Clin Exp Immunol 1994; 95:209-214

The costimulatory signal CD28 is fully functional but cannot correct the impaired antigen response in T cells of patients with common variable immunodeficiency

M. B. FISCHER, H. M. WOLF, H. EGGENBAUER, V. THON, E. VOGEL, J. LOKAJ*, J. LITZMAN*, J. W. MANNHALTER† & M. M. EIBL *Institute of Immunology, University of Vienna, Vienna, Austria, *Institute of Clinical Immunology, Masaryk University, Brno, The Czech Republic, and †Immuno AG, Vienna, Austria*

(Accepted for publication 6 October 1993)

SUMMARY

A wide spectrum of different immunologic abnormalities have been postulated as being responsible for the impairment of specific antibody production and the decrease in all or selected immunoglobulin isotypes present in common variable immunodeficiency (CVID). These abnormalities include impaired B cell differentiation and/or function, defective macrophage function, and significant T cell defects. The aim of the present study was to delineate whether the accessory molecule CD28 is involved in the impaired antigen response of T cells from patients with CVID. Our results demonstrate that CD28 costimulation was functional in T cells stimulated with anti-CD3 or anti-TCR MoAb, but could not correct the impaired response of patients' peripheral blood T cells to tetanus toxoid. Analysis of patients' long-term cultured T cells further confirmed these results. Exogenous rIL-2, another costimulus, augmented but did not correct the defective proliferation and lymphokine production in patients' antigen-driven peripheral blood T lymphocytes or in long-term cultured T cells. These findings indicate that the CD28 signalling pathway in these patients' T cells is unimpaired, and that costimulation via CD28 cannot correct the defect occurring in the course of TCR-mediated T cell activation.

Keywords common variable immunodeficiency antibody deficiency effect of CD28 in CVID early T cell activation defect impaired response to recall antigens in CVID T cells

INTRODUCTION

Common variable immunodeficiency (CVID) represents a heterogeneous group of disorders characterized by decreased levels of some or all immunoglobulin isotypes, due to either intrinsic B cell defects [1,2] or impairment of antigen presentation [3,4]. More recently, subsets of CVID patients with significant T cell dysfunctions including cutaneous anergy [5], decreased or absent proliferation, and/or lymphokine production in response to recall antigens, mitogens, anti-CD3 or phorbol myristate acetate (PMA) have been described [6–11]. Decreased levels of IL-2, IL-4 and interferon-gamma (IFN-γ) following mitogen stimulation in CVID T cells have been reported [9,10], and one paper described defective IL-2, IL-4, IL-5 and IFN-γ gene expression in phytohaemagglutinin (PHA)-activated T cells [12].

In a previous study we characterized a subgroup of patients with CVID whose T cells were unable to proliferate and produce IL-2 and IFN-γ mRNA message or protein in response to the recall antigen tetanus toxoid (TT) [13]. Furthermore, patients' T cells showed a significantly decreased Ca^{++} flux after superantigen stimulation, indicating a defect in the early phase of T cell activation when coupling of the TCR to membrane enzymes results in the generation of second messenger products (Fischer *et al.*, manuscript submitted). However, a decreased or even absent costimulatory signal as a possible cause of the impaired antigen response in patients' T cells could not be ruled out.

In the current study we examined the effect of costimulation by triggering freshly isolated peripheral blood lymphocytes and long-term cultured T cells of patients with CVID via CD28. Our results show that the CD28 signalling pathway is functionally intact, but cannot correct the impaired antigen response observed in these patients.

Correspondence: Martha M. Eibl MD, Institute of Immunology, University of Vienna, Borschkegasse 8A, A-1090 Vienna, Austria.

PATIENTS AND METHODS

Patients

Nine patients with CVID as defined by the WHO classification of primary immunodeficiencies [14] participated in this study after informed consent was obtained. These patients showed

hypo- or agammaglobulinaemia of some or all immunoglobulin isotypes, and a consistent impairment of the production of specific antibodies, despite normal numbers of circulating B cells (Table 1). Their T cells failed to respond to recall antigens (e.g. TT and *Escherichia coli*), as reported earlier [13]. None of the male patients had a family history of X-linked humoral immunodeficiency, and all had B cells within the normal range, supporting the diagnosis of CVID and making X-linked agammaglobulinaemia (XLA) unlikely, although genetic analysis was not performed. Patients' monocytes were normal in numbers, expressed HLA-DR normally, and were functionally intact in their capacity to present antigen to histoidentical and haploidentical antigen-specific T cell lines and T cell clones of healthy control individuals (H. Eggenbauer *et al.*, manuscript in preparation). During the course of this study all patients were on regular gammaglobulin replacement therapy, and none of the patients was suffering from severe infections requiring hospitalization. All patients received TT vaccination or boosters within the last 6–12 months before the study. Age-matched healthy volunteers of both sexes were investigated in parallel and served as controls.

