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A Regulatory Role for CD37 in T Cell Proliferation[1]

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CD37 is a leukocyte-specific protein belonging to the tetraspanin superfamily. Previously thought to be predominantly a B cell molecule, CD37 is shown in this study to regulate T cell proliferation. CD37-deficient ($CD37^{-/-}$) T cells were notably hyperproliferative in MLR, in response to Con A, or CD3-TCR engagement particularly in the absence of CD28 costimulation. Hyperproliferation was not due to differences in memory to naive T cell ratios in $CD37^{-/-}$ mice, apoptosis, or TCR down-modulation. Division cycle analyses revealed $CD37^{-/-}$ T cells to enter first division earlier than wild-type T cells. Importantly, proliferation of $CD37^{-/-}$ T cells was preceded by enhanced early IL-2 production. We hypothesized CD37 to be involved in TCR signaling and this was supported by the observation that CD4/CD8-associated $p56^{Lck}$ kinase activity was increased in $CD37^{-/-}$ T cells. Remarkably, CD37 cross-linking on human T cells transduced signals that led to complete inhibition of CD3-induced proliferation. In the presence of CD28 costimulation, CD37 engagement still significantly reduced proliferation. Taken together, these results demonstrate a regulatory role for CD37 in T cell proliferation by influencing early events of TCR signaling. ***The Journal of Immunology,*** **2004, 172: 2953–2961.**

The leukocyte-specific protein CD37 is a member of the tetraspanin superfamily or transmembrane 4 superfamily, which is characterized by the presence of four conserved transmembrane domains. Tetraspanin members have functional roles in a wide array of cellular processes, including cell adhesion, motility, differentiation, proliferation, and tumor invasion (1–3). It is proposed that they influence signal transduction by the organization of multimolecular complexes of integrins and lineage-specific proteins in cell membranes (2, 4–6). However, the precise molecular mechanisms that underlie tetraspanin function are not well understood.

Many tetraspanin proteins (CD9, CD63, CD81, CD151) have a broad tissue expression, whereas others are restricted to leukocytes (CD37, CD53) and hematopoietic cells (Tssc6). Tetraspanin involvement in leukocyte function has been widely documented (7) and is likely related to their association with other cell surface proteins, including CD2, CD4, CD8, MHC class I, MHC class II, and integrins (2, 5). CD81 has a role in humoral immune responses, IL-4 production by T cells, and T cell-B cell interaction, which is partially mediated by its activation of LFA-1 (8–12). CD53 is involved in T cell development and signaling (13–15). The tetraspanins CD9, CD53, CD81, and CD82 have been implicated in costimulation of T cells (16–19). Furthermore, in APCs, tetraspanin microdomains may have a functional role in presentation of peptide by MHC class II molecules (20).

In humans, CD37 expression is restricted to B and T lymphocytes, monocytes, macrophages, neutrophils, dendritic cells (DC)[3], and certain leukocyte-derived malignant cells (21, 22). CD37 is not expressed by NK cells, platelets, or erythrocytes. Northern blot analyses of organs and cell lines revealed broadly similar CD37 expression in mice (23). CD37 on human B cells associates noncovalently with other tetraspanins (CD53, CD81, and CD82) and MHC class II molecules (24). It has, furthermore, been detected on exosomes secreted by APCs, together with MHC class II, costimulatory molecules, and other tetraspanins (CD53, CD63, and CD81) (25). Exosomes are able to prime T cell-dependent antitumor responses in vivo (26).

Very little is known about the function of CD37. Its leukocyte-restricted expression pattern suggests a specific role in the immune system. In vitro studies have documented homotypic aggregation of B cells upon CD37 cross-linking (27). To gain insight into CD37 function in vivo, CD37-deficient ($CD37^{-/-}$) mice have been generated (28). Development of the immune system was normal in the absence of CD37. However, impaired T cell-dependent B cell responses have been described in these mice (28). In this paper, we report the first evidence of a regulatory role for CD37 in T cells. The absence of CD37 results in dysregulation of TCR signaling, enhanced proliferation, and altered cytokine production.

Materials and Methods

Antibodies

Anti-mouse Ab used were KT31.1 (CD3), 37.51 (CD28), M1/70 (CD11b), RB6.8C5 (Gr-1), RA3.6B2 (B220), TER119 (erythrocyte), M5/114 (MHC class II), T24/31.7 (Thy1), YTS169.4 (CD8α), GK1.5 (CD4), FGK45.5 (CD40), 7D4 (CD25), H1.2F3 (CD69), IM7.81 (CD44), and Mel-14

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[3] Abbreviations used in this paper: DC, dendritic cell; MFI, mean fluorescence intensity; Lck, $p56^{Lck}$; WT, wild type.

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(CD62L) (Ref. 29 and references in this study). Anti-human Ab included OKT3 (CD3; ascites kindly provided by Dr. M. Sandrin, Austin Research Institute, Victoria, Australia), CD28.2 (CD28; BD PharMingen, San Diego, CA), WR17 (CD37; Serotec, Oxford, U.K.), and W6/32 (MHC class I). Abs were dialyzed before use in proliferation assays.

Mice

$CD37^{-/-}$ mice were generated by homologous recombination (28) and backcrossed 10 times to C57BL/6 background. C57BL/6 and wild-type (WT) and BALB/c mice were obtained from the Walter and Eliza Hall Institute (Melbourne, Victoria, Australia). OT-II mice (30), expressing OVA-specific transgenic TCR, were crossed with $CD37^{-/-}$ mice and used in TCR-CD3 down-modulation experiments. Mice were bred at the Austin Research Institute animal facility (Heidelberg, Victoria, Australia) and used at 6–10 wk of age. Animal studies were approved by the animal ethics committee of the Austin & Repatriation Medical Centre.

Murine T cell isolation

Cell suspensions of pooled lymph nodes were prepared in PBS with 2% FCS. T cells were purified by Ab-bead depletion using Ab against the following surface molecules: CD11b, GR-1, B220, TER119, and MHC class II. To purify $CD4^+$ or $CD8^+$ T cells, Abs against CD8α and CD4 were additionally used. Two rounds of magnetic bead depletion were performed using goat anti-rat Ig-coated beads (Paesel & Lorei, Frankfurt, Germany).

