZEN 2011 blue edition software (Zeiss, Jena, Germany). Nuclei were stained with DAPI. The cells were then washed 3 times with 1× PBS and mounted with mounting medium for fluorescence analysis (Vectashield; Vector Laboratories, Burlingame, CA). Isotype-matched controls were included. Pictures were captured with a confocal laser scanning microscopy (CLSM) 700 (Zeiss).

Flow cytometry
For the analysis of cell surface Ags, cells were washed and resuspended in staining buffer (1× PBS containing 1% BSA and 0.02% NaN₃). Subsequently, the cells were incubated for 20 min at 4°C with a fluorochrome-conjugated mAb. To control immunofluorescence staining, the cells were additionally incubated with secondary conjugates for 20 min at 4°C. Before analysis, the cells were washed with staining buffer. Flow cytometry was performed using a LSR II (BD Biosciences) and analyzed with FlowJo version 8.8.6 for Macintosh or FlowJo version 7.2.5 for Windows.

Luciferase assays
To assess the IL-2 promoter activity and NFAT binding to DNA, IL-2 luciferase- and NFAT-reporter Jurkat T cells were assayed, respectively. Briefly, the reporter cells (2 × 10⁴) were either kept unstimulated or were stimulated for 6 h in triplicates in CD3 mAb OKT3–coated (1 µg/ml) 96-well plates with or without soluble CD28 mAb Leu-28. The cells were washed with PBS and subsequently lysed in 100 µl luciferase lysis reagent (Promega, Madison, WI) for 30 min on ice. Seventy-five microliters of lysate were transferred into white 96-well microplates, and luminescence was measured after addition of the reaction reagent at a Mitras LB940 Elisa Reader (Berthold Technologies, Bad Wildbad, Germany). The protein concentration was determined via Bradford assay (Bio-Rad, Hercules, CA), and the luminescence intensity was normalized to the protein content of each sample.

Cytokine measurement
Culture supernatants (30 µl) of primary T cells were harvested after 24 h of stimulation and analyzed via the Lumien xMAP suspension array technology. Briefly, the cells were stimulated with plate-bound OKT3 (1 or 5 µg/ml) and soluble Leu-28 (2 µg/ml) or kept unstimulated. Standard curves were generated using recombinant cytokines (R&D Systems, Minneapolis, MN). Experiments were performed in GraphPad Prism. In general, data are expressed as mean ± SEM (or mean ± SD, when indicated).

Calcium mobilization assay
Cells (1 × 10⁶) were washed, resuspended in 100 µl RPMI 1640 medium containing 1 µmol/l Indo-1 AM (Invitrogen Life Technologies) and incubated for 30 min at 37°C. The cells were washed with medium, incubated in 1 ml medium for next 30 min at 37°C, and rested thereafter on ice until data acquisition at a LSR II flow cytometer. For the analysis of intracellular calcium flux, 300 µl Indo-1–loaded cells were prewarmed for 5 min at 37°C, the baseline response was recorded for 30 s, before the cells were stimulated with a 1:300 dilution of the hybridoma supernatant of the anti-TCR mAb C305 for 3 min to analyze the increase in calcium mobilization. The indo-violet/indo-blue ratio was calculated with the FlowJo software and plotted against the time. Ionomycin was used to check the overall responsiveness and cell viability.

PCR analysis
For real-time PCR analysis, primary human T cells, both resting and stimulated for 1 d with plate-bound CD3 mAb OKT3 (1 µg/ml) together with soluble CD28 mAb Leu-28 (0.5 µg/ml), were lysed in TRIzol (Invitrogen Life Technologies), and RNA was extracted according to the manufacturer’s instructions. cDNA was synthesized from 500 ng total RNA with SuperScript VILO cDNA Synthesis Kit (Invitrogen Life Technologies). Gene expression was measured by the 2–ΔΔCT method (24), based on quantitative real-time PCR using the CFX96 Real-Time PCR system (Bio-Rad) with TaqMan primer sets for human CD222, and YWHAZ as an endogenous control. For conventional PCR, 20 ng genomic DNA purified by standard isopropanol precipitation was used as a template of PCR amplitization with Taq polymerase (40 cycles at 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s) and with primer sets for human CD222 bridging an exon–exon junction, and intronless gene endosomalin, as an endogenous control (TaqMan Gene Expression Assay; ABI, Life Technologies).

