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Original Paper

Flow Cytometric Analyses of the Specific Activation of Peripheral Blood Mononuclear Cells from Healthy Donors after *In Vitro* Stimulation with a Fermented Mistletoe Extract and Mistletoe Lectins

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Immunostimulatory properties of mistletoe extracts derived from *Viscum album* L. (VAL) are well described, demonstrating activation especially of T, T-helper cells and monocytes/macrophages. In order to characterise in detail the communication between different cell populations, we studied mistletoe-induced expression of co-stimulatory signals and their ligands by flow cytometry. Peripheral blood mononuclear cells (PBMC) from 15 healthy controls were incubated for 7 days with a fermented VAL extract. VAL significantly upregulated the expression of the co-stimulatory molecule B7.1 (CD80) on monocytes/macrophages, but not B7.2 (CD86). No significant changes in the expression of either molecules on B cells could be found, suggesting that only monocytes/macrophages act as antigen presenting cells (APCs) in this *in vitro* system. Purified mistletoe lectins, components of most VAL extracts were also analysed, but did not induce similar responses of monocytes/macrophages. The receptor for B7 molecules, CD28, but not CTLA-4 (CD152), was also found to be significantly enhanced on $CD4^+$ cells after VAL simulation. There was no evidence for activation of a B cell response via the CD40/CD40L pathway. Our data support the concept that stimulation by VAL extracts induces a specific T-helper cell reaction with monocytes/macrophages acting as APCs and purified lectins do not exert the same effects.

Key words: co-stimulation, flow cytometry, mistletoe, PBMC, specific immune system, *Viscum album*
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INTRODUCTION

FROM CLINICAL and experimental studies it is well known that extracts from *Viscum album* L. (VAL) exert cytotoxic and immunostimulatory properties [1–9]. Subcutaneous (s.c.) treatment of cancer patients leads to stimulation of natural killer (NK)-cells, monocytes and eosinophilic granulocytes [10–12] or activation of T, T-helper cells and B cells [1, 13].

We recently published that a fermented VAL extract was able to stimulate proliferation of human peripheral blood mononuclear cells (PBMC) *in vitro* [8] and that activation of naive T cells and monocytes were mainly involved in this process [13]. Thus, we were interested to characterise more precisely stimulatory and co-stimulatory signals expressed on the different cell populations. In addition to the stimulatory signals, the co-stimulatory molecules are crucial for the induction of a specific T cell response [14, 15] and these have to be expressed on antigen presenting cells (APCs). Apart from the professional APCs such as dendritic cells or macrophages, B cells can present antigens to T cells by binding them via their surface immunoglobulins, followed by internalisation, processing and presentation within HLA molecules [16–18]. In this respect, it may be that mistletoe antigens bind to B cells, which then function as APCs for T cells. Alternatively, T cell dependent B cell antigens could activate a B cell response via the CD40/CD40L pathway

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[16, 19]. Thus, in our study, we analysed the effects on cells involved in the stimulation procedures induced by the fermented VAL extract and mistletoe lectins (ML), which are components of most commercially available VAL preparations [8].

MATERIALS AND METHODS

Subjects

The reactivity of PBMC from 15 healthy controls (8 females, 7 males, average age 35.6 ± 12 years; range 25–56 years), which had been shown to react strongly to the fermented mistletoe extract, Iscador Pini, in cell cultures, were investigated. None of the subjects had ever received any mistletoe treatment. All individuals agreed and gave their consent to this experimental *in vitro* study.

Materials

A commercially available mistletoe extract from VAL grown on pines (Iscador Pini, IP; lot no. 304/3227, kindly provided by Dr Werner, Forschungsinstitut Hiscia, Arlesheim, Switzerland) was used: it is an aqueous extract fermented with *Lactobacillus plantarum*. Lectins were present in this preparation at a concentration of < 10 ng/ml and viscotoxins at a concentration of 85 μg/ml, according to the information given by the manufacturer. ML-1 was purchased from Sigma (St. Louis, Missouri, U.S.A.) ML-2 and ML-3 were a kind gift from U. Pfüller (University of Witten/Herdecke, Germany).

Antibodies to the different cell surface markers (CD, cluster of differentiation) used in this study for flow cytometry were obtained from Immunotech (Hamburg. Germany), Ancell (CD86; Alexis, Grünberg, Germany) or Pharmingen (CTLA-4; Hamburg, Germany). Examinations were performed by three colour staining using the combinations shown in Table 1.

