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Defective integration of activating signals derived from the T cell receptor (TCR) and costimulatory molecules in both $CD4^+$ and $CD8^+$ T lymphocytes of common variable immunodeficiency (CVID) patients

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SUMMARY

CVID is characterized by hypogammaglobulinaemia and impaired antibody production. Previous studies demonstrated defects at the T cell level. In the present study the response of purified $CD4^+$ and $CD8^+$ T lymphocytes to stimulation with anti-TCR monoclonal antibody (the first signal) in combination with anti-CD4 or anti-CD8, anti-CD2 and anti-CD28 MoAbs (the costimulatory signals) was investigated. Both $CD4^+$ and $CD8^+$ T cells from the patients showed significantly reduced IL-2 release following stimulation via TCR and costimulation via CD4 or CD8 and CD2, respectively. However, normal IL-2 production following TCR plus phorbol myristate acetate (PMA) costimulation and normal expression of an early activation marker, CD69, after TCR + CD28 stimulation indicated that TCR was able to transduce a signal. Furthermore, both IL-2 and IL-4 release were impaired in $CD4^+$ lymphocytes following TCR + CD28 stimulation. In addition, stimulation via TCR + CD28 resulted in significantly decreased expression of CD40 ligand in the patients. These results suggest that the integration of activating signals derived from the TCR and costimulatory molecules is defective in CVID patients; the defect is not confined to costimulation via a single molecule, or restricted to cells producing Th1-type cytokines such as IL-2, and is expressed in both $CD4^+$ and $CD8^+$ T cell subsets.

Keywords common variable immunodeficiency activation costimulation T cell receptor CD40 ligand

INTRODUCTION

CVID comprises a heterogeneous group of diseases with primary immunodeficiency due to as yet undifferentiated genetic defects. All patients with CVID have in common a profound defect in antibody production, and usually serum levels of one or more immunoglobulin isotypes are decreased [1]. B cells from CVID patients appear to be immature but functionally intact [2–4], consistent with the assumption that a defect at the T cell level is the cause of this humoral immunodeficiency. Several studies have indicated defects in mitogen-induced T cell activation in patients with CVID [5–7]. More recent studies showed that stimulation of CVID T cells via the TCR with antigen, superantigen or anti-TCR MoAb in the presence of accessory cells led to reduced proliferation, IL-2 and interferon-gamma (IFN-γ) gene transcription and release, and impaired Ca^{2+} flux [8–10]. These results suggested a defect in early signalling events following triggering of the TCR in patients with CVID [10].

Following TCR ligation costimulatory signals are triggered by interaction of structures such as CD28, CD4 or CD8 and CD2 on the T cell with B7 (CD80, CD86), MHC class II or class I, and CD58 (LFA-3), respectively, on the antigen-presenting cell (APC), leading to maximal T cell stimulation [11,12]. In the present study we wanted to clarify further defective T cell activation in CVID by investigating TCR-mediated T cell activation in purified $CD4^+$ and $CD8^+$ T cells. Crosslinking of the TCR and simultaneous delivery of defined costimulatory signals with MoAbs in the absence of accessory cells [13] allowed the examination of TCR-mediated activation in an antigen-independent mode.

CD40 ligand (CD40L) is expressed on activated $CD4^+$ T cells, and plays an important role in T–B interaction required for antibody production [14–21]. A recent study described defective CD40L expression in a subset of CVID patients [22]. While this finding was instrumental for the understanding of how the

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observed T cell abnormalities may lead to the humoral defect predominant in CVID, further investigation of CD40L expression was required to demonstrate a defect upon triggering of well defined biologically relevant surface structures. In addition, the functional relevance of defective CD40L expression in CVID patients had to be examined by showing that these patients' $CD4^+$ cells have an impaired capacity to provide help for IgG production in normal B cells.

PATIENTS AND METHODS

Patients

Patients with well documented CVID according to the diagnostic criteria of the WHO expert group for primary immunodeficiency diseases [1] were included in the study. Patients were on regular substitution therapy with IVIG (400 mg/kg body weight at 3–4-week intervals). Blood samples were always collected before IVIG infusion was given. Healthy blood donors were studied in parallel with the patients.

Preparation of T cell subpopulations

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood (5 U/ml of preservative-free heparin) by density gradient centrifugation (Lymphoprep; Nycomed Pharma AS, Oslo, Norway), and $CD4^+$ and $CD8^+$ T cells were isolated by negative immunoselection. In brief, PBMC were incubated at 37°C in plastic tissue culture plates (Macroplate; Greiner, Gmünd, Austria) to remove adherent monocytes. Non-adherent cells (1×10^7/ml) were incubated (30 min, 4°C, continuous rotation) with MoAbs against glycophorin A (final concentration 1·5 μg/ml), CD16 (final concentration 2 μg/ml), CD20 (final concentration 1·5 μg/ml), all from Immunotech S.A. (Marseilles, France), and CD11b (MAS 439, final concentration 1·5 μg/ml), CD14 (MAS 072, final concentration 10 μg/ml), all from Sera-Lab Ltd (Crawley Down, UK; microdialysed before use), and CD8 (MAS 436; Sera-Lab, final concentration 10 μg/ml) or CD4 (IOT4a; Immunotech, final concentration 10 μg/ml), respectively. Cells were washed twice, mixed with sheep anti-mouse IgG-coated beads (Dynabeads M-450; Dynal, Oslo, Norway, 2×10^7 beads/1×10^7 cells) and incubated under continuous rotation for another 30 min at 4°C. Cells bound to the magnetic beads were removed with a cobalt-sumarium magnet (HPC-1; Dynal). The remaining cells were collected, washed and analysed by flow cytometry. Purity was >95% for the $CD4^+$ or $CD8^+$ cell preparations, which were also $CD3^+$.