Lipid raft preparation
Cells (2–3 × 10⁶) were lysed for 30 min on ice in lysis buffer containing 1% detergent Brij-58 and protease inhibitors. Lysates were adjusted with 80% sucrose solution to 40% sucrose in 1 ml, placed on a 1 ml 60% sucrose layer and overlaid by 20, 10, and 5% sucrose fractions (all 1 ml containing 0.5% detergent Brij-58 and protease inhibitors). Gradients were ultracentrifuged for 16–18 h at 150,000 × g at 4°C. Fractions (500 µl) were taken from the top to the bottom and denatured at 95°C with sample buffer and analyzed by SDS–PAGE, followed by Western blot analysis.

Gel filtration analysis
The GE Healthcare AKTA FPLC system was used for the gel filtration experiments. Cell lysates were prepared by solubilization with 1% detergent Triton X-100 (Promega). Samples were loaded at 4°C in a volume of 500 µl onto a Superose 6 HR 10/300 GL column (GE Healthcare) equilibrated with Triton X-100 (0.5%) in PBS. The absorbance at 280 nm was monitored, and 500 µl fractions were collected and analyzed by immunoblotting. The following standards of molecular mass (all from GE Healthcare or Sigma-Aldrich) were used: Blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa).

Membrane–cytosol separation
Cells (6 × 10⁵) were incubated in 2 ml hypotonic buffer (42 mmol/l potassium chloride, 5 mmol/l magnesium chloride, 10 mmol/l HEPES, and protease inhibitor mixture (pH 7.5)) for 30 min on ice and thereafter passed nine times through a 30-g needle. Cell fragments were centrifuged twice at 300 × g, and the resulting supernatant was centrifuged for 40 min at 35,000 × g at 4°C. The cytosolic supernatant was collected and the pellet containing membranous cellular parts was solubilized in extraction buffer (1× lysis buffer containing 1% Triton X-100, 0.1% NaDodSO₄, 0.5% sodium deoxycholate, and the protease inhibitor mixture) for 30 min on ice and centrifuged at 300 × g to remove debris. The fractions were analyzed via Western blotting.

Statistical analysis
Data analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA). Different experiments were analyzed by one-sample or standard Student’s t test or two-way ANOVA, followed by the Bonferroni posttest. A p value < 0.05 was considered as significant (*). Statistical analyses were performed in GraphPad Prism. In general, data are expressed as mean ± SEM (or mean ± SD, when indicated).

Results
CD222 surface expression on human T cells increases upon TCR stimulation
CD222 is upregulated on the cell surface of rodent T cells upon T cell activation (17). Therefore, we hypothesized that this molecule might play a role in T cell activation. We first investigated the expression of CD222 on human lymphocytes. Isolated human PBMC were labeled with CFSE and analyzed for surface expression of CD222 via flow cytometry using a specific CD222 mAb. Upon cross-linking of the TCR complex with an activatory CD3 mAb, CD222 was upregulated on the cell surface on days 2, 3, and 4 (Fig. 1A). However, analysis of CD222 mRNA levels in resting...
and CD3/CD28 mAb–stimulated T cells revealed no significant increase in CD222 expression upon activation (Fig. 1C). This suggests the regulation of CD222 via subcellular distribution and not via gene expression. Regardless of the cell type, ~90% of CD222 have been reported to be localized in the TGN and endosomal compartments at steady state (25). Therefore, the majority of CD222 is kept intracellular and only a small proportion is displayed, dependent on the cellular condition, at the cell surface.

Investigation of the T cell proliferation kinetics showed that CD222 upregulation on the plasma membrane preceded T cell division cycles. Both stimulated, yet not blasting T cells and blasting T cells displayed more CD222 on the surface compared with unstimulated T cells (Fig. 1B). Notably, the majority of proliferating cells was positive for CD222, which pointed toward a possible involvement of CD222 in T cell activation and/or differentiation.

