DEFECTIVE VAV EXPRESSION AND IMPAIRED F-ACTIN REORGANIZATION IN A SUBSET OF COMMON VARIABLE IMMUNODEFICIENCY PATIENTS WITH T-CELL DEFECTS

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ABSTRACT

Common variable immunodeficiency (CVID) is a primary immune disorder characterized by impaired antibody production, which is in many instances secondary to defective T-cell function (T-CVID). We have previously identified a subset of T-CVID patients characterized by defective TCR-dependent protein tyrosine phosphorylation. In these patients ZAP-70 fails to be recruited to the TCR as the result of impaired CD3ζ phosphorylation, which is however not dependent on defective Lck expression or activity. Here we show that neither Fyn or CD45 are affected in these patients. On the other hand, T-CVID T-cells show dramatic defects in the Vav/Rac pathway controlling F-actin dynamics. A significant deficiency in Vav protein was indeed observed, which in three out of four T-CVID patients was associated with reduced Vav1 mRNA levels. The impairment in Vav expression correlated with defective F-actin reorganization in response to TCR/CD28 coengagement. Furthermore, TCR/CD28 dependent upregulation of lipid rafts at the cell surface, which requires F-actin dynamics, was impaired in these patients. The actin cytoskeleton defect could be reversed by reconstitution of Vav1 expression in the patients' T-cells. The results provide the demonstration of an essential role of Vav in human T-cells and strongly suggest Vav insufficiency in T-CVID.
INTRODUCTION

Common variable immunodeficiency (CVID) is a primary dysfunction of the immune system, encompassing a heterogenous group of disorders whose unifying feature is late-onset hypogammaglobulinemia. As a consequence of the impaired antibody production, CVID patients suffer from increased susceptibility to bacterial infections and show a trend to develop autoimmune disorders and cancer\(^1\). Establishing the molecular basis of CVID has to date been hampered by the heterogeneity in phenotypical features of the disease. Major progress towards elucidating CVID has been achieved with the identification of defects in lymphocyte populations other than B-cells. Although B-cell dysfunctions account for a significant proportion of CVID, B-cells from many patients are functional \textit{in vitro}, but are not activated \textit{in vivo} into mature antibody-secreting plasma cells due to defective T-cell help. Defects both in cytotoxic T-cells and in antigen presenting cells have also been identified in a subset of CVID patients, suggesting that all cells implicated in the orchestration of the immune response are potential targets of the genetic lesion(s) in this disease\(^2,3\).

In CVID patients with defective T-cell function (T-CVID), decreased proliferative responses to mitogens, impaired production of cytokines such as IL-2, IL-4, IL-5, IL-10 and IFN\(\gamma\)\(^{4-11}\) and defective expression of activation markers, including CD40 ligand\(^12\), attractin\(^13\) and L-selectin\(^14\), have been observed. Furthermore, homozygous loss of ICOS, an inducible costimulator expressed on activated T-cells, has been recently documented in a subset of T-CVID\(^15\). While distinct genetic lesions affecting different stages of T-cell activation and differentiation may underlie the impairment in helper T-cell function, defects in the molecular machinery which controls initiation of TCR signaling could account for many of
the abnormalities described in T-CVID. Impairment of early signaling events, such as calcium mobilization and phospholipid breakdown, has indeed been reported in a subset of T-CVID patients\(^7,16\). Furthermore, a decrease in the levels of the tyrosine kinase Lck, resulting from aberrant splicing of the gene transcript, has been described in one T-CVID patient\(^17\).

We have previously shown a correlation between impaired proliferative responses to TCR engagement and defective triggering of the early tyrosine phosphorylation cascade in T-cells from T-CVID patients\(^18\), suggesting a defective coupling of the TCR to downstream tyrosine kinases. ZAP-70 activation, an event crucial to productive TCR signaling\(^19\), is indeed impaired in these patients as the result of defective ZAP-70 recruitment to the activated TCR, which appears, in turn, secondary to a defect in CD3\(\zeta\) phosphorylation\(^20\).

Here we present a structural and functional analysis of the molecular components implicated in initiation of TCR signaling in four T-CVID patients characterized by defective ZAP-70/CD3\(\zeta\) interaction. The data indicate that impaired reorganization of the actin cytoskeleton, resulting from a reduction in Vav expression, underlies the TCR signaling defect in these patients.
METHODS

Patients and cells

Patients were classified as CVID according to the WHO classification of primary immunodeficiencies\textsuperscript{21}. The hematological and immunophenotypical characterization of patients T-CVID1, T-CVID2, dis.ctr1 and dis.ctr2 has been previously presented\textsuperscript{18,20} (see footnote). Patients T-CVID3 and T-CVID4 were newly recruited. They were both classified as T-CVID based on their impaired proliferative responses to TCR/CD3 ligation (control, 100\%±2.9; T-CVID3, 50.2\%±2.7 T-CVID4, 41.4\%±1.9\%). Similarly to patients T-CVID1 and T-CVID2\textsuperscript{20}, immunoblot analysis of ZAP-70-specific immunoprecipitates of PBL with anti-phosphotyrosine antibodies revealed a defective interaction of ZAP-70 with phospho-ζ and an impairment in ZAP-70 activation in response to TCR engagement in these patients, which correlated with defective CD3ζ phosphorylation (supplemental figure 1), while CD3ζ, Lck or ZAP-70 expression were unimpaired (supplemental figure 2, Table I). Controls were sex- and age-matched healthy donors. Peripheral blood was obtained after informed consent, and sample size was kept small according to the guidelines of the ethical committee. No overt infectious disease was present at the time of blood sampling. To limit sampling from the patients, PHA T-cell lines were derived following standard procedures and used as a source of RNA for cDNA sequencing. PBMC were isolated from whole blood by density centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech Italia, Milan) and subsequently depleted of monocytes by adherence.