Splenocyte and T cell proliferation

Splenocyte preparations were made in RPMI 1640 medium (Life Technologies, Grand Island, NY) with 10% FCS and 0.1 mM 2-ME (complete RPMI 1640) using 100 μm cell strainers (BD Biosciences, Franklin Lakes, NJ). Erythrocytes were lysed by ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2 EDTA, pH 7.2). Splenocytes (1×10^5 cells/well) were stimulated with 1.5 μg/ml Con A (Sigma-Aldrich, St. Louis, MO) in 96-well plates for various times. Purified $CD4^+$ and $CD8^+$ T cells (2×10^4 cells/well) were stimulated with KT31.1 (coated at 1 μg/ml) with or without anti-CD28 (1 μg/ml in solution) in complete RPMI 1640. In additional experiments, $CD4^+$ and $CD8^+$ T cells were cultured in KT31.1-coated plates with 10 ng/ml recombinant IL-2 (BD PharMingen). T cells were pulsed for 6 h with 1 μCi of [^{3}H]thymidine (Amersham, Little Chalfont, Buckinghamshire, U.K.) and incorporation was measured using a scintillation counter (Top Count; Packard Instrument, Meriden, CT).

DC isolation and MLR

DC were isolated from BALB/c spleens using Nycodenz centrifugation and immunomagnetic bead depletion as previously described (29). In MLR, 5×10^3 DC were incubated with 2×10^4 $CD37^{-/-}$ or WT purified T cells in complete RPMI 1640 in 96-well plates. Control wells included DC and T cell cultures only. T cell proliferation was assayed as previously described.

CSFE studies

Purified lymph node T cells (1×10^7 cells/ml) were labeled with 10 μM CSFE (Molecular Probes, Eugene, OR) in PBS with 0.1% (w/v) BSA for 15 min at 37°C. Cells were washed in complete RPMI 1640 and stimulated in 24-well plates (2×10^5 cells/well) coated with KT31.1 (1 μg/ml). Flow cytometric analysis was performed twice daily on propidium iodide-negative cells. Division cohort analysis was performed as described (31). Briefly, the proportion of cells within each division was divided by 2^i (where i is the division number) and normalized to give a total of 100%. These values were plotted against a division number generating a Gaussian distribution with a mean division number. The mean division numbers were plotted against time resulting in a linear relationship with the inverse of the slope representing the average division cycle time, and the intercept with division 1 representing the average time to reach the first division.

Cytokine ELISA

Cytokines present in supernatants of stimulated T cell cultures were analyzed by sandwich ELISA using MaxiSorp 96-wells plates (Nunc, Roskilde, Denmark). All Ab and recombinant mouse IL-2, IL-4, and IFN-γ were obtained from BD PharMingen. Anti-IL-2 (3 μg/ml), anti-IL-4 (3 μg/ml), and anti-IFN-γ (1 μg/ml) were coated overnight at 4°C, plates were blocked with 1% FCS and 1% (w/v) BSA in PBS, and supernatants were added for 2 h at 20°C. Plates were incubated with biotinylated anti-IL-2 (1 μg/ml), anti-IL-4 (0.5 μg/ml), and anti-IFN-γ (0.5 μg/ml), followed by Streptavidin-HRP (Amersham) and ABTS diammonium salt (Sigma-Aldrich) as substrate. Absorbance was read at 405 nm.

ELISPOT assays

All Abs were obtained from BD PharMingen. 96-Well nitrocellulose filter plates (Multiscreen HA, Millipore, Bedford, MA) were coated with anti-IL-4 (7.5 μg/ml) overnight at 4°C. $CD4^+$ T cells from 3 day-stimulated cultures were added at 2×10^4 and 2×10^5 cells/well. After a 5-h incubation at 37°C, biotinylated anti-IL-4 (1 μg/ml) was added followed by streptavidin-alkaline phosphatase (Sigma-Aldrich) and alkaline phosphatase substrate kit (Bio-Rad, Hercules, CA). Blue spots were enumerated using an ELISPOT reader equipped with AID 2.9 software (AutoImmun Diagnostika, Strassberg, Germany).

Flow cytometry

FITC-conjugated anti-CD62L and biotinylated anti-CD44 were used in combination with Streptavidin-PE (BD PharMingen) to measure percentages of memory vs naive $CD4^+$ and $CD8^+$ T cells in spleens and lymph nodes. Spontaneous apoptosis in T cell cultures and induced apoptosis (5 Gy gamma irradiation of 1.5×10^6 cells/ml) was analyzed using Annexin V-FITC (BD PharMingen) in combination with propidium iodide staining.

In CD3 down-modulation assays, purified lymph node T cells of WT and $CD37^{-/-}$ OT-II mice were stimulated in KT31.1-coated plates as previously described. TCR expression was determined using Vα2TCR-biotin (BD Biosciences) in combination with Streptavidin-FITC (BD PharMingen). The percentage of TCR-CD3 down-modulation was determined using the following formula: Percentage of TCR down-modulation (at time t) = $100 \times$ [mean fluorescence intensity (MFI) of unstimulated T cells (at time t) − MFI of stimulated T cells (at time t)/MFI of unstimulated T cells (at $t = 0$)] as described (32).

To analyze $p56^{Lck}$ (Lck) expression, purified T cells of WT and $CD37^{-/-}$ mice were stained with KT31.1 conjugated to Cy5, permeabilized by 0.1% (w/v) saponin in PBS with 0.5% FCS, and incubated with anti-Lck 3A5 (Upstate Biotechnology, Lake Placid, NY), followed by anti-mouse IgG $F(ab')_2$ -FITC (Chemicon International, Temecula, CA). Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (BD Biosciences).

Lck in vitro kinase activity

Purified T cells of pooled spleens and lymph nodes of WT and $CD37^{-/-}$ mice were biotinylated at 0.5 mg/ml (sulfo-NHS-biotin; Pierce, Rockford, IL), and lysed in 1% Brij-58 lysis buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1 mM Na_3VO_4, 5 mM NaF, and protease inhibitors). Immunoprecipitations (2.5×10^7 T cells/i.p.) of Lck (3A5), CD4 (GK1.5), CD8 (YTS169.4), and control rat IgG (Caltag Laboratories, Burlingame, CA) were performed essentially as described (33). In kinase reactions, immunoprecipitates were reacted with 10 μM ATP (Sigma-Aldrich) in 0.1% Brij-58 (25 mM HEPES, pH 7.4, 1 mM Na_3VO_4, 5 mM NaF, 10 mM $MnCl_2$) for 20 min at 20°C. Immune complexes were separated by 10% SDS-PAGE and immunoblotted with Streptavidin-HRP (Amersham) to detect biotinylated proteins, with phosphotyrosine Ab PT66-HRP (Sigma-Aldrich) to detect phosphorylated Lck, or with 3A5 Ab followed by anti-mouse IgG-HRP (BD Biosciences) to visualize total Lck protein. Densitometry analysis was performed using Scion image software (Frederick, MD). Ratios of phosphorylated Lck to total Lck were normalized to the intensity of biotin bands for CD4 and CD8.