Cell cultures

Isolation of PBMC from heparinised blood was performed as previously described [8]. Briefly, PBMC were isolated by Ficoll density centrifugation and suspended in RPMI FG1640 (Biochrom, Berlin, Germany) supplemented with gentamicin (Refobacin, Merck, Germany, 0.1 mg/ml) at a concentration of 3×10^6 cells/ml. Five hundred microlitres of this cell suspension, 250 μl of autologous plasma and 250 μl

Table 1. *Antibodies used to label specific cell subsets*

Specificity of the monoclonal antibodies*	Definition of cell subsets
CD80/CD86/CD14	Co-stimulatory signals on monocytes/macrophages
CD80/CD86/CD19	Co-stimulatory signals on B cells
CD80/CD86/CD3	Co-stimulatory signals on T cells
CD28/CTLA-4/CD4	Receptors for B7 molecules on T-helper cells
CD40/-/CD19	Receptor for co-stimulatory signals on B cells
CD40L/-/CD4	Co-stimulatory signals on T-helper cells

*FITC-conjugated antibody is listed first, PE-conjugated antibody second, and PE-CY-5-conjugated antibody third. FITC, fluorescein-isothiocyanate; PE, phycoerythrin; PE-CY-5, phycoerythrin-cyanin 5.

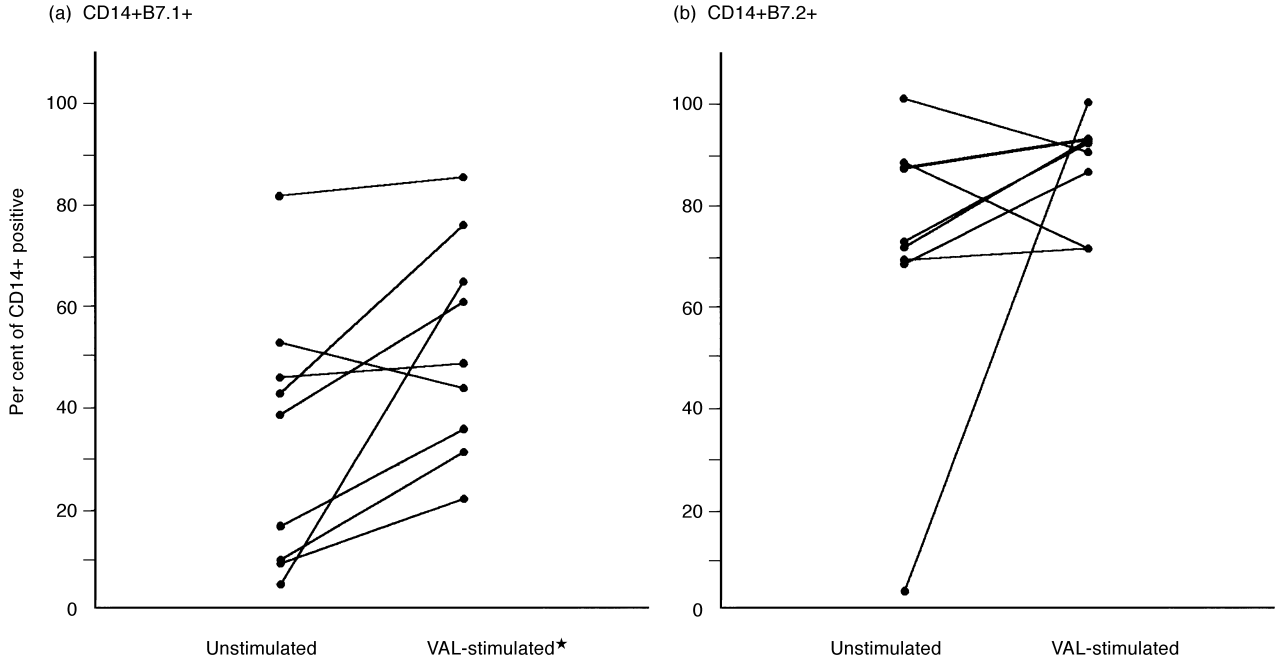

Figure 1. Expression of the co-stimulatory signals B7.1 (CD80) (a) and B7.2 (CD86) (b) on $CD14^+$ monocytes ($n = 9$) in 7 day cultures of peripheral blood mononuclear cells from healthy individuals stimulated with the fermented *Viscum album* L. (VAL) extract. Mean values (± SD): $CD14^+$ $B7.1^+$ unstimulated, 33.5 ± 25.5; VAL stimulated, 51.9 ± 21.2; $CD14 + B7.2^+$ unstimulated, 71.8 ± 27.3; VAL stimulated, 87.1 ± 9.8; *significant $P < 0.05$ (Wilcoxon signed rank test).