Cell lines included Jurkat T-lymphoma cells, the Lck-defective Jurkat variant JCaM1\textsuperscript{22} and the CD45-defective variant J45.01\textsuperscript{23}. Mammalian expression vectors encoding either F505Lck\textsuperscript{24} or F528Fyn\textsuperscript{25} and carrying the genes encoding neomycin and hygromycin
resistance, respectively, were introduced into JCaM1 cells by electroporation. Stably transfected cells were selected in medium containing 1 mg/ml G418 (Gibco BRL, Life Technologies Italia srl, Milan) or 50 μg/ml hygromycin (Sigma Italia srl, Milan). Stable transfectants were checked for CD3 expression by flow cytometry. Peripheral blood T-cells were transiently transfected using the Amaxa nucleofector device (Amaxa Biosystems, Cologne, Germany) and the conditions for T-cell transfection recommended by the manufacturer. Endotoxin-free plasmid DNA was purified using a kit from Qiagen GmbH (Hilden, Germany). Flow cytometric analysis of PBL transfected with the Amaxa control GFP reporter and subsequently labeled with fluorochrome-conjugated anti-CD3 mAb confirmed that only CD3+ cells had been transfected and showed that the transfection efficiency was consistently ≥50%. PBL from the four T-CVID patients and from four matched healthy donors were cotransfected with 1 μg GFP reporter/sample and 3 μg of either empty vector (pEF-BOS) or the same vector encoding myc-tagged Vav1. 24 hr after transfection cells were processed for confocal microscopy as described below. Lysates of transfected cells were tested for recombinant Vav expression by immunoblotting with anti-myc mAb.

**Antibodies and reagents**

Phosphospecific polyclonal antibodies against the active form of p38, JNK and ZAP-70 (Y493) were purchased from Cell Signaling Technologies (Beverly, MA). Anti-phosphotyrosine, anti-ZAP-70, anti-Vav, anti-Lck, anti-Shc, anti-LAT and anti-Rac polyclonal and monoclonal antibodies were purchased from Upstate Biotechnology Inc.; polyclonal anti-ZAP-70, anti-p38 and monoclonal anti-CD3ζ antibodies, as well as an antibody specific for phosphorylated CD3ζ, from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Fyn, anti-Grb2 and anti-myc mAb from BD Biosciences (Heidelberg, Germany); anti-tubulin and anti-actin mAb from Amersham Pharmacia Italia. A mouse mAb suitable
for immunoprecipitation of tyrosine phosphorylated CD3ζ was kindly provided by M. Banyiash. Anti-CD3 IgG from OKT3 hybridoma supernatants were affinity-purified on Mabtrap (Amersham Pharmacia Italia srl) and titrated by flow cytometry. Anti-human CD28 mAb was purchased from BD Biosciences. Fluorochrome-labeled anti-human CD3, CD22, CD4, CD8, CD45RA and CD45RO were purchased from BD Biosciences, fluorochrome-labeled anti-mouse Ig from DAKO SpA (Milan, Italy), fluorochrome-labeled phalloidin from Sigma Italia. Secondary unlabeled antibodies were purchased from Cappel (Durham, NC), secondary peroxidase-labeled antibodies from Amersham Pharmacia Biotech. Cholera toxin B was purified from Vibrio sp. 60 harboring pATA13 as reported. The anti-CtxB mouse mAb was previously described.

Activations, immunoprecipitations, immunoblots and in vitro kinase assays

Activations by cross-linking of anti-CD3 mAb in solution were carried out as described. Cells (1-5x10⁶/sample for immunoblot analysis, 1-2x10⁷/sample for immunoprecipitations) were lysed in 1% (v/v) Triton X-100 in 20 mM Tris-HCl pH8, 150 mM NaCl (in the presence of 0.2 mg/ml Na orthovanadate, 1 µg/ml pepstatin, leupeptin and aprotinin and 10 mM PMSF) and postnuclear supernatants were probed as such or immunoprecipitated using the appropriate polyclonal or monoclonal antibodies and either protein A-Sepharose or agarose-conjugated anti-mouse Ig (Sigma-Aldrich srl, Milan, Italy). Immunoblots were carried out using a chemiluminescence detection system (Pierce, Rockford, IL). In vitro autophosphorylation assays of Lck-specific immunoprecipitates were carried out in 20 µl of 20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 10 mM MnCl₂, 10µCi of γ-[³²P]ATP, at room temperature for 10 min. The reaction products were subjected to SDS-PAGE, transferred to nitrocellulose, and exposed to a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The filters were subsequently probed with anti-Lck mAb as immunoprecipitation control.
CD3/CD28 costimulation for confocal microscopy and flow cytometry was carried out by cell incubation on ice for 30 min in the presence of saturating amounts of OKT3 and 5 μg/ml anti-CD28 mAb, followed by transfer to plate-bound secondary antibodies.