Isolation of MNC and preparation of a T-enriched lymphocyte population

Mononuclear cells were isolated from heparinized whole blood (10–20 U/ml heparin) by buoyant density gradient centrifugation [15]. Monocytes were prepared by adherence to plastic surfaces as described previously [13]. Cell purity was 80–90% monocytes as defined by flow cytometry with a CD14 MoAb. Non-adherent cells were removed and fractionated into T-enriched cells (> 95% CD2 and > 90% $CD3^+$ positive) and non-T cells by rosetting with 2-amino ethyl isothiouronium bromide (AET)-treated sheep erythrocytes as described elsewhere [16].

Peripheral blood T lymphocyte cultures

Triplicate cultures containing adherence-purified monocytes (1×10^5/well) and T-enriched cells (1×10^6/well) were stimulated with recall antigen TT (10 LF/ml; Swiss Serum and Vaccine Institute, Berne, Switzerland) with or without rIL-2 (10 U/ml; Amersham, Aylesbury, UK) and anti-CD28 MoAb (10 ng/ml Kolt-2; Janssen Biochem., Geel, Belgium), anti-CD3-specific MoAbs (10 ng/ml OKT3; Ortho Diagnostic Systems Inc., Raritan, NJ) and anti-TCR $\alpha\beta$ (2 μg/ml BMA-031 were coated on 4×10^8 beads/ml (Dynabeads M450; Dynal, Oslo, Norway) and used in a final concentration of 1×10^6 beads/1×10^6 T cells (5 ng/ml BMA-031)). After incubation periods of 7 days for antigen and 3 days for the MoAbs, cells were pulsed with 0·2 μCi ^{3}H-thymidine (Amersham) and harvested 16 h later. ^{3}H-thymidine incorporation was determined by using a liquid scintillation counter (Packard Instruments Corp., Tri-Carb, CT). Supernatants of the individual cultures were harvested 24 h and 48 h post-stimulation, sterile-filtered and stored at −20°C until lymphokine determinations were performed.

Long-term cultured T cells

T-enriched cells (1×10^6/ml) were cocultured with irradiated (60 Gy) autologous monocytes (1×10^5/ml) in the presence of TT (10 LF/ml) and exogenously added rIL-2 (100 U/ml). Cultured T lymphocytes (1×10^6) were restimulated every third week with irradiated (60 Gy) autologous monocytes (1×10^5) in the presence of TT (10 LF/ml) and rIL-2 (10 U/ml). Eleven days after each restimulation, cultures were split and stimulated with 10 U/ml highly purified IL-2 (Biotest, Dreieich, Germany). The resting period of 10 days, during which the T cells are cultured in medium containing IL-2 alone, has been recommended to avoid refractoriness of the cultured T cells to further antigenic stimulation. After 12 weeks of cultivation, long-term cultured T cells (1×10^6/ml) were stimulated with antigen (10 LF/ml), rIL-2 (10 U/ml) and anti-CD28 MoAb (10 ng/ml Kolt-2) in the presence of autologous monocytes (1×10^5/ml), and proliferative responses and lymphokine release were assessed. After 48 h of incubation, culture supernatants were harvested, sterile-filtered and stored at −20°C until lymphokine determinations were performed. After 3 days, ^{3}H-thymidine incorporation was assayed as described above.

Assessment of IL-2, IFN-γ and IL-4 activity in culture supernatants

IL-2 concentration in culture supernatant was determined by a bioassay using a CTLL-2 cell line according to the method of Gillis & Smith [17]. Concentrations of IFN-γ and IL-4 were determined with commercial ELISA kits (IFN-γ, Medgenix Diagnostics, Fleurus, Belgium; IL-4, British Biotechnology, Oxford, UK).

Statistical analysis

For statistical evaluation of the difference between patients and controls, the Mann–Whitney *U*-test was employed. A difference was considered to be statistically significant at a level of $P < 0{\cdot}05$.