Table 2. Influence of the fermented Viscum album *L. (VAL) extract on the counts of CD14$^+$ monocytes in cultures of peripheral blood mononuclear cells from healthy controls* (n = 9) *after stimulation for 7 days* in vitro

	Individuals								
Antigens (μg/ml)	1	2	3	4	5	6	7	8	9
Unstimulated	348	98	61	450	436	665	1150	957	392
VAL 10 000	2007	1693	480	486	n.t.	466	318	995	478
VAL 1000	1091	429	338	1372	2046	980	536	1398	474
VAL 100	416	275	177	427	635	1043	1651	1549	422

n.t. not tested

of the respective antigen solution or medium control were incubated for 7 days in 24-well flat bottom microtitre plates (NUNC, Roskilde, Denmark) in a humidified 5% CO_2 atmosphere at 37°C. For kinetic studies, cells were cultured for 1, 4 or 7 days.

Different concentrations of the VAL extract were added: 100, 1,000 and 10 000 μg/ml (final concentration; corresponding to the weight of fresh plant). ML concentrations were 0.01, 0.1, 1 and 10 ng/ml.

Sample preparation and flow cytometric analysis

At the end of the incubation time, PBMC were analysed by flow cytometry, as previously described [13]. Briefly, half the supernatant of the spontaneous and of the VAL-activated cell cultures was removed and the cells were carefully resuspended. Aliquots of 100 μl were transferred into tubes containing 20 μl of each antibody directed against the respective surface marker (following the instructions given by the manufacturer) and incubated for 30 min (4°C, in the dark). Cells were lysed and fixed with a commercial lysing reagent (FACS lysing solution, Becton Dickinson, Heidelberg, Germany) for 10 min, washed twice with phosphate buffered saline (PBS) resuspended in a fixative (Cellfix, Becton Dickinson) and analysed.

All experiments were performed by use of a single laser flow cytometer (FACScan) from Becton Dickinson and the 'Lysis II' software. Analysts of the cell subsets and activation

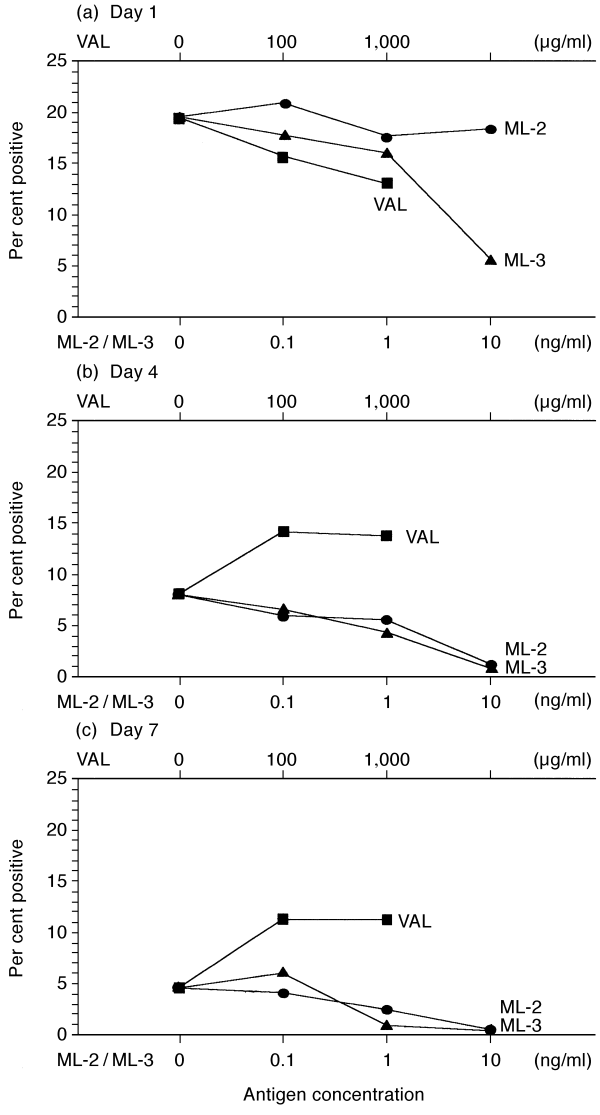

Figure 2. Kinetic studies of the influence of the fermented *Viscum album* L. (VAL) extract, the mistletoe lectins (ML) ML-2 and ML-3 on CD14$^+$ monocytes in cultures of peripheral blood mononuclear cells from a healthy donor measured by flow cytometry.