T cell stimulation and measurement of cytokine release

Negatively selected T cell subpopulations ($1·5\times10^5$/well) were incubated without accessory cells in 96-well microtitre plates (Falcon Microtest II; Becton Dickinson, Lincoln Park, NJ) under standard conditions (37°C, 5% CO_2) in RPMI 1640 medium containing 10% heat-inactivated human AB serum, 2 mM L-glutamine (JRH Biosciences, Sera-Lab), 100 U/ml penicillin and 100 μg/ml streptomycin (JRH Biosciences). Cells were stimulated for 60 h with MoAb anti-TCR αβ (BMA-031; Behring, Marburg, Germany, coated onto Dynabeads (70 ng/1×10^6 beads; 5×10^5 beads were added per well)), anti-CD4 (Leu-3a; Becton Dickinson, San Jose, CA, final concentration 15 ng/ml), anti-CD2 (OKT11; Ortho Diagnostic Systems, Neckargemund, Germany, final concentration 15 ng/ml), anti-CD28 (Immunotech S.A., final concentration 10 ng/ml), anti-CD8 (Leu-2a; Becton Dickinson, final concentration 15 ng/ml) or with phorbol myristate acetate (PMA; Sigma-Chemie, Deisenhofen, Germany, final concentration 10 ng/ml). For measurement of IL-2 release, cell cultures contained anti-Tac MoAb (CD25 IL-2 Receptor Blocking; Immunotech S.A., final concentration 1·5 μg/ml). IL-2 release was measured with the Interleukin 2 Enzyme Immunoassay Kit (Immunotech S.A.), and IL-4 release was determined with the IL-4 Quantikine Immunoassay Kit (R&D Systems, Minneapolis, MN).

Flow cytometry

Flow cytometric analysis was performed using a FACScan (Becton Dickinson). Directly conjugated MoAbs TCR αβ (BMA-031; Behring), CD2 (OKT11; Ortho Diagnostic Systems), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD8 (Leu-2a), CD69 (Leu-23) (all from Becton Dickinson), CD28 (Immunotech S.A.) and CD40L (anti-CD40L/FITC; Ancell, Bayport, MN) were used in two- or three-colour fluorescence staining following standard methodology.

Examination of CD40L (gp39) and CD69 expression on activated T cells

PBMC (2×10^6) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS; HyClone Labs, Logan, UT), 2 mM L-glutamine (JRH Biosciences), 100 U/ml penicillin and 100 μg/ml streptomycin (JRH Biosciences). Cells were stimulated with PMA (Sigma-Chemie, final concentration 20 ng/ml) plus ionomycin (IM; Calbiochem, San Diego, CA, final concentration 1 μg/ml) or with anti-TCR (BMA-031; Behring, coated onto Dynabeads, 5×10^6 beads/ml) alone or in combination with anti-CD4 (Leu-3a; Becton Dickinson, final concentration 100 ng/ml), anti-CD2 (OKT11; Ortho Diagnostic Systems, final concentration 50 ng/ml) or anti-CD28 (Immunotech S.A., final concentration 50 ng/ml). IL-2 (recombinant human IL-2; Boehringer, Mannheim, Germany) was used at a final concentration of 100 U/ml. Since exposure of T lymphocytes to phorbol esters such as PMA induces endocytosis of CD4 [23], CD40L expression was analysed after 4 h and 16 h by three-colour flow cytometry on the $CD5^+CD8^-$ PBMC population, consisting predominantly of $CD4^+$ T cells.

Reverse transcriptase-polymerase chain reaction assay and analysis of data by densitometry

PBMC (2×10^6/ml per well) were stimulated with MoAb anti-TCR-coated beads (5×10^6 beads/ml per well) plus CD28 MoAb (final concentration 50 ng/ml) for 4 h as described above. Unstimulated cells were cultured in medium alone. Total cellular RNA was then isolated according to the method of Chomczynski [24]. Equal amounts of total RNA were reverse-transcribed and then amplified on a BIO-MED Thermocycler 60 using Taq polymerase (USB Corp., Cleveland, OH) and the oligonucleotide primer pair specific for gp39 (sense: GCATGATCGAAACATACAAC-CAAACTTCTC; antisense: GAGTTTGAGTAAGCCAAAG-GACGTGAAGC) [25]. Co-amplification of the ribosomal protein S14 (sense: GGCAGACCGAGATGAATCCTCA; antisense: CAGGTCCAGGGGTCTTGGTCC) [26] served as internal control. The amplification profile involved 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 2 min. Aliquots of polymerase chain reaction (PCR)-generated products were fractionated on 1·5% ethidium bromide–agarose gels and validated by the predicted size. The relative density of the fragments was determined by image-analysing densitometry (OD*MM) (Pharmacia LKB Biotech., pdi Quantity One, Uppsala, Sweden). The densitometric