RESULTS

CD28 costimulation is functional in freshly isolated T cells of CVID patients activated with anti-CD3 MoAb or with anti-TCR MoAb

To investigate the function of CD28 in the course of T cell activation in CVID, we measured T cell proliferative response and IL-2 and IFN-γ release following stimulation with anti-CD3 MoAb (10 ng/ml OKT3) or anti-TCR MoAb (5 ng/ml BMA-031) with or without costimulation with a MoAb directed against CD28 (10 ng/ml Kolt-2). As depicted in Table 2, costimulation with CD28 significantly increased the proliferative response of patients' and controls' freshly isolated peripheral blood T cells activated with anti-CD3 MoAb or anti-TCR MoAb. Furthermore, CD28 costimulation also increased IL-2 and IFN-γ production following stimulation with anti-CD3 MoAb or anti-TCR MoAb in both patients and controls (Table 3).

CD28 costimulation cannot correct impaired antigen responsiveness in CVID T cells

Patients' T cells failed to respond with adequate proliferation and IFN-γ release upon antigen stimulation [13]. Figures 1 and 2 show that signalling via CD28 could not correct the deficient antigen response of patients' freshly isolated peripheral blood T cells. A costimulatory signal provided by the CD28 receptor did not increase the proliferative response (Fig. 1) or IFN-γ production (Fig. 2) of these cells. In contrast, addition of exogenous rIL-2 to patients' antigen-driven T cell cultures significantly increased the proliferative response ($P = 0{\cdot}000\,079$, Fig. 1), and augmented but did not correct IFN-γ production

Table 1. Characterization of patients' serum immunoglobulin levels, peripheral blood lymphocyte subsets and T cell proliferation

Patient	Sex	Age (years)	Serum immunoglobulin (g/l)			Lymphocyte phenotypes			T cell proliferation (ct/min × 10^3)		
			IgG	IgA	IgM	CD3	CD3+CD28/CD3	CD19	Tetanus toxoid	PHA	Medium
1	F	58	0·6	0·1	0·07	72	76	15	2·8	92·0	1·7
2	F	52	2·2	0·2	0·01	73	65	6	0·9	78·7	0·7
3	F	41	0·7	0·5	0·01	85	66	7	1·1	43·5	0·5
4	F	21	0·05	0·01	0·1	81	65	5	1·6	104·4	1·5
5	F	40	0·6	0·01	0·01	85	39	6	1·2	68·8	0·7
6	M	28	1·7	0·4	0·1	72	72	6	1·9	93·1	0·8
7	M	26	0·1	0·01	0·01	76	68	20	2·1	126·5	1·5
8	M	17	0·2	0·02	0·1	84	91	6	0·6	164·1	0·1
9	M	41	0·7	0·01	0·1	74	85	12	0·5	56·2	0·3
Mean ± s.e.m.											
Patients			0·8 ±0·2	0·1 ±0·03	0·06 ±0·02	78 ±2	69 ±5	9 ±2	1·4 ±0·2	91·9 ±12·2	0·9 ±0·2
Controls*			11·4 ±0·6	2·4 ±0·2	1·5 ±0·2	71 ±2	78 ±6	10 ±2	25·2 ±4·3	104·2 ±9·2	0·8 ±0·2

Serum immunoglobulin levels were determined before IgG replacement therapy was started. The proliferation assay was performed by stimulating T-enriched cells (1×10^6/ml) and autologous monocytes (1×10^5/ml) with tetanus toxoid (10 LF/ml) or phytohaemagglutinin (PHA) (1:125) as described earlier [13]. Cell cultures were set up in triplicate, and the s.d. of the triplicate values never exceeded 20% of the mean.

* Normal values represent 10–16 healthy adults of both sexes and are expressed as mean ± s.e.m.

Table 2. The costimulatory effect of CD28 on the proliferative response of anti-CD3 or anti-TCR-stimulated T cells

	^{3}H-thymidine incorporation (ct/min)	
	Controls ($n=5$)	Patients ($n=9$)
CD3 (10 ng/ml)	32 396 ± 4323	33 606 ± 3779
CD3 (10 ng/ml) + CD28*	98 436 ± 10 739	63 740 ± 974
TCR (5 ng/ml)	19 863 ± 2243	25 907 ± 3861
TCR (5 ng/ml) + CD28*	62 211 ± 5761	65 772 ± 16 264

T-enriched cells (1×10^6/well) and autologous monocytes (1×10^5/well) were cocultured and stimulated with anti-CD3 MoAb (10 ng/ml OKT3) and anti-TCR $\alpha\beta$ MoAb (2 μg/ml BMA-031) coated on beads (Dynabeads M450, 4×10^8 beads/ml (Dynal, Oslo, Norway) at a final concentration of 1×10^6 beads/1×10^6 T cells (5 ng/ml BMA-031)) with and without anti-CD28 MoAb (10 ng/ml Kolt-2). After 3 days ^{3}H-thymidine incorporation was determined as described in Patients and Methods. ^{3}H-thymidine incorporation of cells cultured in medium or anti-CD28 MoAb never exceeded background levels (< 1500 ct/min). Values represent mean ± s.e.m.