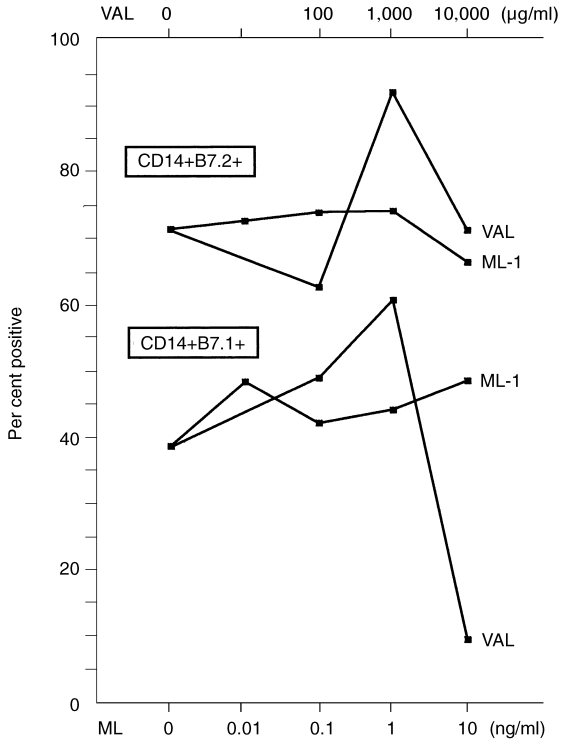

Figure 3. Dose-dependent expression of the co-stimulatory signals B7.1 (CD80) and B7.2 (CD86) on CD14$^+$ monocytes in 7 day cultures of peripheral blood mononuclear cells from a healthy control stimulated with the fermented *Viscum album* L. (VAL) extract or mistletoe lectin ML-1. Data from one of four experiments are given.

markers were carried out by counting 10 000 cells. A gate was set using the two parameters, forwardscatter (FSC, defining cell size) and sidescatter (SSC, defining granularity), to exclude cells other than lymphocytes, lymphoblasts and monocytes, which may cause a high background binding of the antibodies. Quadrants were set with respect to unspecific binding of the control antibodies (Immunotech) for evaluation of changes, in the proportion of the different subsets, activation markers or co-stimulatory signals.

Statistical analysis

Statistical analysis of the flow cytometric experiments was performed using the Wilcoxon matched pairs signed rank test.

RESULTS

Activation of antigen presenting cells

It has recently been shown that *in vitro* stimulation with the fermented VAL extract induced an increase in the proportion of HLA-DR$^+$ monocytes [13]. As shown in Figure 1, monocytes/macrophages were activated by VAL to express the co-stimulatory signals B7.1 (CD80, significant, $P < 0.05$) and B7.2 (CD86, not significant) which is clear evidence that the immune response is induced by the presence of VAL epitopes via APCs. Results are presented for the optimal VAL concentration. Similar investigations on the expression of B7.1 and B7.2 on B cells revealed no significant changes induced by VAL (data not shown).

By further flow cytometric analysis, a dose-dependent VAL-induced increase in the number of monocytes/macrophages became evident, as compared with the unstimulated control cultures (Table 2). This effect could be substantiated in kinetic studies, demonstrating that the proportion of monocytes remained almost unchanged in the VAL stimulated cell cultures during the 7 day culture period, while it decreased in the unstimulated cultures (Figure 2). Similar results were obtained for the expression of the co-stimulatory signals B7.1 and B7.2 on these cells (data not shown). ML-2 and ML-3 did not exert this protective effect on monocytes/macrophages (Figure 2).

Comparative studies on the influence of the VAL extract and ML-1 revealed that while VAL stimulated the molecules B7.1 and B7.2 on monocytes/macrophages, ML-1 activated only B7.1 with a minor influence on B7.2, as shown in Figure 3. This was the case in three of the four experiments, while in the fourth it was the opposite effect with stimulation of B7.2, but not of B7.1, by ML-1.