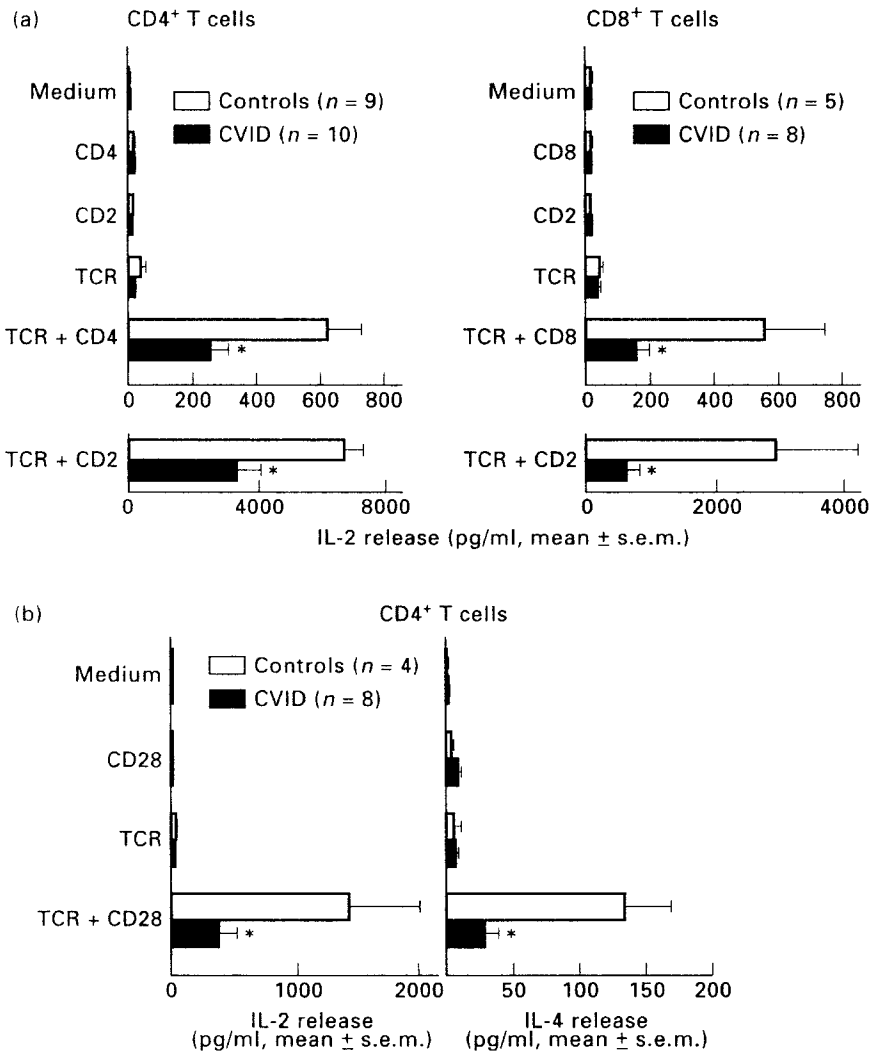

Fig. 1. Both $CD4^+$ and $CD8^+$ T cells from patients with CVID show a defect in T cell activation via TCR and a costimulatory signal, and the defect affects IL-2 and IL-4 production. Purified $CD4^+$ and $CD8^+$ T cells from CVID patients and healthy controls were stimulated with MoAbs anti-TCR coated onto beads in combination with anti-CD2, anti-CD4, anti-CD8 or anti-CD28 as described in Patients and Methods. Supernatants were harvested after 60 h of incubation. IL-2 and IL-4 release were measured with ELISA. *Statistically significant differences between patients and controls (IL-2 release, (a): $CD4^+$ cells, TCR + CD4, $P = 0{\cdot}011$; TCR + CD2, $P = 0{\cdot}002$; $CD8^+$ cells, TCR + CD8, $P = 0{\cdot}028$; TCR + CD2, $P = 0{\cdot}014$; (b): TCR + CD28, IL-2, $P = 0{\cdot}044$; IL-4, $P = 0{\cdot}03$). One patient of the eight tested produced 1558 pg/ml IL-4 following TCR + CD28 stimulation and was not included in the data given in (b).

reading of gp39 in a particular test was related to the corresponding densitometric reading for the internal control S14, and fold increase in gp39 mRNA expression (SI_{gp39}) in TCR + CD28-activated cells was calculated according to the following formula:

$$SI_{gp39} = \frac{(OD^*MM_{gp39}/OD^*MM_{S14})_{TCR+CD28\text{-stimulated cells}}}{(OD^*MM_{gp39}/OD^*MM_{S14})_{unstimulated\ cells}}$$

Measurements of IgG secretion

Highly purified B cells (>99% $CD19^+$ cells) from healthy donors prepared by immunoselection were seeded in duplicates in microtitre plates at a cell density of 2×10^4/well in a total volume of 200 µl together with autologous monocytes (6×10^3/well) and were cultured with or without added $CD4^+$ T cells (4×10^4/well) in RPMI 1640 medium containing 10% heat-inactivated FCS (HyClone Labs), 2×10^{-5} M 2-mercaptoethanol (2-ME; Sigma-Chemie), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (JRH Biosciences). Cells were stimulated with pokeweed mitogen (PWM; Gibco, Grand Island, NY, final dilution 1:200) and incubated under standard conditions (37°C, 5% CO_2). For quantification of IgG production, culture supernatants were collected on day 14 and IgG concentrations were measured using an isotype-specific ELISA as described earlier [10].