Human T cell isolation and proliferation

PBMC were isolated from heparinized venous blood of healthy volunteers by Ficoll-Histopaque (Amersham) density gradient centrifugation. Monocytes were removed by adherence to petri dishes for 2 h at 37°C. B cells were depleted by CL100 human T cell column (Cedarlane, Ontario, Canada) according to manufacturer's protocol. Purified human T (4×10^5 cells/well) were incubated in 24-well plates coated with anti-CD3 (1:4000) with or without anti-CD37 (coated at 2 μg/ml) and anti-CD28 (1 μg/ml in solution) in complete RPMI 1640. Controls included stimulation with anti-CD3 plus anti-MHC class I (coated at 2 μg/ml). Proliferation was assessed as previously described.

Phosphorylation assays

Purified human T cells (2×10^5/condition) were washed in PBS and stimulated with anti-CD37 (2.5 μg/ml), or anti-CD3 (1:4000) plus anti-CD28 (5 μg/ml) for 3 min at 20°C, followed by cross-linking with anti-mouse IgG $F(ab')_2$ (1:200; Chemicon) for 5 min at 37°C. Cells were immediately lysed in 1% Brij-58 lysis buffer for 30 min at 4°C. After removing insoluble material, samples were separated by 10% SDS-PAGE and immunoblotted with phosphotyrosine Ab PT66-HRP.

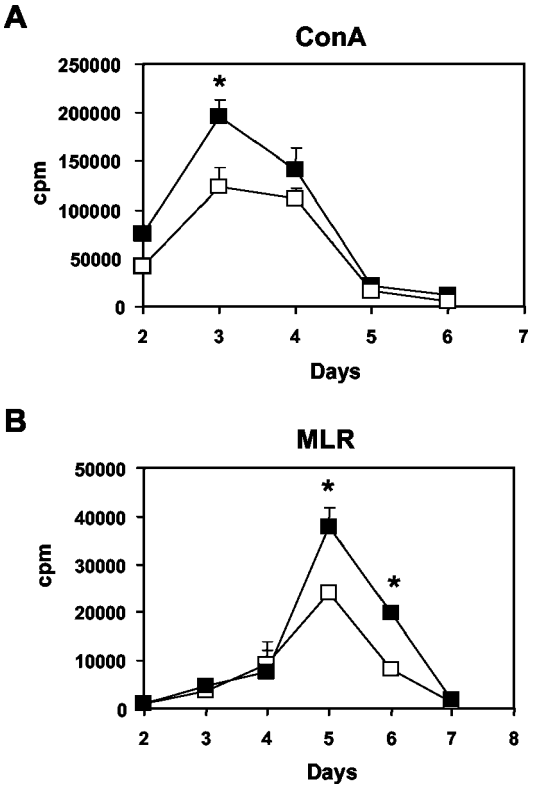

FIGURE 1. T cells of CD37$^{-/-}$ mice are hyperproliferative to Con A and in MLR. *A*, Splenocytes of WT (□) and CD37$^{-/-}$ (■) mice were stimulated with the T cell mitogen Con A. *B*, In MLR, BALB/c DC were incubated with purified WT (□) or CD37$^{-/-}$ (■) T cells. Proliferation was assessed by [^{3}H]thymidine incorporation. Unstimulated cells did not proliferate at any stage. Data represents mean ± SD (n = 4). Experiments were repeated three times yielding similar results. ∗, Significant difference in proliferation compared with WT, $p < 0.05$.

Statistical analyses

Data are expressed as mean of quadruplicate samples ± SEM unless otherwise stated. Statistical differences were determined using unpaired Student's t test. Significance was accepted at the $p < 0.05$ level.

Results

CD37$^{-/-}$ T cells are hyperproliferative

T cell-dependent Ab responses to model Ags are impaired in CD37$^{-/-}$ mice (28). Therefore, we evaluated T cell responses to various stimuli in the absence of CD37$^{-/-}$. CD37$^{-/-}$ splenocytes showed significantly higher proliferation upon Con A stimulation than WT cells (Fig. 1*A*). T cell proliferation in response to allostimulation by DC revealed similar hyperproliferation of CD37$^{-/-}$ T cells (Fig. 1*B*). Hyperproliferation of CD37$^{-/-}$ T cells in MLR was observed using a range of different DC to T cell ratios (data not shown). Next, we studied proliferation of purified CD4$^+$ and CD8$^+$ T cells after stimulating the CD3-TCR complex. CD37$^{-/-}$ T cells proliferated significantly more than WT T cells in response to anti-CD3, or anti-CD3 and anti-CD28 stimulation, with the effect particularly striking in the absence of costimulation (Fig. 2). Moreover, activation of CD37$^{-/-}$ T cells through an alternative costimulatory pathway, anti-CD3 with anti-CD9 stimulation, also led to higher levels of proliferation (data not shown).

Memory T cell compartment is normal in CD37$^{-/-}$ mice

Normal numbers of CD4$^+$ and CD8$^+$ T cells in spleen and lymph nodes of CD37$^{-/-}$ mice have been reported (28), so a changed CD4 to CD8 ratio cannot explain our findings. Because memory T cells are more easily activated than naive T cells, we studied numbers of memory and naive T cells in CD37$^{-/-}$ mice. No differences in percentages of CD62L$^-$CD44$^+$ (memory/activated) and CD62L$^+$CD44$^-$ (naive) were found between CD37$^{-/-}$ and WT T cell subsets (Fig. 3*A*). These results rule out the possibility that hyperproliferation is caused by an altered memory to naive T cell ratio in CD37$^{-/-}$ mice.

CD37$^{-/-}$ T cells undergo normal apoptosis

Another explanation for the hyperproliferative phenotype could be increased resistance to apoptosis. We studied spontaneous apoptosis of purified CD37$^{-/-}$ and WT T cell cultures using Annexin V staining. No differences in the percentage of apoptotic cells between CD37$^{-/-}$ and WT T cells were found at any time (Fig. 3*B*). Similar results were obtained with purified CD4$^+$ and CD8$^+$ T cells (data not shown). Moreover, irradiation-induced apoptosis of