**CD222 knockdown dampens T cell effector functions**

To investigate the putative role of CD222 in T cell activation, primary human PBMC were silenced for the expression of CD222 via the lentiviral-based introduction of shRNA. Total CD222 protein content was analyzed for shCTR and CD222-silenced...
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followed by expressing gating. We found that silencing of CD222
1.6 that we reconstituted before silencing with a GFP-tagged Lck
silenced T cells, we used the Lck-deficient Jurkat T cells JCaM
Indo-1. To enable an equal Lck expression in control- and CD222-
influenced the Ca 2+ flux to a lesser extent, which might be
changed (Fig. 2D). Silencing of CD222 at the less efficient P2 site
significantly reduced, whereas the baseline values remained un-
experiments showed that the peak values of the 390/495 ratio and
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To determine whether the cytokines were less synthesized or the
transport/secretion was hampered upon CD222 downregulation, we
used human luciferase reporter T cell lines: first, CD222- and
control-silenced Jurkat T cells that expressed luciferase under the
control of the IL-2 promoter were stimulated via CD3 or CD3/
CD28, and the lysates were analyzed for luciferase activity. Si-
lencing of CD222 at two distinct positions (shCD222/P1 and
shCD222/P2) led to a reduction of the luciferase activity upon
T cell stimulation via CD3 (Fig. 2A). Even activation of the co-
stimulatory pathway via CD28 ligation, which quantitatively en-
hances TCR-induced signals (26), could not rescue the IL-2 pro-
moter activity in the reporter cells silenced at the first position
(P1) and resulted in a significant luciferase reduction. Although
silencing at the second position (P2) did not reduce CD222 tran-
scripts as effective as P1 (Fig. 2A, inset), the corresponding cells
still displayed a reduced IL-2 promoter activity, although not
significant. Second, we used human reporter Jurkat T cells that
expressed the luciferase gene driven by an artificially designed
promoter region containing multiple binding sites for the tran-
scription factor NFAT (27). The NFAT-driven luciferase expres-
sion was significantly reduced in shCD222/P1 cells compared with
shCTR cells upon stimulation with CD3/CD28 mAb (Fig. 2B). These data revealed that CD222 knockdown affected cyto-
kine production at the transcriptional level.

To analyze whether decreased Ca2+ mobilization was respon-
sible for reduced NFAT promoter activity, we measured intracel-
lar Ca2+ fluxes by flow cytometry with the Ca2+ sensitive dye
Indo-1. To enable an equal Lck expression in control and CD222-
silenced T cells, we used the Lck-deficient Jurkat T cells JCaM
1.6 that we reconstituted before silencing with a GFP-tagged Lck
followed by expressing gating. We found that silencing of CD222
at P1 and P2 led to a significant reduction in the cytosolic Ca2+
mobilization capacity compared with control-silenced Lck-GFP+
JCaM 1.6 T cells (Fig. 2C). Detailed analysis of Ca2+ flux
experiments showed that the peak values of the 390/495 ratio and
the overall mean values of shCD222/P1-silenced cells were sig-
nificantly reduced, whereas the baseline values remained un-
changed (Fig. 2D). Silencing of CD222 at the less efficient P2 site
influenced the Ca2+ flux to a lesser extent, which might be
explained via a dose-dependent mode of action of CD222. Indeed,
this assumption was supported by other experiments: titration of
virus, carrying the silencing construct, correlated with lower ex-
pression of CD222 and reduced Ca2+ mobilization in Jurkat
T cells (Fig. 2E) suggesting a direct correlation between the total
amount of the CD222 protein and the T cell response to TCR
stimuli. Furthermore, the artificial overexpression of CD222 led to
significantly higher intracellular Ca2+ flux in Jurkat T cells and
CD222-overexpressing cells reacted faster than cells expressing
the control vector (Fig. 2F). However, it has to be mentioned that
cells highly overexpressing CD222 are more susceptible for apop-
tosis compared with control cells (28). Furthermore, high
amounts of CD222 lead to a decreased cellular growth rate (29).
Therefore, just cells slightly overexpressing CD222 survived and
grew adequately. Finally, to verify the data obtained via RNA
interference, genmic CD222 was targeted and cut via sequence-
specific zinc finger nucleases in Jurkat T cells (Supplemental Fig.
1A). Analysis of three independently sorted CD222 knockout
populations (C20, C21, and C25; Supplemental Fig. 1B), revealed
that each of them displayed a reduction in intracellular Ca2+ fluxes
compared with CTR cells (Supplemental Fig. 1C). Importantly,
this phenotype was rescued in knockout cells that were stably
transduced with recombinant human CD222 (C20 cells recon-
tituted with CD222; Supplemental Fig. 1D–F). Taken together,
these data imply that CD222 is an essential regulator of the TCR
signaling cascade.

CD222 is crucial for early T cell signaling

To entangle the mode of operation of CD222 in TCR signaling, we
stimulated control- and CD222-silenced Jurkat T cells with the TCR-
specific mAb C305 and evaluated the tyrosine phosphorylation in the
cell lysates via Western blot analysis; several molecules were less
phosphorylated in CD222-silenced cells, particularly in molecular
mass regions corresponding to the TCR proximal signaling mole-
cules ZAP70, LAT, and CD3 (Fig. 3A), suggesting a very early
effect of CD222 on T cell signal transduction. To verify this, we
used specific mAb in a multicolor fluorescence multiplexing ap-
proach. Indeed, the earliest T cell signaling proteins CD3ζ and
ZAP70 displayed lower tyrosine phosphorylation upon CD222
downregulation. Accordingly, all ensuing signaling molecules, such
as LAT and ERK, were less phosphorylated in shCD222 cells
(Fig. 3B). Statistical evaluation of multiple experiments analyzing
pCD3ζ and pERK1/2 revealed that the phosphorylation of both
(i.e., early and late signaling molecules) was significantly reduced
upon CD222 knockdown (Fig. 3C). In addition, Lck’s serine 59,
a substrate of active ERK1/2 (30), was less phosphorylated in
CD222 knockdown cells, which possibly could underlie an insuf-
ficient signal amplification to sustain T cell activation (Supplemental
Fig. 2A). Supporting evidence for the implication of CD222 in
TCR-proximal signaling was provided via CLSM: upon 15-min
stimulation of JCaM 1.6 T cells with superantigen SEE-loaded
Raji B cells, CD222 redistributed from the cell surface and/or
intracellular pericentromosomal compartments (31) to the interface
of the IS in ~70% of JCaM 1.6 T cells that were in contact to Raji
B cells (n = 24; Fig. 3D).