Statistical analysis

Results are given as mean of $n \pm$ s.e.m. of the respective study group and in n every patient and healthy control subject is represented only once. For statistical evaluation of the difference between patients and controls the Mann–Whitney U-test was employed at a level of $P < 0{\cdot}05$. Linear regression of the correlation between the log-transformed TCR plus CD28-induced IL-2 release and CD40L expression or CD69 expression in the patients (linear regression analysis) was calculated by Fischer's z transformation.

RESULTS

Both $CD4^+$ and $CD8^+$ T cells from patients with CVID show a defect in T cell activation via TCR and costimulatory molecules

Purified $CD4^+$ and $CD8^+$ cells were stimulated with TCR MoAb bound to beads and soluble MoAbs to costimulatory molecules such as CD4, CD8 and CD2, respectively. The results obtained in healthy controls confirm previous reports [11,12,27] by showing that in purified resting peripheral blood $CD4^+$ or $CD8^+$ T cells crosslinking of TCR in the absence of accessory cells triggers only minimal IL-2 production (Fig. 1a) or T cell proliferation (data not shown). A second signal by MoAbs to CD4 or CD8 and CD2, respectively, is required to induce the production of significant levels of IL-2 (Fig. 1a) and considerable T cell proliferation (data not shown). Compared with controls, patients' purified $CD4^+$ or $CD8^+$ T cells released significantly lower amounts of IL-2 following activation via TCR and costimulation via CD4/CD8 or CD2 in the absence of accessory cells (Fig. 1a). In addition, the results depicted in Fig. 1b demonstrate an impaired IL-2 release in the patients' $CD4^+$ T cells following stimulation via TCR and CD28, a costimulatory molecule that has been directly implicated in the expression of the IL-2 gene [28–30]. Furthermore, the defect in T cell activation via TCR and CD28 was not limited to IL-2-producing $CD4^+$ T cells (i.e. cells producing Th1-type cytokines), but also involved T cells producing IL-4, a Th2-type cytokine (Fig. 1b).

The TCR can induce a signal in the patients' T cells if a receptor-independent cosignal is given via direct activation of protein kinase C by PMA

Applying both TCR stimulation and activation of protein kinase C (PKC) by PMA induced vigorous production of IL-2 in $CD4^+$ cells of controls and patients alike, whereas neither of the two signals was effective by itself (Fig. 2). These findings indicate that direct activation of PKC was able to bypass the defect in the patients, and that TCR-mediated signalling was unimpaired under these conditions.

CD40L expression following TCR + CD28 costimulation is impaired in CVID patients and recombinant IL-2 cannot restore the defect

Previous studies described the induction of CD40L expression on T cells following receptor-independent activation with PMA plus IM [14–16]. The present results show that costimulation of PBMC from healthy controls with MoAb to TCR coated onto beads and soluble anti-CD28 led to a significant expression of CD40L in the $CD4^+$ population ($CD8^-$ $CD5^+$ cells) as examined by flow cytometry in three-colour immunofluorescence staining (Fig. 3a–c). Stimulation via TCR alone or in combination with CD4 or CD2 induced only low or no CD40L expression (data not shown). These findings are in good agreement with a previous study [31] reporting that activation via CD28 promotes CD3-induced CD40L expression.

As patients with CVID have a profound B cell defect and a previous paper demonstrated an impairment in CD40L (gp39) expression on T cells from these patients activated with PMA plus phytohaemagglutinin (PHA) [22], we were interested in further clarifying the biological relevance of this observation and therefore examined CD40L expression upon stimulation of the TCR + CD28. CD40L expression on patients' $CD4^+$ cells following TCR + CD28 stimulation was decreased in comparison with