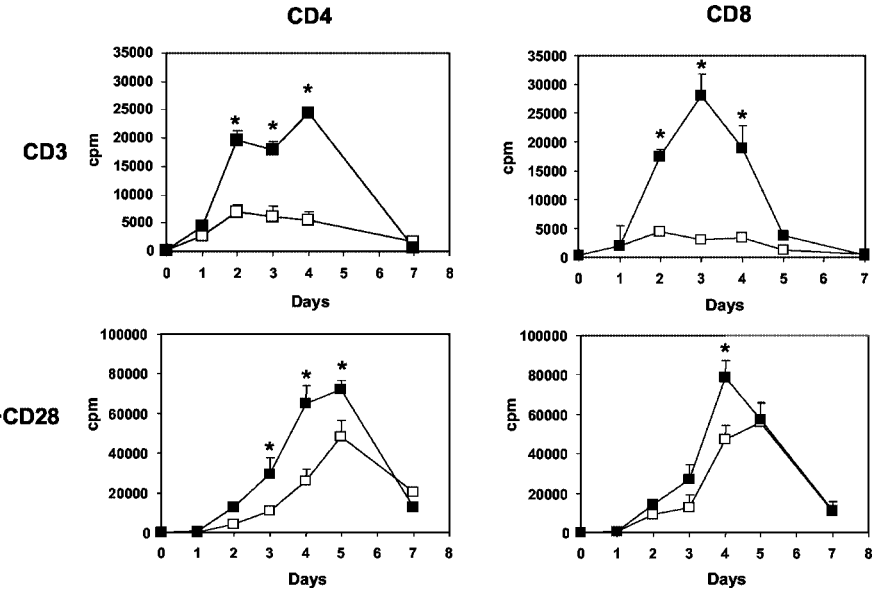

FIGURE 2. Enhanced proliferation of CD37$^{-/-}$ T cells upon CD3-TCR stimulation. Purified CD4$^+$ and CD8$^+$ T cells of WT (□) and CD37$^{-/-}$ (■) mice were stimulated with surface-adsorbed anti-CD3, or anti-CD3 + anti-CD28. Proliferation was assessed by [^{3}H]thymidine incorporation. Experiments were repeated five times yielding similar results. Unstimulated T cells did not proliferate at any stage. ∗, Significant difference in proliferation compared with WT, $p < 0.05$.

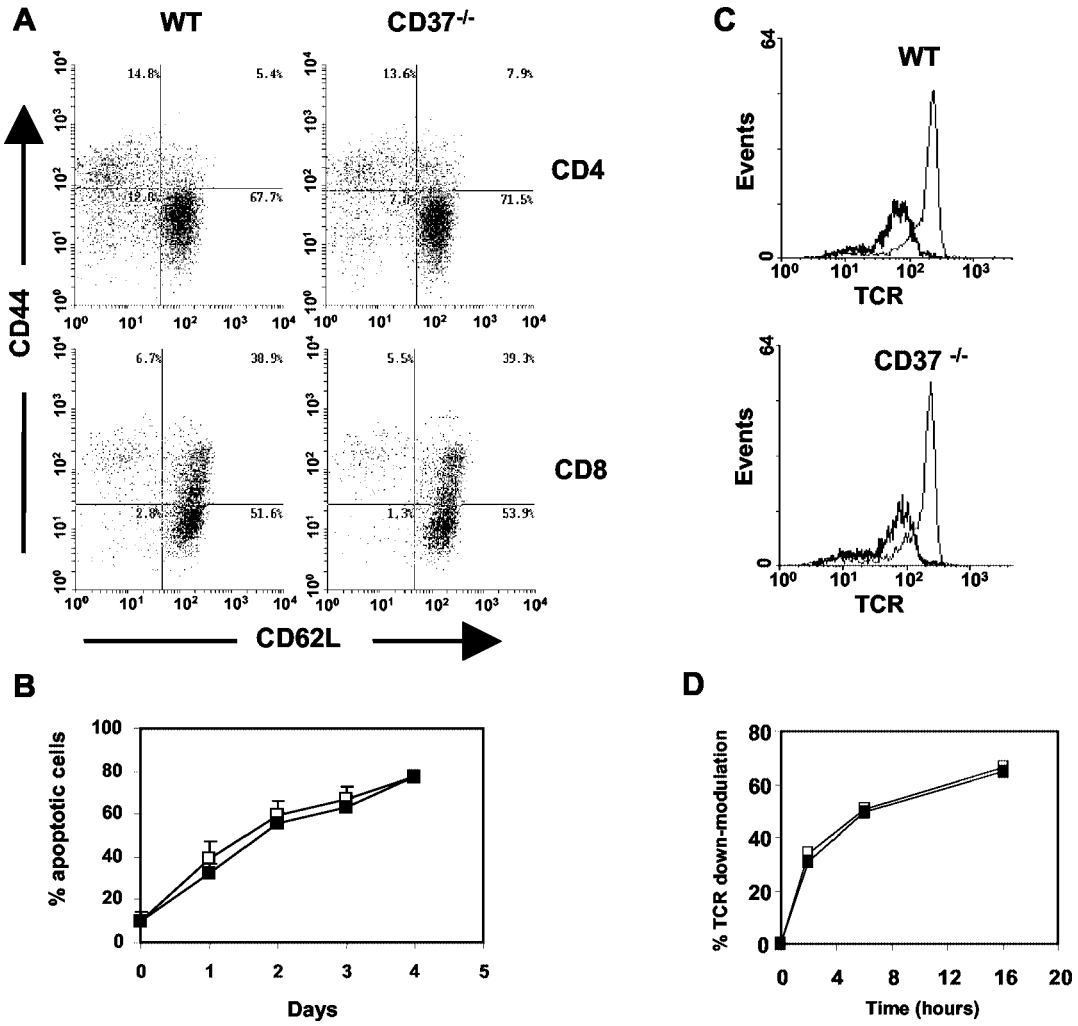

FIGURE 3. Hyperproliferation is not due to differences in memory to naive T cell ratios in CD37$^{-/-}$ mice, apoptosis, or TCR down-modulation. *A*, Purified T cells of WT (*left*) and CD37$^{-/-}$ (*right*) mice were stained for CD4, CD8, CD62L, and CD44 expression. Dot plots show CD62L and CD44 expression on gated CD4^{+} (*upper*) and CD8^{+} (*lower*) T cells. Quadrant percentages of T cell subpopulations are indicated. *B*, Purified T cells of WT (□) and CD37$^{-/-}$ (■) mice were cultured for different days and double-stained with Annexin V and propidium iodide to analyze the percentage of apoptotic cells. *C*, Purified T cells of WT (*upper*) and CD37$^{-/-}$ (*lower*) mice were stimulated with surface-coated anti-CD3 (bold line) for 6 h and analyzed for TCR surface expression. TCR expression level on unstimulated cells is shown as control (thin line). *D*, Graph demonstrates kinetics of TCR down-modulation on WT (□) and CD37$^{-/-}$ (■) T cells after stimulation with anti-CD3. Results are representative of three independent experiments.

CD37$^{-/-}$ and WT T cells was comparable (e.g., after 4 h; 22.4% vs 24.3%).