**Flow cytometry and confocal microscopy**

Immunophenotyping was carried out by two- or three-color flow cytometry using fluorochrome-conjugated mAb from BD Biosciences. To analyze GM1 expression, cells were incubated on ice for 1 hr with 15 μg/ml CtxB, then washed, added with a saturating amount of anti-CtxB mAb and incubated on ice for further 30 min. Bound mAb was revealed using fluorochrome-conjugated anti-mouse Ig. Samples were processed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Calcium flux was measured using Fluo-4 FF (Molecular Probes Europe BV, Leiden, the Netherlands). Cells (10⁶/sample) were suspended in 200 μl RPMI w/o phenol Red (Invitrogen srl) added with 25 mM Hepes pH 7.4, 5 μM Fluo-4 and 500 μM sulfinpyrazone (Sigma-Aldrich srl), incubated 45 min at 37°C, pelleted by centrifugation and resuspended in 500 μl of the same medium without Fluo-4. Baseline fluorescence was recorded for 40 sec at 37°C. Cells were subsequently added with saturating amounts of anti-CD3 mAb and 50 μg/ml secondary antibody. Fluorescence was continuously recorded for 512 sec at 37°C.

To analyze cortical actin reorganization, cells were fixed and permeabilized in Orthopermeafix™ (Orthodiagnostic Systems Inc, Raritan, NJ) for 45 min at RT. After washing, cells were incubated in the dark for 40 min with 0.77μM FITC-labeled phalloidin., GM1 surface expression was analyzed using CtxB/anti-CtxB as described above. Confocal microscopy was carried out on a Leica Microsystems confocal microscope (Heidelberg, Germany).
RT-PCR, cDNA sequencing, genomic DNA PCR

Total RNA was extracted from either T-cell lines or PBL using the RNA WIZ™ isolation reagent (Ambion Inc., Austin, TX). RT-PCR was carried out using AMV reverse transcriptase and the Expand High Fidelity PCR system from Roche Diagnostics SpA (Milan, Italy). The primer for the first strand was oligo-dT (Promega Italia srl, Milan, Italy), while pairs of specific primers were used for cDNA amplification. cDNA was amplified in an Eppendorf Mastercycler Thermal Cycler (Eppendorf srl, Milan, Italy). PCR products were separated by agarose gel electrophoresis and recovered using the Nucleo Spin extraction kit from Macherey-Nagel Srl (Hoerdt, France). Automatic sequencing was performed on both DNA strands on RT-PCR products from the four T-CVID patients, one or both disease controls and at least one healthy control. The nucleotide sequence of Vav was identical to the published sequence. A conserved substitution (C to T transition at position 3 of the codon for alanine 1137) compared to the published sequence was found in CD45 cDNA from the four T-CVID patients, one disease control and two healthy controls.

Semiquantitative RT-PCR analysis of Vav1, Vav2 and Vav3 expression was carried out using RNA from purified PBL from the four T-CVID patients, one disease control (dis.ctr.1) and a pool of RNA from three healthy controls. GAPDH was used as housekeeping control. The RT-PCR products were separated by agarose gel electrophoresis, and the identity of the band amplified with the Vav-specific primers was checked by sequencing. The intensities of the Vav- and GAPDH-specific bands was quantitated by laser densitometry (Kodak Digital Science™ Electrophoresis Documentation and Analysis System 120), and the relative levels of Vav mRNA were normalized to the levels of GAPDH mRNA.

Genomic DNA was extracted from purified PBL from the four T-CVID patients, one disease control (dis.ctr1) and a pool of three healthy controls. Cells were washed twice in PBS, resuspended in 500 µl lysis buffer (see above) containing 3% Triton X-100 and incubated on
ice for 5 min. Nuclei were recovered by centrifugation and lysed in 0.5% SDS, 20 μg/ml RNase in TE. After 1 hr incubation at 37°C, the nuclear extract was added with 100 μg/ml proteinase K and incubated for further 3 hr at 50°C. DNA was purified by standard phenol-chloroform extraction and ethanol precipitation. The region of genomic DNA spanning positions -312 to +137 of human Vav1, as well as exons 1, 3-4, 5-6, 7 and 27 of Vav1, were amplified by PCR. In the latter case, the primers were designed to pair with noncoding sequences flanking the exons. The identity of the resulting fragments was confirmed by automatic sequencing.
RESULTS

Normal expression and cDNA sequence of Fyn and CD45 in T-CVID T-cells

The failure of ZAP-70 to be recruited to the activated TCR in the four T-CVID patients who participated in this study appears secondary to a defect in CD3ζ phosphorylation, however the cDNA sequence, protein expression, activity and subcellular localization of Lck is unaffected in these patients (supplemental figures 1 and 3, and ref.20). We have assessed the integrity of two other components implicated in the regulation of CD3ζ phosphorylation, the tyrosine kinase Fyn and the tyrosine phosphatase CD45.