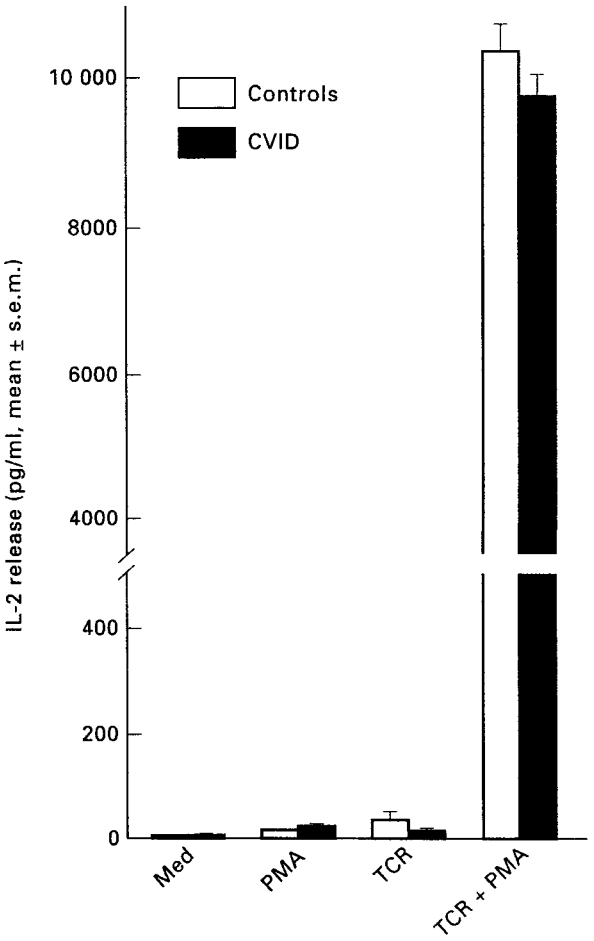

Fig. 2. The TCR is able to transduce a signal in CVID patients. Purified $CD4^+$ T cells ($1{\cdot}5 \times 10^5$ cells/well) from patients ($n = 8$) and controls ($n = 5$) were stimulated for 60 h with anti-TCR MoAb coated onto beads (5×10^5 beads/well) in combination with phorbol myristate acetate (PMA; 10 ng/ml). IL-2 release was measured by ELISA.

healthy controls, as depicted in the FACS histograms in Fig. 4, and the difference was statistically significant (Fig. 3a–c). In contrast to patients with hyper-IgM syndrome [19–21], CD40L expression following activation with PMA + IM (applying optimal conditions) was normal in the CVID patients studied (Fig. 3a,b, 4 h of incubation). Significantly decreased TCR-dependent CD40L expression could still be observed in patients after 16 h following activation, at a time when CD40L expression started to decline in the controls as well (Fig. 3c), indicating that defective CD40L expression in the patients was not due to altered kinetics. Addition of rIL-2 (100 U/ml) could not restore the defective CD40L expression in patients' activated cells (Fig. 3c). Furthermore, a defect in the induction of CD40L in CVID patients following stimulation via TCR could also be detected at the mRNA level (Fig. 3d).

Expression of the early activation marker CD69 following TCR + CD28 costimulation is unimpaired

Although CD40L expression following TCR + CD28 stimulation was impaired, patients' T cells were able to respond to TCR-dependent stimulation, since the expression of CD69 following stimulation via TCR and CD28 was comparable in patients and controls (CD28 alone is ineffective), as was CD69 expression