Normal TCR down-modulation in CD37$^{-/-}$ cells

The induction of T cell activation is directly correlated to the number of engaged TCR molecules on the cell surface and its subsequent down-modulation (34). T cell hyperproliferation has recently been linked to impaired CD3/TCR down-modulation (32). We studied TCR down-modulation on CD37$^{-/-}$ and WT T cells upon CD3-TCR engagement. Unstimulated CD37$^{-/-}$ and WT T cells had comparable surface TCR expression levels (Fig. 3*C*). Moreover, CD37$^{-/-}$ T were able to down-modulate their TCR as efficiently as WT T cells upon CD3-TCR stimulation. We observed the kinetics of TCR down-modulation in CD37$^{-/-}$ and WT T cells to be similar (Fig. 3*D*).

CD37$^{-/-}$ T cells enter first division earlier than WT T cells

We investigated the basis of the hyperproliferation by quantitative analysis of T cell division at the single cell level. CD37$^{-/-}$ and WT T cells were labeled with CSFE, stimulated with anti-CD3, and analyzed over time. CSFE labeling of WT and CD37$^{-/-}$ T cells 24 h after stimulation, when division has not started yet, was similar (Fig. 4*A*). However, 20% of CD37$^{-/-}$ T cells has already entered the third division compared with only 6% of WT T cells 66 h after stimulation (Fig. 4*A*). We observed that CD37$^{-/-}$ T cells had completed more rounds of cell division than WT T cells at all times. Earlier entrance into the first division or a faster division rate could be responsible for the hyperproliferation of CD37$^{-/-}$ T cells. To study these two possibilities, which are not mutually exclusive, we performed division cohort analysis (31). Plotting mean division number of WT and CD37$^{-/-}$ T cells vs time resulted in two linear relations with a similar slope (representing average division cycle time) and a different intercept at division 1 (Fig. 4*B*). This demonstrates that CD37$^{-/-}$ T cells take a shorter average time to go into first division than WT T cells upon CD3 cross-linking. In addition, these results reveal that CD37$^{-/-}$ and WT T cells have a comparable average division time through further divisions.

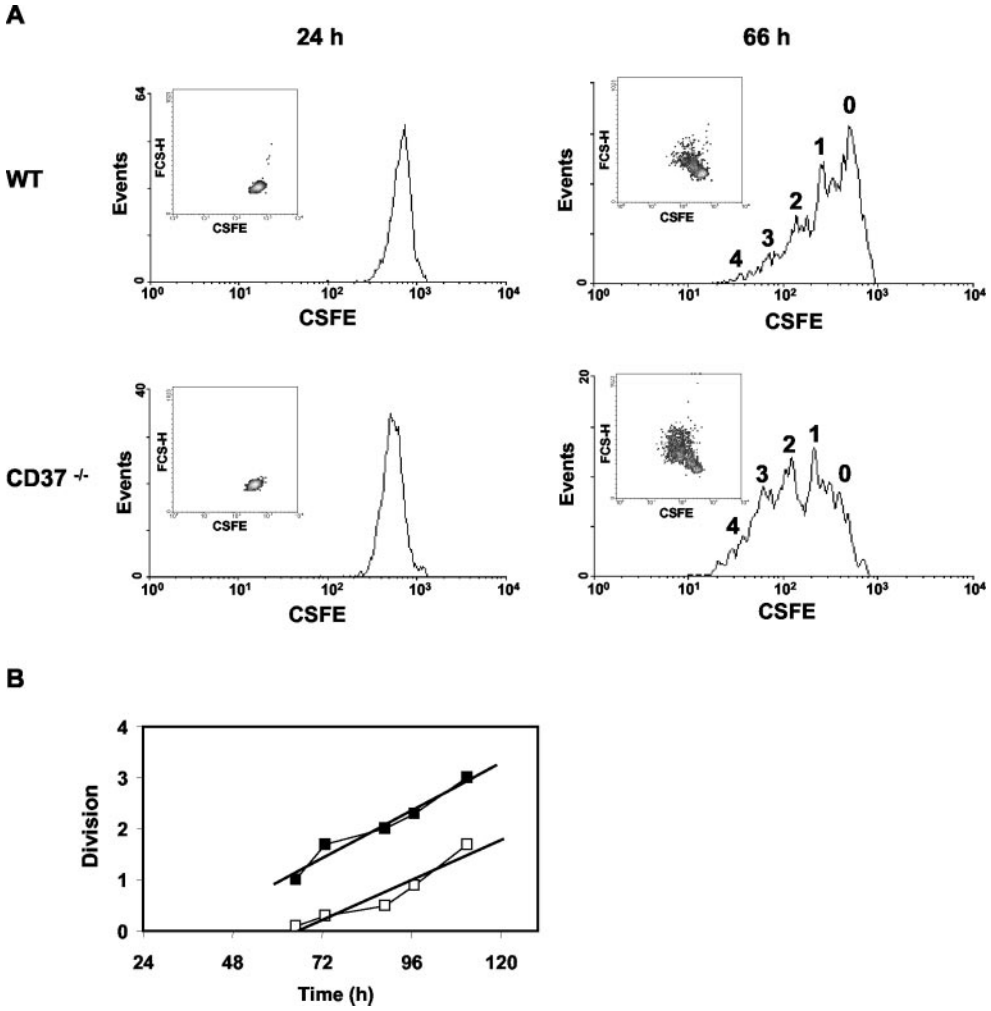

FIGURE 4. CD37$^{-/-}$ T cells reach higher number of division than WT T cells. Purified T cells of WT and CD37$^{-/-}$ mice were labeled with 10 μM CSFE and stimulated with anti-CD3 after which cells were harvested and analyzed for division number. *A*, CSFE profiles of WT (*top*) and CD37$^{-/-}$ (*bottom*) T cells 24 and 66 h after CD3-stimulation. *Inset* shows contour plots of CSFE labeling. Values indicate division numbers 0–4. *B*, Division cohort analysis was performed as described (31). Mean division number of WT (□) and CD37$^{-/-}$ (■) T cells is plotted against time. Experiments were repeated four times yielding similar results.

Altered cytokine production by CD37$^{-/-}$ T cells

Next, we investigated cytokine production by CD37$^{-/-}$ and WT T cells upon CD3-TCR stimulation. CD37$^{-/-}$ CD4^{+} T cells produced significantly higher levels of IL-2 than WT cells in response to CD3 stimulation (Fig. 5*A*). Importantly, this increased IL-2 production was already detectable within 24 h, when proliferation had not yet started (see Fig. 2*A*). Costimulation and IL-2 have been reported to reduce the time to first division (31). To elucidate the role of IL-2 in the hyperproliferation of CD37$^{-/-}$ cells, we added exogenous IL-2 to CD3-stimulated T cell cultures. This excess of IL-2 resulted in comparable proliferation of WT and CD37$^{-/-}$ T cells (Fig. 5*B*).