* Statistically significant increase of anti-CD3 MoAb or anti-TCR MoAb-induced T cell activation by costimulation with MoAb against CD28 in both patients and controls ($P < 0.05$, Mann–Whitney U-test).

($P = 0.000077$, Fig. 2) of patients' peripheral blood T cells compared with antigen alone. Stimulation with exogenous rIL-2 plus antigen was also significantly higher compared with stimulation with rIL-2 alone, as demonstrated in the proliferative response ($P = 0.0142$, Fig. 1) and in IFN-γ release ($P = 0.0019$, Fig. 2). Signalling via CD28 further increased the IFN-γ production of patients' peripheral blood T cells stimulated with antigen and exogenous rIL-2 ($P = 0.0188$, Fig. 2).

Table 3. The costimulatory effect of CD28 on the IL-2 and IFN-γ production of anti-CD3 or anti-TCR-stimulated T cells

Stimulation	24 h		48 h	
	Controls ($n=5$)	Patients ($n=9$)	Controls ($n=5$)	Patients ($n=9$)
IL-2 release (U/ml)				
CD3	39 ± 18	56 ± 17	13 ± 5	19 ± 3
CD3 + CD28*	98 ± 17	95 ± 18	44 ± 4	28 ± 7
TCR	32 ± 8	17 ± 7	20 ± 5	4 ± 1
TCR + CD28*	106 ± 15	67 ± 16	41 ± 7	15 ± 3
IFN-γ release (U/ml)				
CD3	27 ± 10	15 ± 4	29 ± 8	16 ± 4
CD3 + CD28*	51 ± 15	49 ± 13	45 ± 11	52 ± 8
TCR	71 ± 37	21 ± 5	17 ± 5	8 ± 2
TCR + CD28*	112 ± 48	73 ± 21	31 ± 8	32 ± 9

The experimental setup was as described in Table 2 and in Patients and Methods. Supernatants were harvested 24 h and 48 h post stimulation and assayed for IL-2 and IFN-γ content. The IL-2 assay was based on proliferation of an IL-2-dependent murine cytotoxic lymphoid cell line, CTLL-2, and was expressed as arbitrary units/ml. The IFN-γ content was detected by using commercial ELISA kits; concentrations were calculated using a standard curve derived by linear regression of the log-transformed concentrations of the cytokine standards supplied with the respective kits. The IL-2 and IFN-γ release of cells cultured in medium or anti-CD28 MoAb never exceeded background levels (< 0·05 U/ml IL-2, < 0·1 U/ml IFN-γ). Values represent mean ± s.e.m.

* Statistically significant increase of anti-CD3 MoAb or anti-TCR MoAb-induced T cell activation by costimulation with MoAb against CD28 in both patients and controls ($P < 0.05$, Mann–Whitney U-test).