Activation of a T cell response

The expression of the receptors for the B7 co-stimulatory molecules CD28 and CTLA-4 (CD152) was studied on CD4$^+$ cells with and without VAL stimulation. As demonstrated in Figure 4, the proportion of CD4$^+$28$^+$ cells increased significantly after VAL incubation, while the CTLA-4 expression in these experiments showed no significant changes. Furthermore, the expression of the co-stimulatory signals B7.1 and B7.2 on T cells was stimulated significantly by VAL *in vitro* (Figure 5).

Activation of a B cell response

The expression of CD40 on B cells and of the respective ligand CD40L on CD4$^+$ T-helper cells were also studied. As shown in Figure 6, no significant reactivity was induced by the VAL extract compared with the unstimulated control culture.

DISCUSSION

The presented data confirm and extend our previous findings of a T-helper cell-mediated immune response towards the VAL extract via antigen presentation by APCs, in this

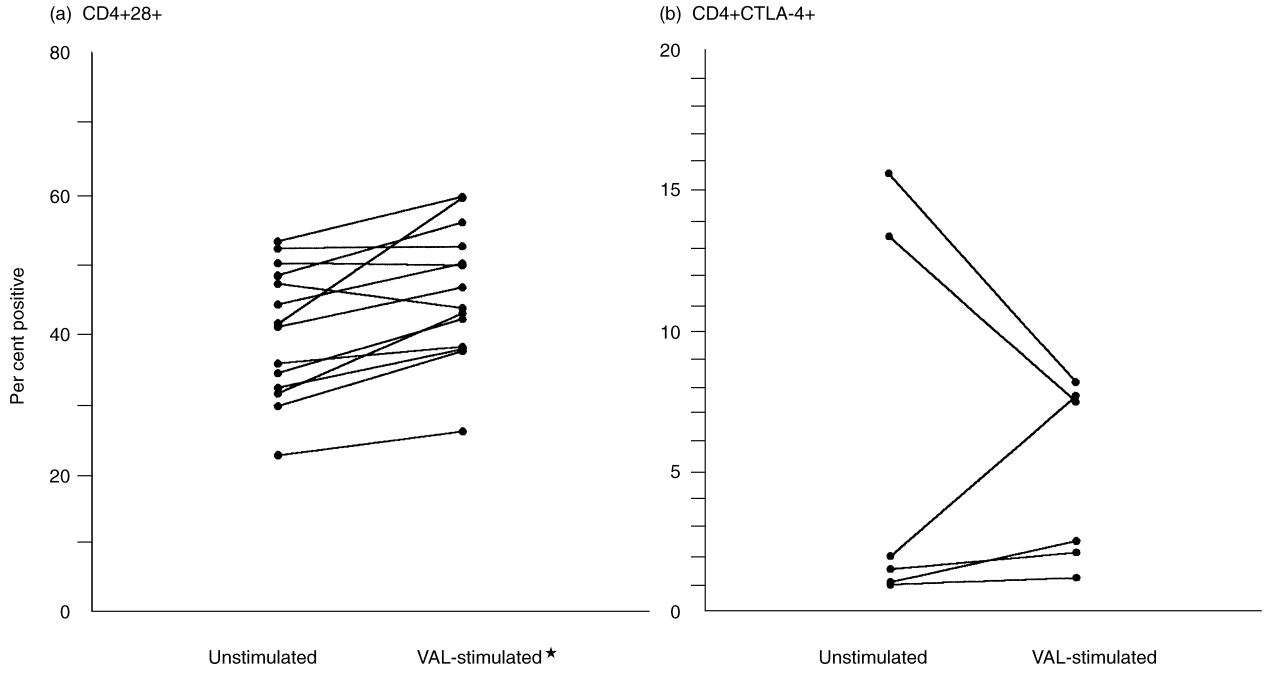

Figure 4. Activation of CD4$^+$ T-helper cells induced by the fermented *Viscum album* L. (VAL) extract in 7 day cultures of peripheral blood mononuclear cells from healthy controls (n=10), depicted as expression of the receptors for co-stimulatory signals CD28 (a) and CTLA-4 (CD152) (b). Mean values (± S.D.). CD4$^+$28$^+$ unstimulated, 40.1±9.5%; VAL stimulated, 45.6±9.5%; CD4$^+$ CTLA-4$^+$ unstimulated, 5.7±6.8%; VAL stimulated, 4.8±3.2%; *significant P<0.01 (Wilcoxon signed rank test).