Lck and CD45 interact with CD222

To unravel possible interaction partners of CD222, we pulled down
CD222 with specific mAb-coupled beads after cell lysis of resting
Jurkat T cells with lauryl maltoside, and analyzed coprecipitated
proteins via LC-MS. We found, along with known CD222 binding
proteins, various T cell–specific proteins. Among these proteins
we found Lck and CD45, molecules implicated in the regulation
of T cell signaling (Table I). To verify the protein–protein inter-
actions, we precipitated CD222 from lysates of resting Jurkat
T cells via CD222 mAb-coupled beads and analyzed coimmuno-
precipitated proteins by Western blotting. We detected both, Lck
and CD45, in the CD222 coimmunoprecipitates (Fig. 4A).

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Moreover, we detected the Lck-CD222 interaction also by a reverse approach performed with resting JCaM 1.6 T cells, expressing Lck fused to the One-Strep-Tag epitope (Lck-OST). Therein, CD222 was reproducibly found in the isolated Lck complexes via LC-MS analysis (Mascot protein score = 486.34). The interaction of CD222 and Lck also was confirmed by coimmunoprecipitation studies with JCaM 1.6 T cells, followed by immunoblotting (Fig. 4B). A truncated nonfunctional mutant of Lck encompassing only the first 10 aa of Lck (LckN10) did not coimmunoprecipitate with CD222 (Supplemental Fig. 2B), suggesting a specific protein–protein interaction. Furthermore, we verified the interaction of CD222 and Lck in nonstimulated primary human T cells isolated from peripheral blood (Fig. 4C). Immunofluorescence-based in situ localization via CLSM revealed that Lck colocalized with CD222 in resting Jurkat T cells (Fig. 4D). However, the majority of interactions seemed to take place in in-
tracellular compartments: Upon conditions allowing the specific immunoprecipitation of cell surface-bound CD222, no Lck was coimmunoprecipitated from lysates of primary T cells (Fig. 4E). Furthermore, CD222 and Lck colocalized with the EEA1 in resting Jurkat T cells (Supplemental Fig. 3). Moreover, when we stimulated JCaM 1.6 T cells that ectopically expressed Lck-GFP with SEE-loaded Raji B cells, we found a coordinated intracellular accumulation of Lck and CD222 submembrane of the active IS (Fig. 4F), suggesting that the cytoplasmic interaction of CD222 and Lck prevails also upon T cell activation. Analysis of consecutive z-stacks of the depicted cells confirmed that CD222 colocalized with Lck almost exclusively within the cell with a Pearson’s coefficient of ~0.3, whereas the Pearson’s coefficient was practically zero at the plasma membrane edges (Fig. 4F, right panel). These findings suggest that the interaction between CD222 and Lck occurs preferentially in cytoplasmic vesicles and that CD222 is involved in the initiation of the early signaling cascade through acting on Lck.

CD222 knockdown leads to an inhibitory phosphorylation signature on Lck

Lck has been reported to exist in resting T cells in four different pools, each constituting ~25% of total Lck and constantly maintaining at this equilibrium (3, 4) with CD45 being an important regulator of the Lck phosphorylation status. Because we found CD222 to interact with both Lck and CD45, we hypothesized that CD222 might be a mediator involved in the regulation of Lck phosphorylation. To test this, we investigated the tyrosine phosphorylation of Lck normalized to total Lck in shCD222/P1, shCD222/P2, and shCTR Jurkat T cells via LiCor infrared fluorescence Western blot analysis (Fig. 5A, 5C). Corresponding CD222 surface expressions were analyzed via flow cytometry (Fig. 5B). Lck was significantly more phosphorylated at Y505 in shCD222/P1 cells compared with shCTR cells (Fig. 5A, 5C). Silencing with the second construct (shCD222/P2) as well increased Lck phosphorylation at Y505 (Fig. 5C), although not significantly, which correlated with the less effective silencing at this position (Fig. 5B). Lck phosphorylation at Y394 was not changed (Fig. 5A, 5D), whereas the amount of Lck not phosphorylated at Y394 (non-pY394) significantly increased upon CD222 knockdown (Fig. 5A, 5E). These findings suggest that silencing of CD222 causes a shift in Lck pools toward the less active form solely phosphorylated at the inhibitory Y505.