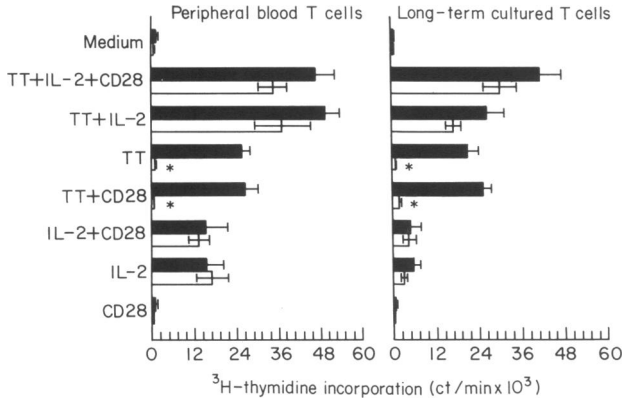

Fig. 1. Effect of CD28 costimulation on the proliferative response of common variable immunodeficiency (CVID) patients' peripheral blood T cells and long-term cultured T cells following stimulation with antigen with or without rIL-2. T-enriched cells (1×10^6/well) of the peripheral blood or long-term cultured T cells (1×10^6/well) were cocultured with autologous monocytes (1×10^5/well) and stimulated with antigen tetanus toxoid (TT; 10 LF/ml) with or without rIL-2 (10 U/ml) or anti-CD28 MoAb (10 ng/ml Kolt-1). Long-term cultured T cells were generated by continuously expanding T cells by stimulation with autologous irradiated (60 Gy) monocytes, antigen TT (10 LF/ml) and rIL-2 (10 U/ml) for 12 weeks as described in Patients and Methods. After 3 days ^{3}H-thymidine incorporation was determined in long-term cultured T cells and after 7 days in T-enriched cells as described in Patients and Methods. Statistical comparison between nine patients (□) and five controls (■) (Mann–Whitney *U*-test): $*P < 0{\cdot}05$.

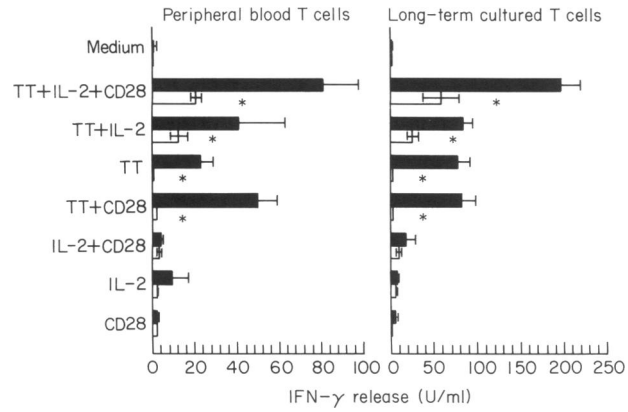

Fig. 2. Effect of CD28 costimulation on the IFN-γ release of common variable immunodeficiency (CVID) patients' peripheral blood T cells and long-term cultured T cells following stimulation with antigen with or without rIL-2. The experimental setup was as described in Fig. 1 and in Patients and Methods. Supernatants were harvested 48 h post-stimulation and assayed for their IFN-γ content as described in Patients and Methods. Statistical comparison between nine patients (□) and five controls (■) (Mann–Whitney *U*-test): $*P < 0{\cdot}05$. TT, Tetanus toxoid.

In the patients' antigen-driven long-term cultured T cells CD28 costimulation required the presence of exogenous rIL-2 to increase antigen-specific proliferative response ($P = 0{\cdot}0127$, Fig. 1). However, IFN-γ release was still significantly lower than in the controls ($P = 0{\cdot}0059$, Fig. 2).

Finally, we were able to demonstrate that patients' long-term cultured T cells were fully functional in the production and

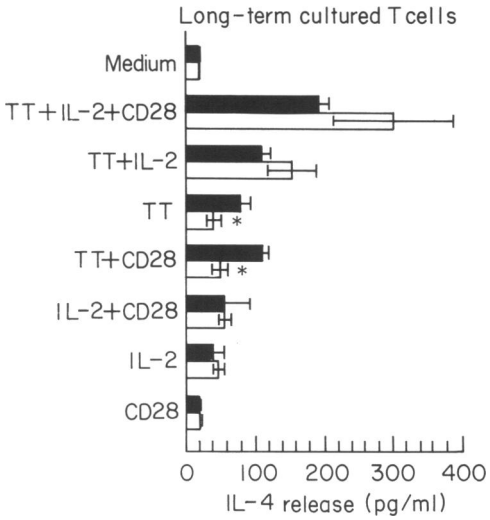

Fig. 3. Effect of CD28 costimulation on the IL-4 release of common variable immunodeficiency (CVID) patients' long-term cultured T cells following stimulation with antigen with or without rIL-2. The experimental setup was as described in Fig. 1 and in Patients and Methods. Supernatants were harvested 48 h post-stimulation and assayed for their IL-4 content as described in Patients and Methods. Statistical comparison between nine patients (□) and five controls (■) (Mann–Whitney *U*-test): $*P < 0{\cdot}05$. TT, Tetanus toxoid.

secretion of IL-4 in response to antigen tetanus toxoid and exogenous rIL-2, while IL-4 response to stimulation with antigen alone was decreased (Fig. 3). An additional costimulatory signal via CD28 increased the IL-4 release, but the difference did not reach statistical significance ($P = 0{\cdot}0708$, Fig. 3).