To investigate whether CD37$^{-/-}$ T cells exhibited a general dysregulation of cytokine production or a dysregulation of IL-2 production specifically, we analyzed IL-4 and IFN-γ production by CD37$^{-/-}$ T cells. Higher amounts of IL-4 were detected in cultures of CD37$^{-/-}$ CD4^{+} cells at days 4, 5, and 6 (Fig. 5*C*). In contrast, IFN-γ production was not significantly different (Fig. 5*D*). To exclude the possibility that increased IL-4 production was attributable to increased CD37$^{-/-}$ T cell numbers after day 1, we studied IL-4 production at the single cell level. ELISPOT assays revealed that the percentage of IL-4 producers among CD37$^{-/-}$ T cells was in fact higher than WT IL-4 producers upon stimulation (Fig. 5*C*, *inset*). Taken together, CD37$^{-/-}$ CD4^{+} T cells produce more IL-2 and IL-4, but not IFN-γ, than WT cells upon CD3-TCR stimulation. Hyperproliferation of CD37$^{-/-}$ T cells was preceded by enhanced IL-2 production, which alludes to CD37 involvement in signaling upstream of proliferation. This was further supported by the observation that CD37$^{-/-}$ T cells expressed higher levels of the activation markers CD25 and CD69 than WT T cells after CD3-TCR engagement before cells had gone into division (data not shown).

In vitro kinase activity of CD4/CD8-associated Lck

As our data infer a role for CD37 in CD3-TCR-induced proliferation, we hypothesized CD37 to be involved in TCR signal transduction. Lck is a tyrosine kinase crucial for early events in TCR signaling. Lck associates noncovalently with the cytoplasmic domains of CD4 and CD8. Total levels of Lck were found to be equal in WT and CD37$^{-/-}$ T cells as determined by intracellular stainings (Fig. 6*A*). We assessed the kinase activity of Lck immunoprecipitated with CD4 and CD8 from purified CD37$^{-/-}$ and WT T cells using in vitro kinase assays. T cells were biotinylated to

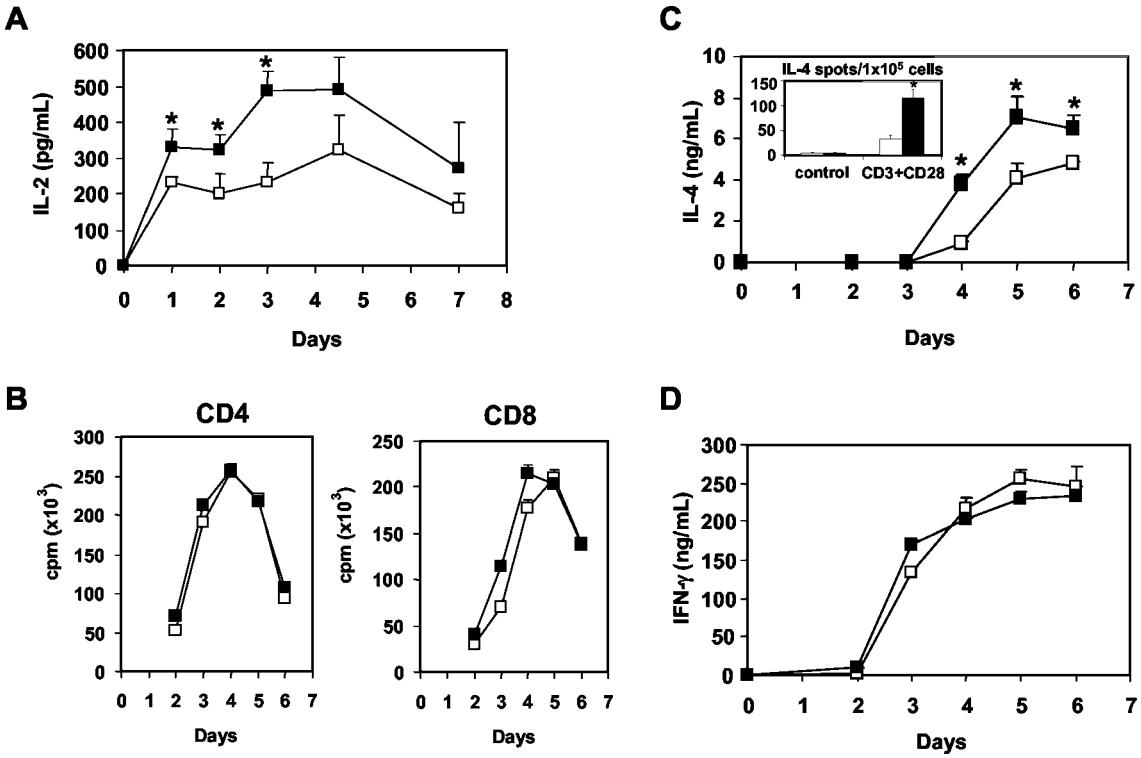

FIGURE 5. Increased production of IL-2 by CD37$^{-/-}$ T cells is involved in hyperproliferation. Purified CD4$^+$ T cells were stimulated with anti-CD3 + anti-CD28, and cytokine levels were measured in supernatants by ELISA. *A*, IL-2 production by WT (□) and CD37$^{-/-}$ (■) CD4$^+$ T cells. *B*, Purified CD4$^+$ and CD8$^+$ lymph node T cells of WT (□) and CD37$^{-/-}$ (■) mice were stimulated with anti-CD3 in the presence of exogenous IL-2, and proliferation was assessed by [^{3}H]thymidine incorporation. *C*, IL-4 production by WT (□) and CD37$^{-/-}$ (■) CD4$^+$ cells. Frequencies of IL-4-producing cells were determined after 3 days by ELISPOT assays (*inset*). *D*, IFN-γ production by WT (□) and CD37$^{-/-}$ (■) CD4$^+$ cells. Unstimulated cells did not produce any cytokines. Experiments were repeated at least three times yielding essentially similar results. ∗, Significant difference compared with WT, $p < 0.05$.

control for total amounts of CD4 and CD8 protein present in pull-downs. As shown in Fig. 6*B*, no major differences were detected in total levels of Lck immunoprecipitated with CD4 or CD8 between CD37$^{-/-}$ and WT T cells. However, CD4 and CD8 molecular complexes precipitated from CD37$^{-/-}$ T cells demonstrated increased Lck phosphorylation in kinase assays (Fig. 6, *B* and *C*).