To pinpoint whether the increased phosphorylation at Y505 was the cause of a blockade in signaling upon CD222 silencing, we used a mutated form of Lck that was made up of a phenylalanine instead of the tyrosine at the position 505 and consequently could not be phosphorylated at this site (32). Lckwt and the mutated form of Lck (LckY505mut) had been ectopically expressed in Lck-deficient

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** CD222 knockdown affects the phosphorylation of distal and proximal TCR signaling molecules. (A) shCTR and shCD222/P1 Jurkat T cells were stimulated with CD3 mAb (MEM-92) for the indicated time points, and lysates were analyzed via Western blotting for the presence of phosphotyrosines. GAPDH was used as a loading control after stripping of the membrane; kiloDalton ranges are indicated on the left. Arrows depict kiloDalton areas of 70, 38, and 22 kDa. (B) For the simultaneous detection of total protein versus the phosphorylated form (green versus red), we analyzed lysates from shCTR and shCD222/P1 Jurkat T cells with specifically labeled Abs directed against the indicated proteins via the LiCor system. (C) The dual fluorescence immunoblots were analyzed by the LiCor system. Phosphorylated proteins were set relative to total protein content of the same molecule (except for CD3), and individual experiments were normalized to the maximum value during the stimulation course, which was set to 100% (% of Max); n = 4 for CD3 and n = 2 for ERK1/2. Data represent mean percentage of Max ± SD. Significance was evaluated via the Bonferroni posttest following two-way ANOVA; *p < 0.05. (D) Upper panels, JCaM 1.6 T cells were stimulated for 15 min with SEE-loaded Raji B cells, fixed, permeabilized, and stained for CD222 (red). Lower panels, JCaM 1.6 T cells without activated B cells were treated the same. Pictures were captured at a CLSM. Phase contrast pictures were overlaid with the CD222-staining (right). Nuclei were visualized with DAPI (blue). Shown is one of at least two representative experiments. Scale bar, 5 μm.
JCaM 1.6 T cells before the cells were silenced for CD222 and intracellular calcium mobilization was measured via flow cytometry. JCaM 1.6 T cells expressing Lckwt showed a reduced calcium flux upon CD222 compared with control silencing. However, silencing of CD222 did not affect the calcium flux in JCaM 1.6 T cells carrying the mutated form of Lck (Fig. 5F), suggesting that the altered phosphorylation of Lck upon CD222-silencing caused the less reactive T cell phenotype.

**CD222 regulates the intracellular distribution of Lck**

To test whether CD222 was responsible for the efficient transport of Lck, we analyzed the distribution and membrane localization of Lck via immunofluorescent staining and CLSM of resting control and CD222-silenced Jurkat T cells. The typical homogenous distribution of Lck but rather directs its cellular distribution. The findings indicate that CD222 does not influence lipid raft partitioning but rather directs its cellular distribution.

**CD222 knockdown impairs the interaction of CD45 with Lck**

Finally, we investigated the interaction of Lck and CD45. Unstimulated JCaM 1.6 T cells ectopically expressing Lck-GFP were stained for CD45 and analyzed for the colocalization of CD45 with Lck via immunofluorescent staining and CLSM of resting control and CD222-silenced Jurkat T cells. The typical homogenous distribution of Lck but rather directs its cellular distribution.
beled the surface of living cells with a CD45 mAb on ice, lysed the cells, immunoprecipitated cell surface CD45, and analyzed the eluates for coimmunoprecipitated Lck (Fig. 7D). The interaction of cell surface CD45 with Lck was reduced by \( \sim 40 \) and \( \sim 20\% \) in shCD222/P1 and shCD222/P2 knockdown cells, respectively (Fig. 7D). In contrast, LckN10, the non-functional mutant variant of Lck consisting of the 10 N-terminal amino acids responsible for plasma membrane anchorage, did not coimmunoprecipitate with surface CD45 (Fig. 7E). These data suggest that CD222 controls the transport of the kinase Lck to the plasma membrane and subsequently its interaction with CD45, the key activatory phosphatase of Lck.