case monocytes. We clearly demonstrated that VAL significantly induced the expression of the co-stimulatory molecule B7.1 on monocytes/macrophages as well as the respective receptor CD28 on T-helper cells, while a B cell response could not be observed. As we had shown in a previous study that $CD8^+$ cells are not involved in the stimulatory process induced by VAL [13], in this study we focused especially on $CD4^+$ cells and APCs. Enhanced survival of monocytes by the fermented VAL extract *in vitro* was also observed. All these findings clearly suggest that there is induction of VAL-specific response of PBMC [20, 21] by APCs during therapy with VAL extracts.

Immune responses towards micro-organisms as well as other antigens and tumours is governed by the two T-helper

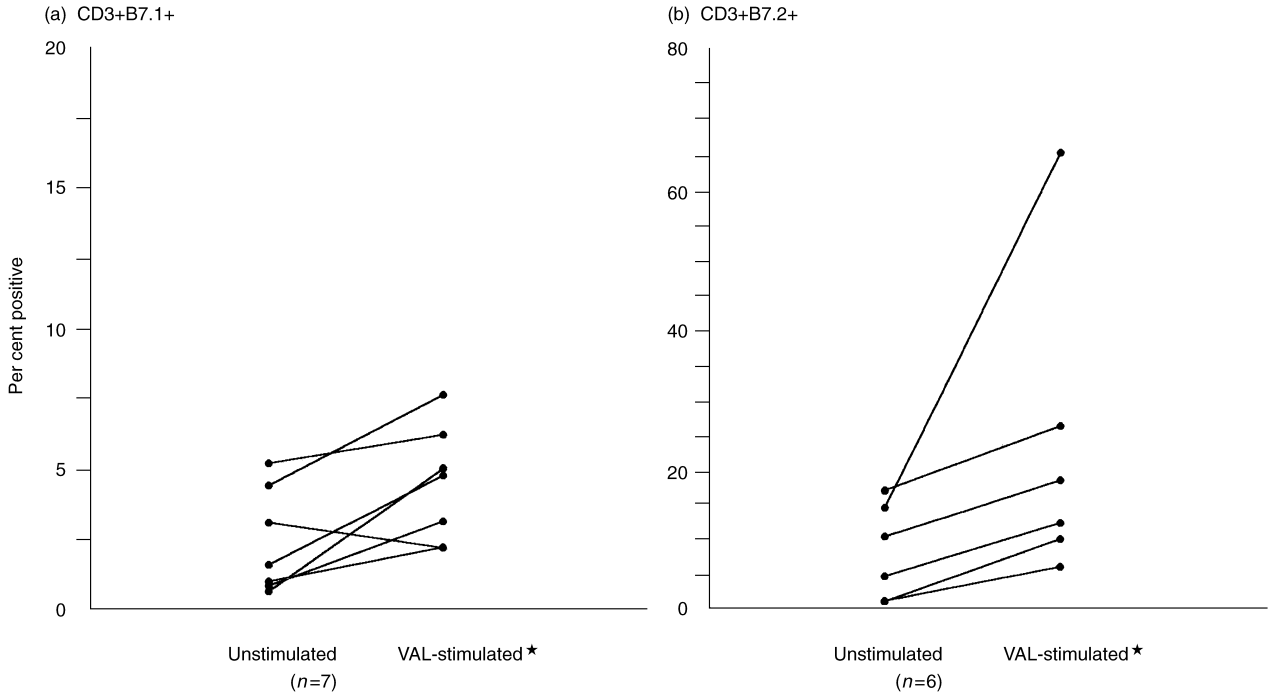

Figure 5. Expression of the co-stimulatory signals B7.1 (CD80) (a) and B7.2 (CD86) (b) on $CD3^+$T cells in 7 day cultures of peripheral blood mononuclear cells from healthy individuals stimulated with the fermented *Viscum album* L. (VAL) extract. Mean values (± S.D.). B7.1 unstimulated, 2.4±1.9; VAL stimulated, 4.9±2.1; $B7.2^+$ unstimulated, 8.2±6.9; VAL stimulated, 23.1±21.8; *significant $P<0.05$ (Wilcoxon signed rank test).