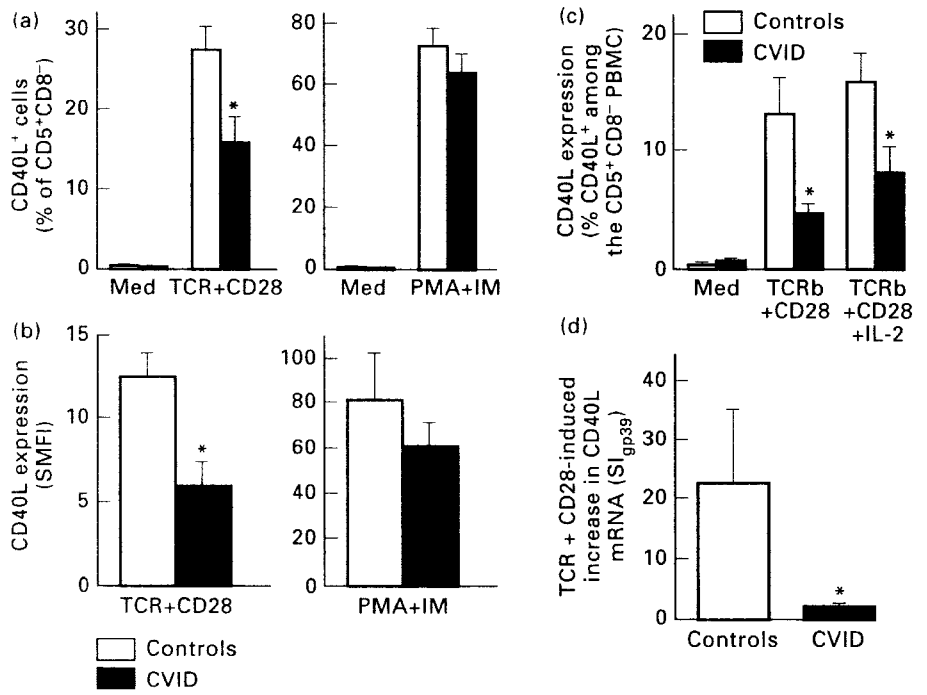

Fig. 3. Defective induction of CD40L expression following stimulation via TCR plus CD28 in CVID patients. Peripheral blood mononuclear cells (PBMC) from CVID patients ($n = 8$) and healthy controls ($n = 8$) were stimulated for 4 h with anti-TCR (coated onto beads) plus anti-CD28 MoAb or with phorbol myristate acetate (PMA) plus ionomycin (IM), as described in Patients and Methods. CD40L expression was examined in the $CD5^+ CD8^-$ PBMC population (mainly $CD4^+$ T cells) by flow cytometry. (a) Values represent mean percentage of CD40L expression ± s.e.m. *Statistically significant difference ($P = 0{\cdot}014$) between patients and controls. (b) Induction of CD40L is expressed as specific mean fluorescence intensity (SMFI), i.e. mean fluorescence intensity (MFI) of CD40L expression on TCR plus CD28 or PMA + IM-activated cells minus MFI of background binding of CD40L-specific MoAb to cells cultured in medium only. Values are expressed as mean ± s.e.m. of eight healthy controls and eight patients, every study subject being tested once. *Statistically significant difference between patients and controls ($P = 0{\cdot}003$). (c) PBMC were stimulated for 16 h with anti-TCR (coated onto beads) plus anti-CD28 MoAb with or without rIL-2 (100 U/ml) before CD40L expression was examined. Values represent percentage $CD40L^+$ cells among the $CD5^+ CD8^-$ PBMC population (mean ± s.e.m.). *Statistically significant difference between patients and controls (TCR + CD28, $P = 0{\cdot}003$; TCR + CD28 + IL-2, $P = 0{\cdot}023$). (d) Defective induction of CD40L gene transcription following stimulation via TCR + CD28 in CVID patients. PBMC from patients ($n = 7$) and healthy controls ($n = 5$) were activated for 4 h with anti-TCR coated onto beads plus anti-CD28 MoAb as described in Patients and Methods. CD40L (gp39) gene expression was then assessed by reverse transcriptase-polymerase chain reaction (RT-PCR), and the resulting PCR products were evaluated by quantitative densitometry analysis of Southern blots. The densitometric reading of gp39 in a particular sample was related to the corresponding densitometric reading for the internal control S14, and fold increase in gp39 mRNA expression (Si_{gp39}) in TCR + CD28-activated cells was calculated according to the following formula: $Si_{gp39} = ((OD*MM_{gp39}/OD*MM_{S14})_{TCR+CD28\text{-stimulated cells}})/((OD*MM_{gp39}/OD*MM_{S14})_{unstimulated\ cells})$. Values represent mean ± s.e.m. Statistically significant difference between CVID patients and controls: $P = 0{\cdot}02$. In unstimulated cells, OD*MM (mean ± s.e.m.) was 0·418 ± 0·036 for S14 mRNA and 0·139 ± 0·101 for gp39 mRNA in controls ($n = 5$), and 0·390 ± 0·028 for S14 mRNA and 0·171 ± 0·034 for gp39 mRNA in patients ($n = 7$).

following activation of the cells with PMA + IM (data not shown). There was a statistically significant correlation between decreased CD40L expression and the impairment in IL-2 release following TCR + CD28 stimulation in $CD4^+$ cells of individual patients (Fig. 5). In contrast, no correlation was found between decreased IL-2 release and CD69 expression (Fig. 5).

T cell help for B cells is impaired in CVID patients

Since CD40L–CD40 interaction together with interleukins is important for normal isotype switching [14–18] and PBMC from our patients did not produce IgG [10], we investigated T cell help for B cells in CVID patients in a functional assay [32,33]. The results depicted in Table 1 confirm previous reports [32,33] by showing that in healthy controls IgG production in this system is entirely dependent on the presence of $CD4^+$ T cells. Thus the results clearly indicate that these CVID patients' $CD4^+$ T cells, shown to be impaired in CD40L expression following TCR-dependent stimulation, have a functional impairment in providing T cell help to induce IgG production in healthy B cells (Table 1).

DISCUSSION

The results presented in this study confirm and extend previous findings by showing that TCR-dependent T cell activation is defective in patients with CVID. Applying an experimental system free of APC, stimulation of the TCR in combination with distinct costimulatory molecules enabled us to analyse further the nature of the defect. Previous studies reported defective T cell responses to recall antigens in patients with CVID [8–10,34,35], and in a recent study impaired antigen response was attributed to a