Although both Lck and Fyn can phosphorylate CD3ζ in heterologous systems, genetic evidence indicates that this function is principally subserved by Lck\textsuperscript{22,30-32}. Nevertheless, to rule out a contribution of Fyn to this process, the Lck-defective Jurkat line JCaM1, where CD3ζ fails to become phosphorylated following TCR engagement, was stably transfected with mammalian expression constructs encoding constitutively active mutants of either Lck (F505Lck) or Fyn (F528Fyn). As shown in Fig.1A (top), the resulting transfectants expressed significant levels of Lck and showed a ~2-fold increase in levels of Fyn, respectively. As expected, expression of F505Lck resulted in full recovery of TCR-dependent CD3ζ phosphorylation, as well as coprecipitation of tyrosine phosphorylated ZAP-70. Conversely, no significant effect on CD3ζ phosphorylation was observed in JCaM1 cells expressing F528Fyn (Fig.1A, bottom), notwithstanding increased protein tyrosine phosphorylation in total cell lysates (data not shown), indicating that Fyn cannot replace Lck in CD3ζ phosphorylation and supporting the notion that CD3ζ is not a major substrate of Fyn \textit{in vivo}. A defect in Fyn is therefore unlikely to underlie the phosphorylation defect in our T-CVID
patients. This is further supported by the finding that Fyn expression, as evaluated by immunoblot analysis of PBL lysates from the four T-CVID patients and two disease controls, was undistinguishable from that observed in T-cells from healthy donors (Fig.1B). The possibility of point mutations was ruled out by sequence analysis of the entire cDNA encoding Fyn.

Src kinases are the earliest components of the TCR signaling machinery, and their activity is positively controlled by the tyrosine phosphatase CD45, which is responsible for dephosphorylation of their C-terminal regulatory tyrosine residue\(^\text{33}\). Accordingly, TCR engagement fails to trigger CD3\(\zeta\) phosphorylation in CD45 deficient T-cells (refs. 23,34, and data not shown). The possibility that alterations in CD45 expression underlie the T-cell defect in our T-CVID patients was ruled out by immunophenotypic analysis of CD45RA/CD45RO expression on T-cells from these patients (Table II). Furthermore, no mutation was found in the nucleotide sequence of the cDNA encoding the transmembrane and intracellular domains of CD45. Collectively, although it has not been possible to measure the activities of CD45 and Fyn due to the limited size of the biological samples available, these data, together with our finding that Lck is unaffected in the same patients (supplemental figure 3 for patients T-CVID3 and T-CVID4 and ref. 20 for the other patients), suggest that the kinases/phosphatases directly responsible for CD3\(\zeta\) phosphorylation are normal in T-CVID T-cells.
Defective Vav expression in T-CVID T-cells

A number of essential components of the intracellular machinery implicated in TCR signal transduction are constitutively segregated to plasma membrane microdomains, known as lipid rafts, including Src kinases, small GTPases such as Ras, and transmembrane adaptors such as LAT\(^\text{35}\). On the other hand, the TCR/CD3 complex itself is only weakly associated with rafts\(^\text{36}\). TCR triggering results in stabilization of the TCR in lipid rafts, possibly as the result of raft clustering. In this location, the CD3/ζ ITAMs are phosphorylated by the resident Src kinases and become competent to initiate the full signaling cascade\(^\text{35,37}\). We and others have shown that reorganization of the actin cytoskeleton is essential for raft coalescence\(^\text{38,39}\). A defect in this step might underlie the failure of T-CVID T-cells to initiate TCR signaling.

F-actin reorganization is regulated in hemopoietic cells primarily by Vav, which promotes guanine nucleotide exchange on Rho family GTPases\(^\text{40}\). The expression of Vav and Rac was evaluated by immunoblot analysis of PBL lysates from T-CVID patients and disease controls (CVID patients without T-cell defects). While no differences in Rac expression were detectable (Fig.2A), a dramatic impairment in Vav expression, exclusive to T-CVID, was consistently observed (Fig.2B). The expression defect was specific to Vav, as T-cells from these patients were comparable to control T-cells in the expression of a panel of proteins implicated in TCR signaling (supplemental figure 2). Semiquantitative RT-PCR showed that in three out of the four T-CVID patients the reduction in Vav protein levels correlated with reduced mRNA levels (Fig.2C). Sequence analysis of full-length Vav1 cDNA from the same patients did not reveal any mutation. The possibility of a compensatory mechanism
involving upregulation of the genes encoding Vav2 or Vav3 in the T-CVID patients was ruled out by semiquantitative RT-PCR (Fig.2D).

Interestingly, two Vav immunoreactive bands, one of which characterized by a faster electrophoretic mobility, was detected in patient T-CVID1, suggesting the possibility of a deletion on one allele. Since no internal deletions were found in the full-length cDNA amplified by RT-PCR, the faster migrating species may be encoded by an allele deleted either at the 5’ or the 3’ end of the coding sequence and therefore unable to pair with one of the primers. This possibility was tested by PCR amplification of the portion of genomic DNA corresponding to the first and last exons of the Vav1 gene, respectively. Controls included DNA from a patient with a single Vav immunoreactive band (T-CVID3) and DNA from a healthy donor. A portion of an unrelated gene (RNA polymerase II), was used as independent control. The amount of template and PCR conditions were calibrated to obtain an amount of PCR product proportional to the amount of template. As shown in supplemental figure 4, while the abundance of PCR product obtained from all DNAs tested was comparable for the last Vav1 exon, the abundance of PCR product obtained from the DNA from patient T-CVID1 was consistently reduced by ~50% compared to the control DNAs for the first Vav1 exon, supporting the presence of a deletion at the 5’ end of one allele. PCR amplification of exons 3-4, 5-6 and 7 showed a reduction in PCR product for all amplified regions with the exception of exon 7, placing the 3’ boundary of the deletion, which appears to extend for at least 50 kb into the Vav1 locus, between exons 6 and 7. The resulting Vav truncation would lack the N-terminal calponin-homology and acidic domains, that are required for activation of IL-2 expression in T-cells. The fact that the size of the second Vav immunoreactive band appears larger than predicted suggests that an in-frame
fusion of the Vav coding sequence with a sequence upstream of the deletion might have occurred.