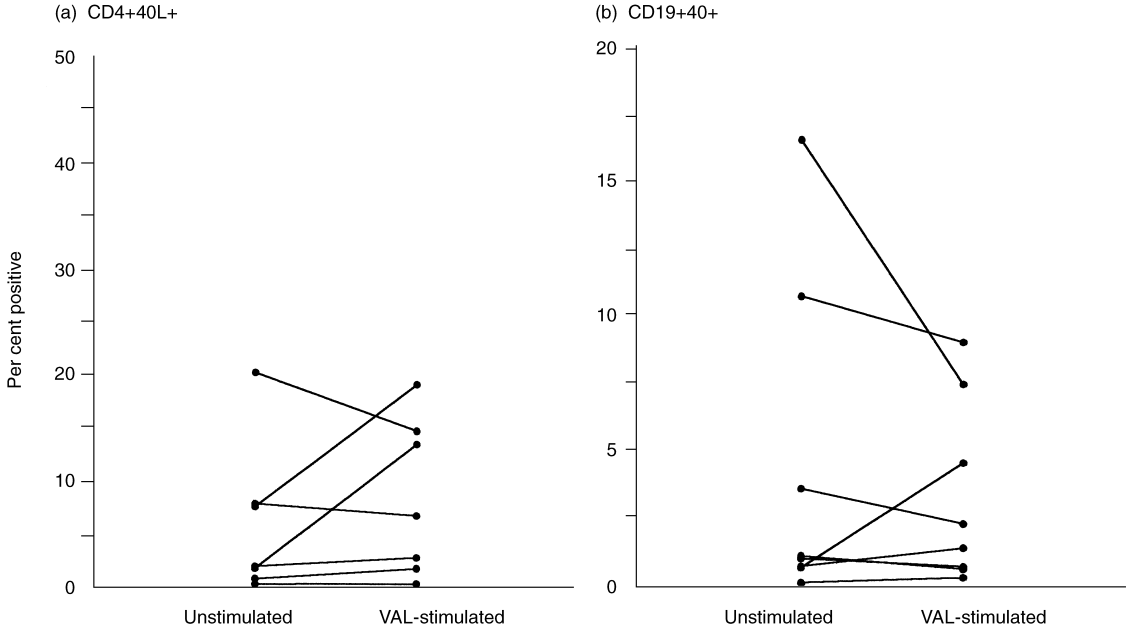

Figure 6. Lack of stimulation of B cells via the CD40/CD40L (CD154) pathway in the absence and in the presence of the fermented *Viscum album* L. (VAL) extract in 7 day cultures of peripheral blood mononuclear cells from healthy controls (n=7), as shown by the expression of CD40L on $CD4^+$ T-helper cells (a) and the respective receptor CD40 on $CD19^+$ B cells (b).

cell populations, the Th1 and Th2 cells, a finding which has been well documented during the last few years [22, 23]. This Th1/Th2 balance can be influenced not only by different microenvironmental factors such as antigen-dose, type of antigen, type of APCs, etc. [24], but also by the differential expression of the co-stimulatory signals [25, 26]. Thus, the preferential activation of B7.1 by VAL seems to favour a Th1 immune response. This observation could be a rational explanation for the beneficial effect of mistletoe therapy observed in some of the tumour patients.

Our further findings that the co-stimulatory signals were induced, not only on APCs but also on $CD3^+$ T cells after *in vitro* exposure to VAL is of interest, but an explanation is difficult. In a previous report, it was suggested that this phenomenon can be taken as a sign of an ongoing immune response [27].

The lack of *in vitro* activation of B cells by the fermented VAL extract may be related to the low content of ML [8] and, interestingly, this kind of therapy does not induce high titres of anti-ML-1 antibodies, in contrast to therapy with extracts containing high amounts of lectins [28, 29].

Another aspect of our study was to investigate whether purified ML exert similar effects as the whole plant extract. We clearly showed that the expression of co-stimulatory signals induced by ML-1, ML-2 or ML-3 was less pronounced compared with the VAL extract. Also, the *in vitro* survival of monocytes/macrophages was not influenced by these lectins. From these data it can be concluded that the fermented mistletoe extract almost devoid of lectins [8] exerts a much more pronounced specific immune response than the purified lectins based on the *in vitro* observations. Further *in vivo* studies are necessary to determine whether these observed differences are of any importance for choosing the appropriate kind of mistletoe therapy for tumour patients.

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