DISCUSSION

The results in this study confirm previous findings [12] by showing that CVID T cells were unable to respond to recall antigen with adequate cell proliferation and IFN-γ release. Furthermore, stimulation with anti-TCR MoAb resulted in reduced IL-2 production ($P = 0{\cdot}003\,09$), while CD3 stimulation induced a normal response. These findings suggest a defect confined to the early phase of T cell activation induced by signals via the TCR; however, a missing or inappropriate costimulatory signal that is needed for fully functional T cell activation after TCR ligation could not be ruled out.

The specificity of the T cell response is determined by recognition of the antigen via the TCR, but engagement of accessory molecules that deliver an additional second signal as well as binding of lymphokines such as IL-2 to their receptor is required for fully functional T cell activation [18–20]. One of the most potent costimulatory structures on T cells is CD28 (for review see [21]). CD28 signalling in synergy with the TCR-generated signal increases the rate of IL-2, IL-3, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) transcription and augments the stability of the cytokine mRNA [22].

In the present study we have addressed the question of whether CVID T cells respond to a costimulatory signal provided by triggering of CD28, and whether CD28 costimulation might correct the T cell activation defect observed in our

patients. Signalling via CD28 increased anti-CD3- or anti-TCR-induced proliferation and IL-2 and IFN-γ production in the patients' peripheral blood T cells, indicating that a costimulatory signal can be delivered by CD28 triggering. However, CD28 stimulation was unable to correct the T cell activation defect in the patients. While CD28 triggering increased antigen-induced IFN-γ release ($P=0{\cdot}0182$) in the controls, CVID T cells failed to respond in this system. Convincing evidence exists that signalling via CD28 alone cannot activate T cells. A functional first signal must be delivered (e.g. by the TCR) to induce a costimulatory effect by CD28 ligation [23]. The finding that CD28 costimulation did not restore impaired antigen responsiveness in the patients' T cells further supports the hypothesis that the activation signal provided via the TCR is not appropriate in this subgroup of patients with CVID.

Recent studies showed that inability to respond to antigen is a main characteristic of anergic T cells (for review see [24]). Addition of IL-2 is known to reverse impaired antigen responsiveness in anergized T cells or T cell lines [25]. In view of these findings it was interesting to note that in our patients' peripheral blood T cells addition of rIL-2 increased T cell proliferative responses and IFN-γ release following antigenic stimulation. Addition of rIL-2 to the patients' T cells, however, was not sufficient to fully restore impaired antigen responsiveness to normal levels, since IFN-γ release following stimulation with antigen plus rIL-2 was still significantly lower ($P=0{\cdot}011\,64$) than in the controls. Furthermore, in contrast to anergized T cells, patients' T cells responded normally to CD3 stimulation. To further rule out the possibility that CVID T cells might be in a state of anergy that could be corrected by addition of IL-2, we established long-term cultured T cell lines stimulated with TT-pulsed autologous irradiated (60 Gy) monocytes in the presence of rIL-2. Although antigen-reactive T cells could be expanded in the presence of antigen plus rIL-2, continuous cultivation in rIL-2 was unable to correct the activation defect. T cell lines only responded to antigen with or without CD28 when rIL-2 was present, but cell proliferation and IFN-γ release were still lower than in the controls. These findings further suggest that anergy is not the proper explanation for the T cell activation defect observed in our CVID patients.

Addition of exogenous rIL-2 to patients' long-term cultured T cells stimulated with antigen increased T cell proliferation ($P=0{\cdot}000\,873$) and IFN-γ production ($P=0{\cdot}0076$) to levels significantly higher than those observed following stimulation with rIL-2 alone. This confirms previous findings that CVID T cells have the capacity to recognize the antigen [13], but do not transduce the proper signal after TCR ligation. Exogenous rIL-2 augments the response to antigen, while triggering of CD28 has no effect. This is not surprising in view of the notion that CD28 costimulation requires a functional first signal while IL-2 does not. Our results indicate that a second or third costimulatory signal (provided by CD28 and/or rIL-2) cannot normalize the defect in antigen-induced T cell activation, but rather drives antigen-reactive T cells along a certain differentiation pathway. This is suggested by the finding that IFN-γ release was impaired, while IL-4 production was normal or even increased in patients' long-term cultured T cells stimulated with antigen plus rIL-2 and/or anti-CD28 MoAb. As IL-2 and IFN-γ producing helper T cells are of crucial importance in B cell differentiation [26], a T cell activation defect resulting in impaired IL-2 and IFN-γ release upon antigenic stimulation that cannot be corrected by costimulatory signals might contribute to the pathogenic mechanisms responsible for the antibody deficiency in the subset of patients with CVID.

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