CD37 signaling in human T cells

To further support the hypothesis that CD37 is involved in T cell signaling, we investigated the effects of CD37 engagement on human T cells. CD37 was readily detected on human peripheral T cells (Fig. 7*A*). Moreover, CD37 cross-linking induced tyrosine phosphorylation in human T cells (Fig. 7*B*). We then studied the effect of CD37 engagement on CD3-TCR-induced proliferation. As illustrated in Fig. 7*C*, T cells proliferated well to anti-CD3 and anti-CD3 plus anti-CD28 stimulation. Strikingly, anti-CD37 mAb completely inhibited CD3-induced proliferation (Fig. 7*C*, *top panel*). As a control, anti-MHC class I mAb was added to anti-CD3-coated plates, and had no effect on CD3-induced activation. In the presence of CD28 costimulation, anti-CD37 mAb partially, but significantly, inhibited T cell proliferation (Fig. 7*C*, *bottom panel*).

Discussion

The involvement of tetraspanin proteins in various cellular processes is well recognized, but incompletely understood. In particular, little is known about the role of leukocyte-specific tetraspanins in the immune system. CD37, previously thought to be predominantly a B cell molecule, is shown in this study to regulate T cell proliferation. The absence of CD37 results in enhanced IL-2 production and decreased time before cells enter the first division upon CD3-TCR stimulation. Increased kinase activity of CD4/CD8-associated Lck in CD37$^{-/-}$ T cells suggests that CD37 couples to TCR signal transduction pathways.

This study demonstrates hyperproliferation of CD37$^{-/-}$ T cells in response to different stimuli, including activation by DC. Consistent with our study, T cells lacking the tetraspanins CD81 and Tssc6 are also hyperproliferative to CD3-TCR stimulation (8, 35). Interestingly, CD37$^{-/-}$ and CD81$^{-/-}$ mice show impaired humoral responses (28, 9), in contrast to Tssc6$^{-/-}$ mice that had normal humoral immunity (35). This suggests that the T cell hyperproliferative phenotype and impaired Ab responses are probably not linked. The mechanism underlying tetraspanin involvement in T cell proliferation had been unknown. Normal numbers of CD4$^+$ and CD8$^+$ T cells in lymphoid organs of CD37$^{-/-}$ mice have been observed (28), which shows that CD37 is not essential for T cell development. Furthermore, we excluded the inherent defect of CD37$^{-/-}$ T cells causing hyperproliferation to be the result of an altered memory to naive T cell ratio or increased resistance to apoptosis. Hyperresponsiveness to anti-CD3 stimulation of T cells deficient in c-Cbl and Cbl-b was recently reported to be the result of impaired TCR down-modulation (32). However, we observed normal TCR down-modulation in CD37$^{-/-}$ T cells. Thus, extension of the functional lifespan of engaged TCR complexes is not causing the hyperproliferative phenotype.

We assessed the origin of hyperproliferation of CD37$^{-/-}$ T cells by quantitative division-tracking at the single cell level. CD3-activated CD37$^{-/-}$ T cells enter the first division earlier in time than WT T cells. The time taken to enter the first division is determined

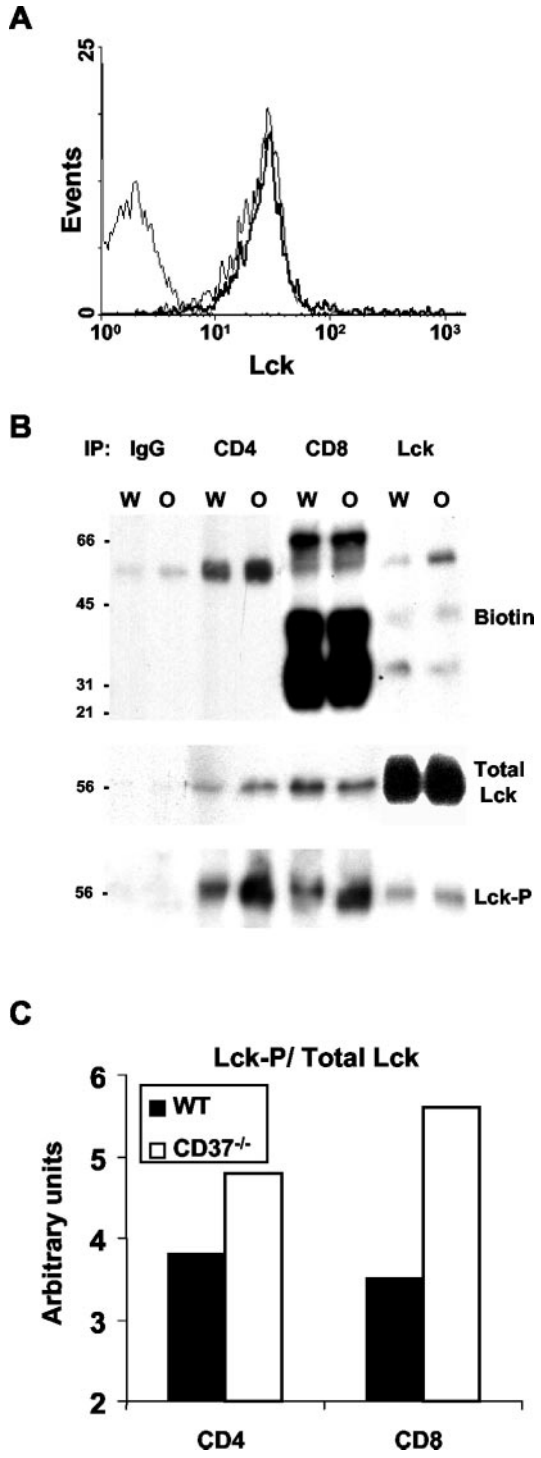

FIGURE 6. Increased $p56^{Lck}$ (Lck) in vitro kinase activity in $CD37^{-/-}$ T cells. *A*, Expression of Lck in permeabilized WT (thin histogram) and $CD37^{-/-}$ (thick histogram) T cells. *B*, Immunoprecipitations of CD4, CD8, Lck, and control IgG of WT (W) and $CD37^{-/-}$ (O) biotinylated T cell lysates were used in in vitro kinase assays. Reactions were immunoblotted with Streptavidin-HRP to visualize biotinylated proteins in pull-downs (*upper*), with Lck Ab to determine total Lck protein (*middle*), and with phosphotyrosine Ab to demonstrate phosphorylated Lck (*lower*). *C*, Ratios (arbitrary units) of phosphorylated Lck to total Lck of WT (■) and $CD37^{-/-}$ (□) present in CD4 and CD8 immunoprecipitations. Values have been normalized to the intensity of biotin bands for CD4 and CD8 using densitometry analysis. Experiments were repeated three times yielding essentially similar results.