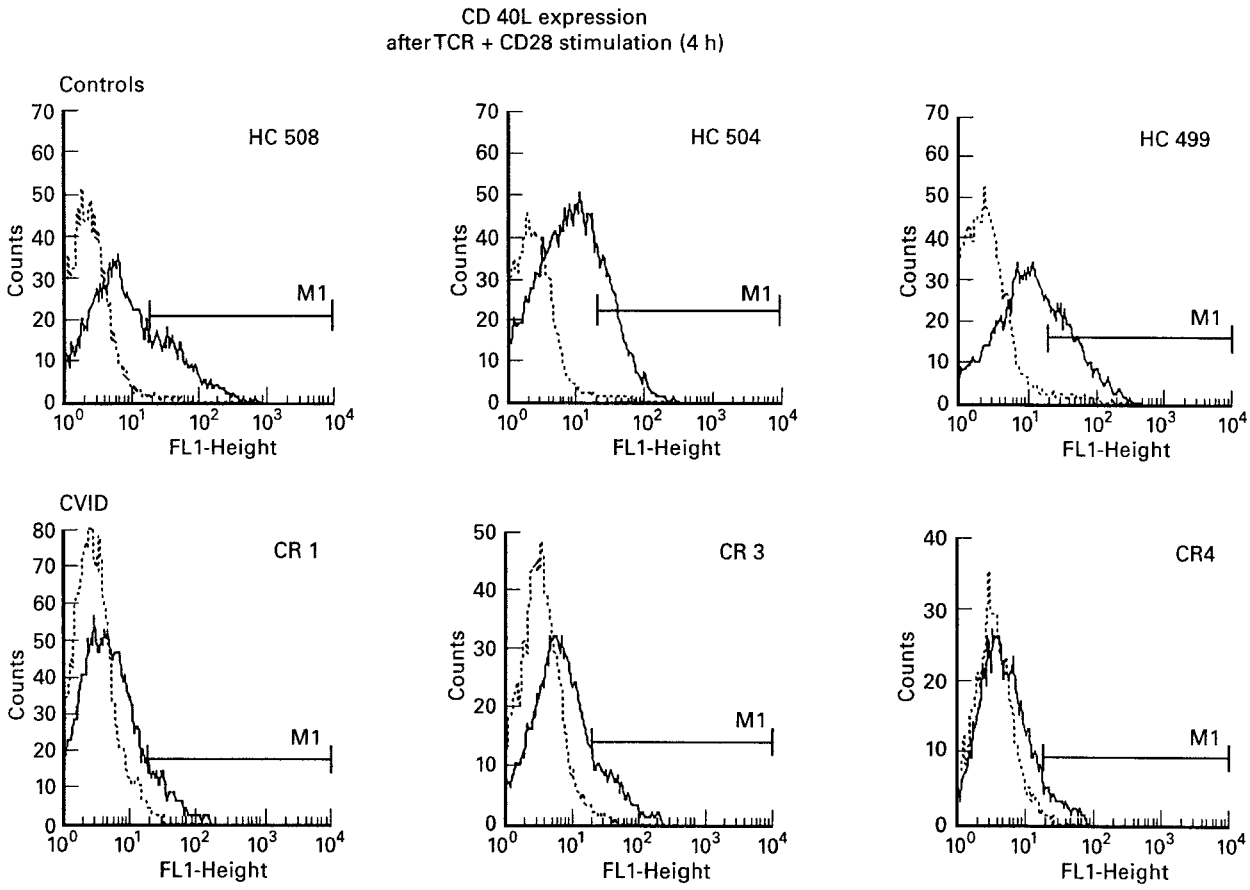

Fig. 4. Flow cytometric examination of CD40L expression. Peripheral blood mononuclear cells (PBMC; 2×10^6/ml) were activated via TCR + CD28 for 4 h. CD40L expression was then analysed by three-colour flow cytometry. Cells were harvested and stained with MoAbs against CD40L (FITC-conjugated), CD5 (PE-conjugated) and CD8 (PerCP-conjugated). A forward scatter *versus* side scatter gate was set to identify lymphocytes, and analysis of lymphocytes for CD5 *versus* CD8 staining enabled the identification of the $CD5^+CD8^-$ cell population (i.e. mainly $CD4^+$ T cells) by fluorescence gating. The histograms show CD40L expression on $CD5^+CD8^-$ PBMC in three representative healthy controls and three CVID patients, as examined by staining with a FITC-conjugated CD40L-specific MoAb and analysis of green fluorescence intensity (i.e. third fluorescence colour). . . . . ., Binding of the FITC-conjugated isotype control MoAb to $CD5^+CD8^-$ lymphocytes; ————————, CD40L expression in this cell population. A marker (M1) was set to identify the percentage of $CD40L^+$ cells.

reduced frequency of antigen-specific T cells [35]. In the present study purified $CD4^+$ T cells were stimulated via TCR, independent of antigen specificity, and the findings presented clearly indicate that a reduction in the number of a T cell subset and/or in the frequency of antigen-reactive T cells cannot be the reason for impairment of TCR-dependent T cell responses. In addition, even though all the respective surface structures including CD28 were expressed normally (data not shown), application of different costimulatory signals following TCR crosslinking always led to impaired activation of T cells, indicating that defective $CD4^+$ T cell stimulation cannot be explained by the defective function of a single costimulatory molecule. Our findings further indicate that impaired IL-2 release in the patients' $CD4^+$ T cells cannot be explained by an imbalance between the Th1 and Th2 subpopulations, since impaired activation of both IL-2 (Th1-type cytokine) and IL-4-producing T cells (Th2-type cytokine) has been observed.

Results obtained on examining the role of $CD8^+$ cells in CVID patients are conflicting. Suppressor activity for immunoglobulin production has been reported [36,37], but several other studies were unable to demonstrate excessive suppression [38]. Recently, suppression of the $CD4^+$ cell function by $CD8^+$ cells in CVID patients has been suggested, but the depletion of $CD8^+$ cells did not restore the purified protein derivative (PPD)-specific response [35]. Other reports on $CD8^+$ T cells in CVID patients described significantly decreased proliferation, c-myc expression, and IL-2 production following stimulation with anti-CD3 MoAb or with PMA [39,40]. Our results extend these latter findings by showing that defective TCR-dependent T cell activation is expressed in $CD4^+$ and $CD8^+$ subsets alike.

Previous reports investigated the costimulatory function of CD28 in CVID [34,41]. Our present results confirm their findings by showing that although T cell responses to TCR + CD28 stimulation were impaired, CD28 was functional in the CVID patients, since activation of purified $CD4^+$ cells with PMA + CD28 was comparable in patients and controls and induced low but detectable levels of IL-2 production (data not shown). TCR triggering in combination with optimal PKC activation (PMA) was also shown to be functional. These results indicate that both the TCR and the costimulatory molecules are capable of delivering functional signals, suggesting that a defect in the amplification of TCR-derived activation by costimulatory signals might be