To understand whether a mutation in the *Vav1* gene promoter could underlie the decrease in the levels of Vav mRNA observed in patients T-CVID1, 2 and 4, a 450 bp fragment spanning positions -312 to +137, containing the minimal promoter region of *Vav* required for tissue-specific expression, as well as the 5' untranslated portion of exon 1, was amplified from genomic DNA from all patients, as well as from a healthy control. Sequence analysis of this fragment in all the patients did not reveal any difference compared to the published sequence, ruling out a defect in the *Vav1* gene promoter as causal to the decrease in *Vav1* mRNA in these patients.

**Defective F-actin reorganization in T-CVID T-cells**

To understand whether the defect in Vav expression could affect Rac activity, and hence actin cytoskeleton rearrangements triggered by TCR ligation, F-actin capping induced by CD3/CD28 coligation was analyzed by confocal microscopy of PBL labeled with fluorochrome-conjugated phalloidin. Costimulation was essential in our hands to achieve consistent levels of actin capping in these cells. Representative micrographs are shown in figure 3A (top). F-actin caps following CD3/CD28 coligation were quantitated in the four T-CVID patients and one disease control (patient dis.ctr1). A significant reduction in F-actin reorganization was reproducibly detected specifically in T-CVID T-cells (Fig.3A).
Activation of Rac, which in T-cells is essential for F-actin reorganization, also results in the activation of the serine-threonine kinase Pak1, which in turn initiates a serine-threonine kinase cascade leading to the activation of the stress-kinases p38 and JNK\(^{43-45}\). In agreement with the impairment in F-actin reorganization, which is presumably correlated to a defective Rac activation, CD3-dependent p38 activation was significantly reduced in T-CVID T-cells (Fig.3B). Furthermore, although the basal phosphorylation levels were relatively high, TCR-dependent increase in JNK phosphorylation was also impaired in patients T-CVID1, T-CVID2, T-CVID4 and, to a lesser extent, in T-CVID3 (Fig.3B). Hence the defect in Vav expression results in defective activation of Rac and downstream events.

Partial impairment in \([\text{Ca}^{2+}]\) mobilization in T-CVID T-cells

T-cells from Vav\(^{-/-}\) mice harbour defects in \([\text{Ca}^{2+}]\) mobilization\(^{46-48}\). To address whether this defect is also observable in T-cells with reduced Vav expression, TCR-dependent \([\text{Ca}^{2+}]\) mobilization was measured in T-CVID T-cells by flow cytometric analysis of PBL loaded with the fluorescent calcium chelator, Fluo-4 FF. As shown in figure 4, a partial but consistent attenuation in \([\text{Ca}^{2+}]\) flux triggered by CD3 engagement was observed in all T-CVID patients.
Defective upregulation of GM1 expression in T-CVID T-cells

CD3/CD28 costimulation has been shown to increase the levels of surface lipid rafts as the result of both de novo synthesis and transport from intracellular stores\textsuperscript{49}. Defective upregulation of surface rafts has been reported in T-cells from WASP patients\textsuperscript{50}, where the genetic defect correlates with impairment of actin reorganization\textsuperscript{51}, suggesting a role for F-actin in lipid raft mobilization. We analyzed the levels of lipid rafts on the T-cell surface in the four T-CVID patients and one disease control using ganglioside GM1 as lipid raft marker. GM1 was measured by indirect immunofluorescence using cholera toxin B (CtxB) and anti-CtxB mAb. Flow cytometric analysis of T-CVID T-cells following CD3/CD28 coligation showed a reduction both in the levels of GM1 and in the relative number of GM1\textsuperscript{+} T-cells compared to both disease control and healthy control T-cells (Fig.5A). In all unstimulated cells the levels of GM1 were not significantly over background levels (data not shown). The differences revealed in the flow cytometric analysis were also observable by confocal microscopy. As shown in figure 5B, unstimulated PBL showed barely detectable levels of diffuse immunoreactivity to CtxB/anti-CtxB complexes in all samples. In control cells, as well as in T-cells from a disease control patient (dis.ctr1), CD3/CD28 costimulation resulted in a significant increase in cell size and a strong increase in GM1 expression, which was detectable as punctuate staining at the cell surface. In T-CVID T-cells consistent differences were observed, particularly in patients T-CVID1 and T-CVID2, with a significant percentage of small cells showing a weak, diffuse CtxB-specific immunoreactivity (Fig.5B). Hence the impairment in F-actin reorganization can be correlated to a defective upregulation of lipid rafts, an important downstream event in productive TCR signaling\textsuperscript{49}. 
Recovery of F-actin reorganization in T-CVID T-cells following reconstitution of Vav expression