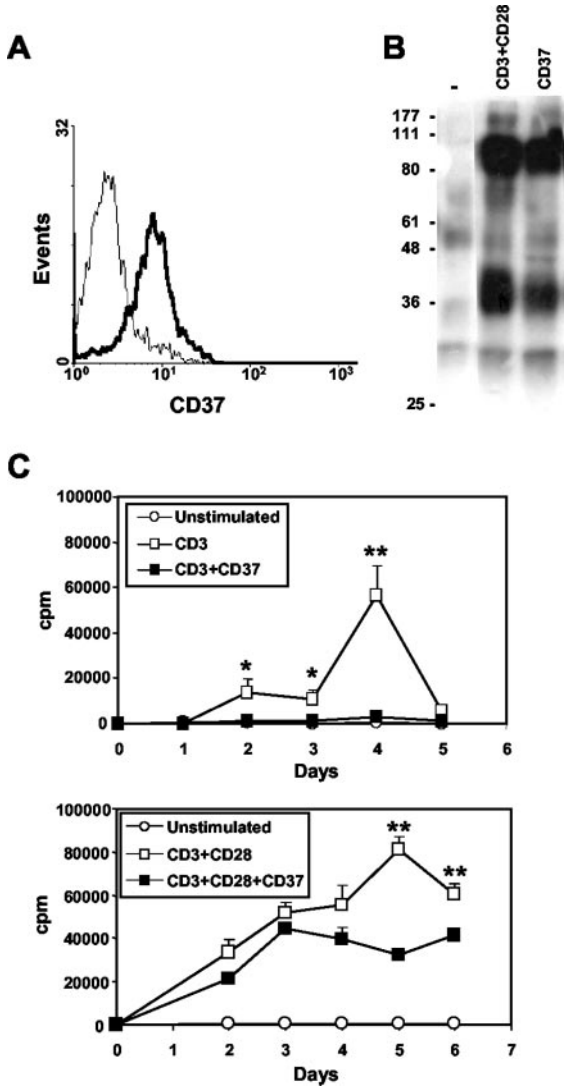

FIGURE 7. CD37 signals and regulates CD3-induced proliferation in human T cells. *A*, CD37 expression (thick histogram) on purified human T cells compared with control IgG (thin histogram). *B*, Tyrosine phosphorylation in human T cells after cross-linking CD37 or CD3 + CD28 for 5 min compared with cross-linker Ab alone (−). *C*, Purified human T cells were stimulated with anti-CD3 (*top*), or with anti-CD3 + anti-CD28 (*bottom*) with or without anti-CD37. Proliferation was assessed by [^{3}H]thymidine incorporation. Unstimulated T cells are shown as control (○). Experiments were repeated four times yielding similar results. Significant inhibition of proliferation by anti-CD37 is shown. ∗, $p < 0.05$, ∗∗, $p < 0.005$).

by the strength of TCR stimulation and by contribution from costimulation or cytokines (31). Our data are consistent with reports documenting heterogeneity in T cell proliferation in WT mice to be primarily due to differences in the time of entry to the first division cycle (31, 36). Division number, a critical element in T cell differentiation, is directly related to cytokine production. IL-4 production is observed only by T cells that have undergone at least four divisions (36). Thus, increased IL-4 production by $CD37^{-/-}$ T cells is a consequence, rather than a cause, of T cell proliferation. Although these results may infer skewing toward Th2 immune responses in $CD37^{-/-}$ mice, normal immunity to the classical Th2 pathogen *Nippostrongylus brasiliensis* has been reported in the absence of CD37 (28). Conversely, T cells from CD81-deficient mice were impaired in IL-4 production (10, 12). However, whether

it is the absence of CD81 from T cells (12) or B cells (10) that is critical for this impairment, appears to depend on the experimental system used.

Importantly, CD3-TCR stimulation led to early enhanced IL-2 production by $CD37^{-/-}$ T cells. IL-2 involvement in $CD37^{-/-}$ hyperproliferation was further supported by the observation that addition of exogenous IL-2 resulted in comparable proliferation of WT and $CD37^{-/-}$ T cells, which shows that the hyperproliferative phenotype does not result from increased sensitivity to IL-2.

We addressed the molecular mechanism underlying CD37 function by studying CD37 involvement in coupling to TCR signal transduction pathways. Firstly, we demonstrated that CD37 engagement on human T cells transduced signals that resulted in complete inhibition of CD3-induced proliferation. CD37 was also able to significantly reduce proliferation in the presence of CD28 costimulation. This ability of CD37 to transduce inhibitory signals correlates with the hyperproliferative phenotype of $CD37^{-/-}$ T cells, and also with the similar T cell phenotype observed in CD81- and Tssc6-deficient mice (8, 35). It is difficult to reconcile these results with several studies showing mAb against tetraspanins CD9, CD53, CD81, and CD82 to deliver costimulatory signals to T cells (16–19 and see Ref. 7 for detailed discussion on this point). However, it should be remembered that mAb against the same molecule can act either as agonists or antagonists, and thus induce different functional outcomes.

Secondly, we observed an increased kinase activity of CD4/CD8-associated Lck precipitated from $CD37^{-/-}$ T cells. The tyrosine kinase Lck plays a critical role in phosphorylation of key T cell signaling molecules, including the TCR ζ-chain and ZAP-70. How can we explain this finding? The first possibility is that in WT T cells CD37 regulates Lck activity by sequestering molecule(s) involved in Lck function away from their site of action. One such molecule might be CD4 itself, as it has been reported that the tetraspanins CD81 and CD82 interact with CD4 in the absence or uncoupling of Lck (37). However, the ratio of CD4 to Lck molecules pulled down in our experiments appears to be comparable between WT and $CD37^{-/-}$ T cells. A second possibility is that CD37 may influence the dynamics of CD4-Lck distribution to microdomains important in TCR signaling. Lck activation is regulated by CD4, which facilitates the rapid recruitment of Lck in the immunological synapse (38). Tetraspanin involvement in the formation of the immunological synapse has been inferred by studies showing accumulation of CD82 (39) and CD81 (40) at the site of TCR engagement, and CD9 localization to lipid microdomains together with TCR signaling molecules (41). A third explanation for the current findings comes from the observation that some tetraspanins associate with an unidentified tyrosine phosphatase, which dephosphorylates Lck (42). Thus, CD37 deficiency may be accompanied by lack of this phosphatase in CD4 and CD8 complexes.

Taken together, the absence of CD37 results in increased kinase activity of CD4/CD8-associated Lck, dysregulation of TCR signaling, enhanced IL-2 production and proliferation. Given the apparent similarity of tetraspanin function in T cell signaling, we postulate a regulatory role for tetraspanin microdomains in T cell proliferation and immune regulation.

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