**Discussion**

Lck is described to exist in four different phosphorylation states, constantly maintained at equilibrium, with \( \sim 40\% \) of consti-
As active Lck represents a high risk for a T cell to get permanently activated resulting in hyperreactive adaptive immune reactions in an organism, the spatial arrangement of Lck has to be accurately organized in time and space. Limiting the access of possible kinase substrates or orchestrating the interaction with regulatory proteins can prevent overwhelming or self-directed immune responses. On the basis of our data, we propose a novel function for the late endosomal transporter CD222 in the early TCR signaling cascade. CD222 is essential for the efficient recruitment of Lck to the plasma membrane, the place where it interacts with its phosphatase CD45. The loss of CD222 leads to accumulation of Lck in its inactive form, which results in diminished T cell activation and effector functions.

Because Lck represents a very powerful kinase that is involved in various T cell signaling pathways (41–44), its transport and regulation has been investigated extensively (45–49). One important molecule implicated in the transport of Lck is the myelin and lymphocyte protein (MAL). Upon knockdown of MAL, Lck was stuck in the TGN, without significant increase in cytosolic Lck. Furthermore, MAL was essential for lipid raft partitioning of Lck, whereas the transport to nonraft membranes was unhampered (45). In contrast, knockdown of CD222 hampered the efficient transport of Lck to the plasma membrane with an apparent accumulation of Lck within intracellular membranes and freely floating in the cytoplasm but without any significant effect on lipid raft partitioning. It has been shown previously via single molecule microscopy and bleaching experiments that single Lck molecules recolonize the cell membrane directly from the cytoplasm, rather than via two-dimensional transversal diffusion (49), which may explain the high amount of non–membrane-bound cytosolic Lck. Lck has been reported previously to be present in endosomes (47, 50). Our confocal microscopy and also gel filtration fractionation experiments revealed an accumulation of Lck and CD222 in the EEA1-positive fractions corresponding to endosomal membranes. Furthermore, via mass spectrometry analysis we identified Rab11, a marker of endosomal membranes critically involved in protein trafficking, to interact with CD222. This strongly suggests that...
The transport of Lck to the cell surface is blocked in CD222 knockdown cells. (A and B) Jurkat T cells silenced for CD222 (shCD222/P1 and /P2) and control-silenced cells (shCTR) were placed on adhesion slides, fixed, permeabilized, and stained for Lck (mAb H-95). For detection, AF488-coupled secondary anti-rabbit Ab was used (green). After blocking, CD222 was stained using AF647-conjugated mAb MEM-238 (blue). Pictures were captured with a CLSM and are representative of at least two individual experiments. Scale bar, 5 μm. (B) Individual cells in each setting (N in A) were analyzed for Lck expression via plotting the surface fluorescence intensity in three dimensions with the ImageJ software (surface plot function). (C–E) Upper panels, Cellular distribution patterns of Lck-GFP, LckN10-GFP, and LckN10-GFP. JCaM 1.6 T cells expressing GFP-tagged versions of Lck were let to adhere on adhesion slides, fixed with 4% formaldehyde, and analyzed for the distribution of Lck and Lck variants via CLSM. Nuclei were visualized with DAPI. Shown are representative pictures out of three independent experiments. Scale bar, 5 μm. Lower panels, JCaM 1.6 T cells expressing GFP-tagged wt full-length Lck (C, Lck-GFP, n = 69), the GFP-tagged 10 N-terminal aa of Lck (D, N10-GFP, n = 20), and the N-terminal deletion (Figure legend continues)
CD222 coordinates the endosome-dependent transport of Lck that was shown to be essential for T cell activation (48). Uncoordinated 119 protein has been shown to activate endosomal Rab11 for the plasma membrane targeting of Lck (48) and activation of Fyn (51). However, unlike MAL and uncoordinated 119 protein, CD222 does not contribute to the IS, but rather builds an early vesicular platform submembrane of the IS. Although several consecutive devices obviously coordinate Lck transport (52–54), the contribution of CD222 seems to be very specific as human CD222 can perform its action on human Lck even in mouse fibroblasts (i.e., irrespective of other T cell–specific molecules). Moreover, the absence of MAL in fibroblasts (55) suggests that CD222 operates upstream of MAL.

To our knowledge, the role of CD222 in T cell signal transduction has yet not been investigated in detail, but CD222 has been shown to enhance TCR-induced signaling via CD26 internalization (56). However, we found no evidence for the contribution of CD26 to the effect of CD222 on Lck because we have observed the effect
of CD22 knockdown on T cell activation in Jurkat T cells that lack CD26 expression (57). Interestingly, ligation of the common costimulatory molecule CD28, which also quantitatively enhances T cell responses to TCR stimuli (26), did not rescue, but even resulted in a more pronounced phenotype induced by silencing of CD22.