The functional defects in Vav-dependent events in T-CVID T-cells, and in particular the impairment in F-actin reorganization, are consistent with the observed reduction in Vav expression in these patients. To establish whether the decrease in Vav protein is causal to the defect in cortical actin reorganization in T-CVID T-cells, a reconstitution experiment was carried out. T-cells from the four T-CVID patients and from four healthy donors were transiently transfected by electroporation with either empty vector or the same expression vector encoding myc-tagged Vav1. A constitutive GFP reporter was used as transfection control. Immunoblotting with anti-myc mAb showed that recombinant Vav1 was expressed in transfected cells (Fig.6). F-actin capping induced by CD3/CD28 coligation was analyzed by confocal microscopy after cell labeling with fluorochrome-conjugated phalloidin. As shown in figure 6, the defect in F-actin reorganization was reversed in T-CVID T-cells following transfection with the Vav expression construct. Hence the defect in Vav expression underlies the impairment in actin cytoskeleton reorganization in T-cells from the four T-CVID patients included in this study.
DISCUSSION

Cortical actin reorganization is crucially required for TCR signaling\textsuperscript{52,53}. In T-cells this process is primarily regulated by activation of Rac, which is triggered by the guanine nucleotide exchanger, Vav\textsuperscript{40,53}. T-cells from Vav\textsuperscript{−/−} mice, as well as Vav-deficient Jurkat T-cells, display defective TCR clustering at the immunological synapse and impaired formation of actin caps\textsuperscript{46,47,54,55}. This defect correlates with impaired T-cell activation. Our finding of an impairment in F-actin reorganization in T-CVID T-cells resulting from defective Vav expression is fully consistent with the notion that Vav is a master regulator of the actin cytoskeleton in T-cell activation. That defects in F-actin dynamics and TCR signaling are observable in T-CVID T-cells even though Vav expression is not abolished indicates that, while the levels of Vav are normally sufficient to meet the requirements of the cell, a decrease below these levels is likely to result in Vav insufficiency. This notion is in agreement with the finding that TCR clustering and F-actin accumulation at the T-cell/APC interface are impaired in mice heterozygous for a Vav\textsubscript{1} null mutation\textsuperscript{55}. Furthermore, the data suggest that other guanine nucleotide exchangers such as Sos, which has been shown to activate both Ras and Rho GTPases\textsuperscript{56}, cannot substitute Vav in T-cells.

Coalescence of lipid rafts, which is essential for full triggering of the TCR signaling cascade\textsuperscript{39,57-59}, is impaired in Vav\textsuperscript{−/−} T-cells\textsuperscript{38}, as well as in T-cells treated with pharmacological inhibitors of actin polymerization\textsuperscript{39}, suggesting a role for Vav-dependent reorganization of cortical actin in this process. Clustering is likely to stabilize the pre-existing weak association of the TCR with lipid rafts\textsuperscript{36} and promote its juxtaposition to Lck, which is primarily responsible for CD3$\zeta$ phosphorylation and subsequent initiation of the TCR signaling
cascade as the result of recruitment and activation of ZAP-70 (refs. 22,31,32 and Fig. 1A). Although T-cells from the T-CVID patients who participated in this study display normal expression of Lck and Fyn, as well as of their positive regulator CD45, ZAP-70 fails to be recruited to the activated TCR as the result of defective CD3ζ phosphorylation20. We propose that a defect in the Vav/Rac pathway controlling lipid raft clustering, due to impaired Vav expression and F-actin reorganization, may underlie the failure of T-cells from our T-CVID patients to correctly initiate TCR signaling. The signaling defects observed in T-CVID T-cells from our patients are largely in agreement with the defects reported both for T-cells from Vav1−/− mice and for Vav-deficient Jurkat T-cells, including defective actin capping, impaired Ca2+ mobilization and -albeit more controvertial- impaired stress kinase activation46-48,54. In contrast, as opposed to our patients, early PTK dependent signaling appears unaffected in Vav1−/− T cells. The possibility of compensation for the loss of Vav1 by upregulation of Vav2 or Vav3 in Vav1−/− T cells has however not been addressed. Furthermore, a different phenotypic outcome of Vav deficiency in mouse and man cannot be excluded. On the other hand, the alternative possibility that the impairment in CD3ζ phosphorylation and ZAP-70 activation in T-CVID T-cells may result from a defect affecting both early events and Vav expression cannot at this stage be ruled out.

The impairment in Vav protein expression is, in three out of the four T-CVID patients, correlatable to a reduction in the levels of Vav-specific mRNA. No mutation was found in any of the patients in the region upstream of the Vav1 gene which has been identified as essential and sufficient for the tissue-specific expression of Vav152, ruling out promoter sequence defects. Alternative intrinsic defects, including decreased mRNA stability or Vav1 haploinsufficiency (as appears to be the case for patient T-CVID1), may underlie the reduction in Vav1 specific mRNA. Alternatively, transcriptional regulation of the Vav1 gene
may be impaired in these patients. Vav is primarily expressed in hematopoietic cells. The regulation of Vav expression has been proposed to be achieved by stage- and tissue-specific transcriptional activators rather than by epigenetic modifications of the gene promoter. This notion is supported by the finding that Vav expression can be reactivated in mouse 3T3 fibroblasts by fusion with human monocytic U937 cells, which express Vav. Analysis of the Vav gene promoter has identified a 23-bp segment essential for tissue-specific regulation, which includes a 7-bp potential CBF/AML-1 binding site. The absence of Vav expression in fetal liver of CBP/AML-1−/− mice supports a role for this transcription factor in the regulation of Vav, at least in primitive hemopoiesis. Whether the defect in Vav expression in T-CVID T-cells is dependent on a defect in the transcriptional machinery remains to be determined. In this context, it is noteworthy that T-cells from one of the T-CVID patients (T-CVID3) show a significant impairment in Vav expression, notwithstanding normal levels of Vav mRNA, which suggests a post-transcriptional defect affecting Vav mRNA translation or Vav protein stability. This feature indicates that different genetic lesions may underlie Vav deficiency in T-CVID.