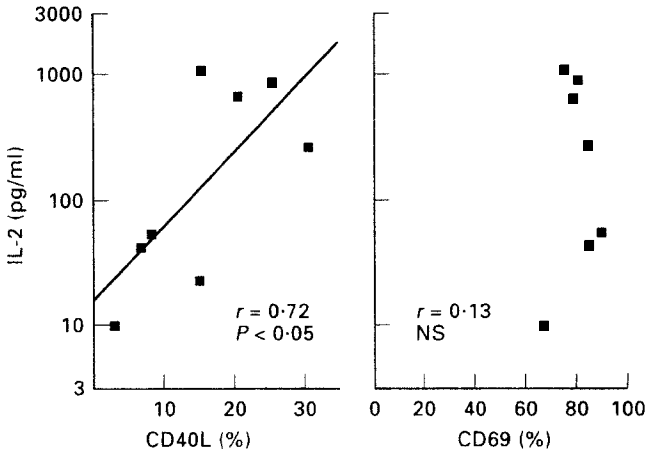

Fig. 5. Correlation between decreased IL-2 production and impaired CD40L, but not CD69 expression in CVID patients' T lymphocytes following TCR + CD28 stimulation. IL-2 production was studied in purified $CD4^+$ cells following stimulation via TCR + CD28 as described in Patients and Methods after 60 h of stimulation. Expression of CD40L and CD69 was studied by flow cytometry on peripheral blood mononuclear cells (PBMC) activated for 4 h via TCR + CD28; CD40L expression is given as percentage (%) of positive cells among the $CD5^+ CD8^-$ PBMC population, CD69 expression as percentage of positivity within the $CD3^+$ PBMC population. A statistically significant correlation could be found between impaired IL-2 production and diminished CD40L expression ($r = 0{\cdot}72$; $P < 0{\cdot}05$), but not between IL-2 production and CD69 expression ($r = 0{\cdot}13$).

Table 1. Defect in the capacity of patients' $CD4^+$ T cells to induce IgG production in allogeneic B cells from healthy controls

	IgG levels (ng/ml, mean ± s.e.m.)	
	Medium	PWM
$Mo_{controls}$	5 ± 3	15 ± 8
$B_{controls}$	6 ± 3	24 ± 8
$(Mo + B)_{controls}$	8 ± 3	16 ± 7
$CD4^+{}_{controls}$	5 ± 3	20 ± 9
$CD4^+{}_{CVID}$	0 ± 0	5 ± 5
$(Mo + B)_{controls} + CD4^+{}_{controls}$	101 ± 68	17798 ± 3140
$(Mo + B)_{controls} + CD4^+{}_{CVID}$	57 ± 32	8894 ± 2228
		$P = 0{\cdot}02$

Purified B cells (2×10^4/well) and macrophages (6×10^3/well) from healthy donors were cultured alone or with purified $CD4^+$ T cells (4×10^4/well) from CVID patients ($n = 7$) or allogeneic healthy controls ($n = 10$). Cells were stimulated with pokeweed mitogen (PWM; final dilution 1:200). Culture supernatants were collected on day 14. IgG production was determined by ELISA.

responsible for impaired TCR-mediated T cell activation in CVID patients.

Three essential pathways are presently known to be involved in TCR-mediated signalling, the $p21^{ras}$-mediated pathway, the calcium/calcineurin-dependent pathway and the pathway involving PKC activation. Recently it has been reported that ras plays a key role in TCR/CD3-mediated CD69 expression in T cells [42–44]. Our finding that CD69 expression in CVID patients following PMA + IM stimulation as well as following TCR-dependent activation is comparable to controls suggests that the ras activation pathway is functional in CVID patients. Previous studies on the mechanisms regulating CD40L expression on activated T lymphocytes indicated that CD40L expression requires activation of PKC and a rise in intracellular calcium concentration [45,46]. In a recent study [47] we described that peripheral blood T cells from the same patients as described here failed to generate normal levels of the second messenger IP_3 following stimulation via TCR. Defective integration of signals derived from the TCR and costimulatory molecules could involve a defect in early signal-transducing elements, e.g. at the level of amplification of one of the TCR-dependent signal transduction pathways, leading to a partial T cell defect with impaired activation of the calcium/calcineurin pathway and/or suboptimal activation of PKC, leading to an impairment in TCR-dependent IL-2 gene transcription and CD40L expression. In our patients, defective TCR-mediated T cell activation could be restored by direct stimulation of PKC, pointing towards a defect upstream of PKC activation. A previous study, however, showed defective T cell responsiveness to phorbol ester stimulation in five out of nine patients with CVID [7], suggesting a defect downstream of PKC activation in these patients and pointing to the possibility that different defects along the same pathway could be responsible for the functional impairment observed.

Attempts have been made to clarify the heterogeneity of patients with CVID by classifying the patients into subgroups, e.g. according to their B cells' capacity to produce different immunoglobulin isotypes *in vitro* [3,48]. Although in our study the extent to which T cell responses were impaired varied between the individual patients (e.g. Fig. 5), the number of patients studied appears too small to make the decision whether this variation represents quantitative differences in the expression of the same or related defect(s), or whether real qualitative differences exist that would allow subdivision of the patients into subgroups. Our results show that $CD4^+$ T cells from the patients studied have an impaired capacity to provide T cell help for IgG production to highly purified allogeneic B cells from healthy controls, thus indicating the functional relevance of impaired gp39/CD40L expression in these patients. The finding that both TCR and costimulatory structures are able to transduce signals, and that certain TCR-dependent signalling pathways such as those leading to CD69 expression and/or IL-2R gene expression [8] are less affected or unimpaired, helps in understanding that important T cell functions such as those required for cell-mediated immunity are operational.

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