The loss of CD22 in resting T cells results in accumulation of Lck single phosphorylated at residue Y505 that represents the closed inactive form of Lck (7, 58). Consequently, the homeostasis of the four proposed conformational forms of Lck (3) is shifted toward the inhibitory closed conformation of Lck. Recently, it has been proposed that the intracellular distribution of Lck is regulated via its phosphorylation state and the corresponding conformations (4, 6): the open active form (pY394) is thought to induce clustering, whereas the closed inactive form (pY505) prevents the formation of clusters, which has been shown to be lipids independent (4). Putting these data together one might suggest that the CD22 knockdown, which results in a 2-fold increase of inhibitory Lck pY505, does prevent Lck from clustering and T cells from getting activated. The finding that the hyperactive mutated form LckY505mut triggers the activation of the secondary messenger Ca2+ also in the absence of CD22 supports this model.

On the basis of our data, CD22 mediates the shift of Lck toward the active dephosphorylated form by complexing it with CD45. We pinpoint the CD22-driven CD45–Lck interaction to the cell membrane, and second, that Csk-induced phosphorylation of Y505 (59) can occur within the cell. Because we found CD22 to interact with both the kinase Lck and its phosphatase CD45, the question arises whether the transport of CD45 might be as well miss-regulated upon silencing of CD22. However, several facts speak against the simultaneous transport deregulation of Lck and CD45 in the CD22 knockdown phenotype. First, we found just a minor downregulation of CD45 at the cell surface. Second, CD45 is not only a positive but also a negative regulator of T cell activation and differentiation (59, 60). Intermediate levels of CD45 surprisingly produce hyperre-active T cells (59), which we could not detect at all in our experimental setup. Third, CD45 dephosphorylates not only Lck but also CD3ζ (4, 59–61). However, the phosphorylation of CD3ζ was unaffected in resting, and notably, even significantly reduced in stimulated CD22 knockdown cells. Taking these facts into consideration, we conclude that the primary function of CD22 in T cell activation is to act as intermediary in the interaction of CD45 with Lck resulting in the correct dephosphorylation of Lck at the inhibitory Y505.

We show in this study that the interaction between CD22 and Lck occurs preferentially inside the cell and disbands at the cell surface, where Lck is “handed over” to CD45. This indicates that CD22 is involved in very early signaling events. However, we do not have at the moment an explanation for CD22’s upregulation on the surface of proliferating and differentiating T cells—in the later phase of T cell activation, shortly before cell division is initiated. One can envisage several possible reasons of the increased CD22 surface accumulation during later phases of T cell activation: 1) It might be necessary to fully confer the capacity of cells to respond. 2) It might result from higher protein transport activity mediated by CD22. Because upon T cell activation the turnover of Lck is enhanced (50), the fusion of CD22-containing transport vesicles with the plasma membrane might result in elevated CD22 on the cell surface. 3) It might be important for the later signaling events as a negative feedback loop to abrogate T cell activation. The latter could be accomplished via regulating the bioavailability of the mitogenic insulin-like growth factor 2 that is both postnatally expressed in thymic epithelial cells (62) and upregulated in diseases (28), namely via internalization and subsequent degradation in lysosomes (12, 63). 4) Furthermore, CD22’s surface upregulation might play a role in T cell differentiation from the double negative to the double positive stage as CD22 Ab was reported to block T cell ontogeny (64). Notably, Lck knockout mice also display a similar reduction in double-positive thymocytes in T lymphocyte development (65) as the one caused by the CD22 Ab treatment. However, because of the lethality of the CD22 knockout in mice (66–68), the function of CD22 in T cell differentiation has never been shown in vivo. 5) An involvement of CD22 in T cell migration can also not be excluded as it has been shown that CD22 negatively regulates cell adhesion via uPAR and integrins (22). 6) Alternatively, it could be the result of a better accessibility of the CD22 mAb caused by altered interactions that unmask the epitope.

Irrespective of the pathways that are amenable in the upregulation of cell surface CD22 upon T cell activation, we show in this study a central function of the endosomal transporter CD22 in the initiation of T cell signal transduction through controlling Lck distribution: CD22 targets Lck to CD45 at the plasma membrane and thereby maintains the equilibrium of Lck phosphorylation at the steady state.

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Disclosures

The authors have no financial conflicts of interest.

References


16: 4983–4998.


14: 70–79.


12: 259–266.


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7: 939–9344.

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3: 939–951.

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68: 3977–3981.


52: 9339–9344.

51: 9372–9381.

50: 9789–9800.

49: 10928–10932.


47: 5392–5397.


45: 1335–1347.

44: 8439–8444.

43: 1113–1123.


41: 1008–1018.

40: 3977–3981.

39: 9329–9341.

38: W462-8.

37: 585–593.

36: 1303–1315.