Vav plays a key role in sustaining TCR signaling after lipid raft clustering and formation of the immunological synapse, suggesting that, in addition to preventing initiation of the TCR signaling cascade, the reduction of Vav in T-CVID T-cells might also affect later signaling events downstream of Rac. In support of this notion, the upregulation of lipid rafts at the cell surface triggered by CD3/CD28 costimulation in T-CVID T-cells (Fig.5), as well as their proliferative response to TCR ligation (ref. 18 and Methods), are significantly impaired in our T-CVID patients. Collectively, the defect in Vav expression shown by our data not only contributes a significant step forward in the understanding the molecular basis of T-CVID but also underlines the essential role of Vav in human T-cell activation.
Acknowledgements. The authors wish to thank Denis Alexander for the gift of CD45-deficient Jurkat cells, Frédérique Michel for generously providing myc-tagged Vav1, Nagaja Capitani, Giacomo Spinsanti and Francesco Nardi for support and advise, and Sonia Grassini for technical assistance.
REFERENCES


FIGURE LEGENDS

Figure 1. Fyn fails to restore correct CD3ζ phosphorylation in Lck-deficient cells and is normally expressed in T-CVID T-cells. A. Top, Immunoblot analysis of Lck and Fyn expression in parental JCaM1 cells and in stable transfectants expressing either F505Lck or F528Fyn. Bottom, immunoblot analysis using anti-phosphotyrosine antibodies of CD3ζ-specific immunoprecipitates from lysates of Jurkat cells, or JCaM1 cells or stable JCaM1 transfectants expressing either F505Lck or F528Fyn. A small amount of tyrosine phosphorylated CD3ζ in JCaM1 cells expressing Fyn was detectable at longer exposure times (data not shown). 0, non stimulated; CD3, activated by CD3 cross-linking with OKT3 mAb for 30 sec at 37°C. The filter was stripped and reprobed with anti-CD3ζ mAb. B. Anti-Fyn immunoblots of postnuclear supernatants of PBL from T-CVID patients (T-CVID1-4), two disease controls (dis.ctr1 and 2) and a representative healthy control (ctr; n=3). The filters were stripped and reprobed with anti-tubulin or anti-actin mAb. The migration of molecular mass markers is indicated.

Figure 2. Defective Vav expression in T-CVID T-cells. Anti-Rac (A) or anti-Vav (B) immunoblots of postnuclear supernatants of PBL from T-CVID patients (T-CVID1-4), one or two disease controls (dis.ctr1 and 2) and a representative healthy control (ctr; n•4). The filters were stripped and reprobed with anti-actin mAb. The migration of molecular mass markers is indicated. C,D. Semiquantitative RT-PCR analysis of Vav1 (C) or Vav2 and Vav3 (D) mRNA levels in T-CVID PBL. Vav-specific RT-PCR products from from T-CVID patients (T-CVID1-4), one disease control (dis.ctr1) and a pool of three healthy controls (ctr) were resolved by agarose gel electrophoresis, quantitated by laser densitometry and normalized to the levels of GAPDH mRNA used as internal control.
The data are expressed as % of the levels of Vav mRNA in PBL pooled from three healthy controls. The raw data of a representative experiment (n=2) are shown in the insert.

**Figure 3. F-actin capping is impaired in T-CVID T-cells.** A. Relative percentage of F-actin cap formation in PBL from the four T-CVID and a disease control (expressed as % capping in PBL from healthy controls, n=6), as measured by confocal microscopy of cells labeled with fluorochrome-conjugated phalloidin. Representative examples of F-actin labeling in cells either non stimulated or activated for 1 hr by CD3/CD28 costimulation are shown at the top. B,C. Immunoblot analysis of p38 (B) and JNK (C) phosphorylation using phosphospecific antibodies of postnuclear supernatants of PBL from T-CVID patients and one or two representative healthy controls (ctr). 0, non stimulated; CD3, activated by CD3 cross-linking with OKT3 mAb for 5 min. The filters were probed with anti-phospho-p38 or anti-phospho-JNK antibodies and, after stripping, with anti-p38 antibodies as loading control.

**Figure 4. [Ca²⁺] mobilization is partially impaired in T-CVID T-cells.** Flow cytometric analysis of intracellular [Ca²⁺] in T-CVID PBL. The arrow indicates the time of addition of anti-CD3 mAb. Data were analysed and plotted using Flowjo (Tree Star, Inc). Representative experiments are shown (n=2).

**Figure 5. T-CVID T-cells fail to upregulate surface GM1.** A. Flow cytometric analysis of GM1 expression in PBL from the four T-CVID patients (T-CVID1-4), one disease control (dis.ctr1) and two representative healthy controls (ctr; n=4). Cells were activated for 27 hr by CD3/CD28 costimulation. The empty histogram shows the negative control (anti-CtxB and fluorochrome-labeled anti-mouse Ig, no CtxB). GM1 labeling in nonstimulated
cells was not significantly higher than the negative control (not shown). The relative percentage of GM1+ cells, as well as their mean fluorescence intensity, are indicated. Representative experiments are shown (n•2). B. Analysis of GM1 expression by confocal microscopy of PBL from T-CVID patients (T-CVID1-4), one disease control (dis.ctr1) and one representative healthy control (ctr; n=4). Cells were activated for 48 hr by CD3/CD28 costimulation. Representative images are shown. Each experiment was performed 2-3 times.

Figure 6. Recovery of F-actin capping in T-CVID T-cells by reconstitution of Vav1 expression. Relative percentage of F-actin cap formation in response to CD3/CD28 costimulation in T-cells from the four T-CVID patients (expressed as % capping in T-cells from four matched healthy controls transfected with empty vector), as measured by confocal microscopy of cells labeled with fluorochrome-conjugated phalloidin. T-cells were cotransfected with a GFP reporter and either empty vector or the same vector encoding myc-tagged Vav1. Capping was quantitated on GFP+ cells (n=100). Two representative examples of F-actin labeling (TRITC-phalloidin) and GFP fluorescence in Vav/GFP cotransfected T-CVID T-cells activated for 1 hr by CD3/CD28 costimulation, together with the respective overlays, are shown at the top (right). An immunoblot analysis with anti-myc mAb of a representative control and T-CVID T-cell lysate transfected with either empty vector or the Vav expression vector is also shown (left).
Footnote

Patients T-CVID1, T-CVID2, dis.ctr.1 and dis.ctr2 were included in previous studies\textsuperscript{18,20}. For reasons of clarity they were however renamed. T-CVID1 and T-CVID2 correspond, respectively, to patients 1 and 2 in both studies. Patient dis.ctr1 corresponds to patient 3 in ref.20 and to patient 5 in ref.18. Patient dis.ctr2 corresponds to patient 4 in ref.20 and to patient 6 in ref.18.
Table I. Status of the early TCR signaling machinery in the T-CVID patients used in this study

<table>
<thead>
<tr>
<th>Activity</th>
<th>Phosphorylation</th>
<th>Expression</th>
<th>Subcellular loc.</th>
<th>cDNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3z*</td>
<td>defective</td>
<td>normal</td>
<td>ND</td>
<td>normal</td>
</tr>
<tr>
<td>ZAP-70*</td>
<td>defective</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Lck*</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Fyn*</td>
<td>ND</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
</tbody>
</table>

*ref.20 for patients T-CVID1 and T-CVID2, supplemental figures 1 and 2 for patients T-CVID3 and T-CVID4
*ref.20 for patients T-CVID1 and T-CVID2, supplemental figures 2 and 3 for patients T-CVID3 and T-CVID4
'data presented in this paper (Fig.1 and data not shown)
Table II. Immunophenotyping of PBMC from CVID patients.

<table>
<thead>
<tr>
<th>CD3</th>
<th>CD4</th>
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<th>CD45RA</th>
<th>CD45RO</th>
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</thead>
<tbody>
<tr>
<td>%(^a)</td>
<td>MFI(^b)</td>
<td>%(^a)</td>
<td>MFI(^b)</td>
<td>%(^c)</td>
</tr>
<tr>
<td>Control</td>
<td>83.8±2</td>
<td>235.4±4.9</td>
<td>44.6±0.2</td>
<td>260.4±56.0</td>
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<td>Patient 1</td>
<td>81.5</td>
<td>190.7</td>
<td>31.4</td>
<td>253.5</td>
</tr>
<tr>
<td>Patient 2</td>
<td>74.4</td>
<td>211.5</td>
<td>28.7</td>
<td>266.0</td>
</tr>
<tr>
<td>Patient 3</td>
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<td>211.8</td>
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<tr>
<td>Patient 4</td>
<td>87.7</td>
<td>243.2</td>
<td>28.1</td>
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<td>Patient 5</td>
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<td>46.2</td>
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<td>Patient 6</td>
<td>91.1</td>
<td>210.7</td>
<td>35.1</td>
<td>260.2</td>
</tr>
</tbody>
</table>

\(^a\) % of peripheral blood lymphocytes
\(^b\) mean fluorescence intensity obtained in a two-color flow cytometric analysis of PBMC from two representative healthy donors and the four CVID patients.
\(^c\) % of CD3\(^+\) peripheral blood lymphocytes
\(^d\) mean fluorescence intensity obtained in a three-color flow cytometric analysis of PBMC from three representative healthy donors and the four CVID patients. The data refer to CD3\(^+\) cells.
FIGURE 1
FIGURE 2
**FIGURE 3**

A

Comparison of F-actin caps in different conditions, showing a significant increase with CD3+CD28 activation.

B

Western Blot analysis of P-p38 and p38 with different treatments:

- **WB anti-P-p38** shows a clear increase in phosphorylated p38 with CD3+CD28 activation.
- **WB anti-p38** exhibits no significant changes.

C

Western Blot analysis of P-JNK and p38 with different treatments:

- **WB anti-P-JNK** shows a moderate increase in phosphorylated JNK with CD3+CD28 activation.
- **WB anti-p38** shows no significant changes.
FIGURE 4
FIGURE 5
FIGURE 6
Defective VAV expression and impaired F-actin reorganization in a subset of common variable immunodeficiency patients with T-cell defects

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