34: 447–461.

33: 665–674.

32: 389–400.

31: 939–951.

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29: 259–266.

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26: 7535–7547.

25: 402–408.

24: 259–266.

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21: 2415–2433.

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Supplemental Material

Pfisterer et al., Supplemental Figure 1

Supplemental Figure 1. Reconstitution of CD222 expression in knock-out T cells rescues downregulated Ca\(^{2+}\) fluxes. (A) The genomic location of the targeted Exon 22 of CD222 on chromosome 6 is denoted by the red line. Recognition sequences and genomic coordinates of the zinc finger nuclease (ZFN) are shown below. (B) Total CD222 and Lck content in control Jurkat T cells (CTR) and different CD222 knock-out cell populations (C20, C21, C25) were analyzed via Western blot. GAPDH served as a loading control. (C) Ca\(^{2+}\) fluxes in control and CD222 knock-out cells were measured upon stimulation with the anti-TCR mAb C305. (D) CD222 surface expression of control cells (black), CD222 knock-out cells C20 (dashed, dark
gray) and C20 cells stably reconstituted with recombinant human CD222 (reconst., light gray). The isotype control is illustrated as light gray filled histogram. (E) Genomic DNA purified from control cells (CTR), CD222 knock-out cells (C20), and C20 cells reconstituted with CD222 (reconst.) was used as a template for the PCR amplification with primer sets for human CD222 and the intron-less short gene endosialin as an endogenous control. In contrast to the control primers recognizing endosialin, CD222 primers bridge an exon-exon junction and thus can only recognize the recombinant form of CD222. (F) Ca\(^{2+}\) flux was measured as described in (C) in control cells (black), CD222 knock-out cells C20 (dark gray) and reconstituted cells (light gray) Shown is one representative experiment out of two.
Supplemental Figure 2. (A) Serine 59 phosphorylation of Lck is decreased upon silencing of CD222. Jurkat T cells were stimulated with the CD3 mAb MEM-92 for the indicated time points and cell lysates were analyzed for total Lck expression by using mAb H-95 and Western blotting. Phosphorylation of Lck serine 56 and Lck serine 59 were measured via the LiCor multiplexing fluorescence immunoblotting system. The mean fluorescence intensity was determined via ImageJ and the Lck serine 56/serine 59 ratios of four independent experiments were evaluated and plotted via GraphPad Prism (mean ratio±SEM). Statistical
significant changes between shCTR (black) and shCD222/P1 cells (gray) were evaluated via 2-way ANOVA followed by the Bonferroni post test, * p<0.05, ** p<0.01, ns, not significant. (B) CD222 interacts with Lck, but not with LckN10. Wild-type JCaM 1.6 T cells that lack endogenous Lck and JCaM 1.6 T cells ectopically expressing either Lck-GFP (approx. 83 kDa) or truncated LckN10-GFP (approx. 28 kDa) were lysed using 1% detergent lauryl maltoside. Then, CD222 was immunoprecipitated using mAb MEM-238. Lysates and immunoprecipitates (IP) were analyzed by Western blotting. CD222 was detected via mAb MEM-238, Lck and the truncated versions of Lck were detected by mAbs directed against the GFP-tag (B-2). (C) Total Lck content in crude cell lysates of control- and CD222-silenced Jurkat T cells. Whole cell lysates of shCTR and shCD222/P1 cells were probed for Lck and LAT by Western blotting. Band intensities were calculated with ImageJ. Shown is one representative experiment out of three.
Supplemental Figure 3. CD222 colocalizes with EEA1 and Lck. Jurkat T cells were let to adhere on adhesion slides, fixed, permeabilized and stained for CD222 (red), EEA1 (blue) and Lck (green). Triple staining of two representative Jurkat T cells are shown. Pictures were captured with a CLSM. Scale bar = 5 µm.
Supplemental Figure 4. CD222 is influencing protein complex formation without affecting lipid raft formation. (A) shCTR and shCD222/P1 Jurkat T cells were lysed using 1% detergent Brij-58. Lipid raft proteins (light fractions, left) were separated from non-raft proteins (heavy fractions, right) via sucrose density centrifugation and the indicated proteins were detected via Western blotting using specific Abs. Data are representative of at least two individual experiments. (B) Jurkat shCTR (upper panel) and shCD222/P1 (lower panel) T cell lysates were prepared using the 1% detergent Triton X-100. The samples were loaded onto a Superose 6 column and consecutive fractions were taken and analyzed by Western blotting. mAbs to CD222 (MEM-238), CD45 (MEM-28), Lck (H-95), CD3ε (FL-207) and EEA1 (C45B10) were probed. kDa ranges are indicated at the right. Shown is one representative experiment out of two.