Anti-Cancer Drugs 1997, **8** (suppl 1):S33–S37

Stimulation of the specific immune system by mistletoe extracts

S. Fischer[1], A. Scheffler[2] and D. Kabelitz[3]

[1]Bessungerstr. 96, D-64347 Griesheim, Germany; [2]Carl-Gustav-Carus-Institute, Niefern-Öschelbronn; [3]Department of Immunology, Paul Ehrlich Institute, Langen, Germany.

We have investigated the *in vitro* responsiveness of T cells from mistletoe-treated cancer patients and untreated donors to various preparations of mistletoe extracts. Proliferation of peripheral blood mononuclear cells from treated but not from untreated patients was observed in response to therapeutically used mistletoe extracts prepared from apple (*mali*) or pine (*pini*) host trees. The strongest proliferation was induced by vesicle preparation of *mali* extract. Using a newly developed flow cytometry assay (standard cell dilution assay), we determined that cell growth was restricted to CD4+ T cells. Analysis with a panel of monoclonal antibodies against variable regions of the T cell receptor β chain (Vβ) revealed an oligoclonal pattern of CD4+ T cell activation. These results indicate that therapeutic administration of mistletoe extracts sensitized a restricted set of CD4+ T lymphocytes in mistletoe-treated patients. Lymphocytes from untreated donors are only stimulated with heat-treated mistletoe extracts. The responding T cells are γδ T cells and express variable T cell receptor elements Vγ9 and Vδ2. The γδ-stimulating activity of heat-treated mistletoe extracts is sensitive to treatment with alkaline phosphatase but not with proteinase K, indicating that the ligands are non-proteinaceous phosphate-containing compounds.

Keywords: CD4 T cells, mistletoe extracts, oligoclonal activation, γδ T cells, T-cell receptor.

Introduction

Viscum album (mistletoe) extracts are widely used in adjuvant cancer therapy in Germany and in other countries. Mistletoe extracts contain different compounds including lectins, viscotoxins, oligo- and polysaccharides, vesicles, and others [1–6]. Oligosaccharides isolated from mistletoe extracts have been shown to increase the cytotoxic activity of natural killer cells [7,8]. Commercially available preparations vary considerably in their relative content of the various compounds. Some of the constituents have been purified and their immunomodulatory effects investigated. The mistletoe lectin-I is a potent inducer of cytokines in macrophages and stimulates the release of tumour necrosis factor-α and interleukin-1 [9,10]. Accordingly, some investigators standardized their extract with respect to mistletoe lectin-I content, while other groups standardized their extract with respect to the process of manufacture in that they always took the same plants at the same time and manufactured the preparations to standardized protocols [11,12].

Although immunomodulatory effects of mistletoe extracts on the *in vitro* activity of non-specific components of the cellular immune system have been documented, the effector cells of the specific immune system (i.e. T lymphocytes) are also activated. Schultze *et al.* [13] have reported that peripheral blood mononuclear cells from mistletoe-treated but not from untreated patients proliferate *in vitro* in response to an aqueous, lectin-containing mistletoe extract. However, proliferation was revealed only in the presence of an autologous plasma containing mistletoe lectin neutralizing antibodies. The same group observed the *in vitro* proliferation of peripheral blood mononuclear cells from untreated allergic and some healthy individuals after stimulation with a fermented extract of *Viscum album* containing only low concentrations of lectin [14].

We have investigated the *in vitro* responsiveness of T lymphocytes from mistletoe-treated and untreated cancer patients, following stimulation with therapeutically administered *Viscum album* extracts prepared from apple (*mali*) or pine (*pini*) host trees, as well as vesicles prepared from *mali* extract [15]. Our aim was to identify the responding T-cell subset and to determine whether stimulation with vesicles induced polyclonal or oligoclonal cell activation. In the second part of our work we demonstrate that a heat-treated mistletoe extract activates human γδ T cells.

Proliferative response of peripheral blood mononuclear cells from mistletoe-treated patients

The *in vitro* proliferation response of peripheral blood mononuclear cells from breast cancer patients treated subcutaneously with mistletoe extracts was monitored by ^{3}H-thymidine uptake after 7 days of culture. It was shown that the vesicles of the *mali* extract ABNOBAviscum mali (0.15 mg/ml), which is largely devoid of mistletoe lectin, induced much stronger proliferation of pe-

Correspondence to S. Fischer

ripheral blood mononuclear cells [15,16]. While the lymphocytes from an untreated control group did not proliferate either following treatment for 4 weeks with the mistletoe extract or vesicles, the lymphocytes from the *mali* 4-treated group proliferated after giving the mistletoe extract and vesicles. Proliferative responses were observed over a wide range (0.5–500 μg/ml) of the extract concentrations, and in response to 2.5–10 μg/ml of the vesicles.

The vesicles were obtained after high-speed centrifugation of the ABNOBAviscum mali extract and resuspension of the green coloured pellet with ultrasonication. The vesicles consist essentially of chloroplast membranes of mistletoe, which are composed of approximately 50% monogalactosyl diglyceride and 50% digalactosyldiglyceride. These results support the report of Schultze *et al.* [13], indicating that the presence of specifically sensitized T cells in mistletoe-treated patients can be revealed by *in vitro* stimulation, but only in the presence of autologous plasma. Thus, the *in vitro* proliferative response to mistletoe extracts depends on the therapeutically administered mistletoe concentrations and on the duration of treatment.

To identify the responding T-cell subset(s), we have used the recently developed standard cell dilution assay to measure the absolute number of various lymphocyte subsets after culture [17]. Stimulation with the mitogen phytohaemagglutinin (PHA) led to the expected expansion of both CD4+ and CD8+ T lymphocytes (Fig. 1). In contrast, stimulation with *mali* or *pini* extracts, and again most notably with the vesicles (in response to their stronger proliferative response), triggered the selective activation and expansion of CD4+ T cells only. No CD8+ T-cell growth above the medium control was observed [15,16].

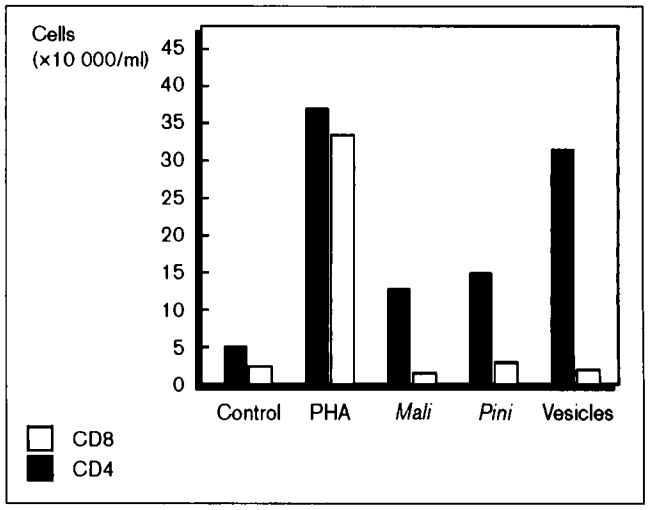

Figure 1. Cellular expansion of CD4+ and CD8+ T cells. Peripheral blood mononuclear cells from mistletoe-treated patients (between 42 and 70 years with pancreatic, colon, lung and adenocarcinoma) were stimulated with phytohaemagglutinin (PHA; 2 μg/ml) or mistletoe preparations for 8 days. Thereafter, the absolute number of viable CD4+ and CD8+ cells was determined by standard cell dilution assay. Means ± SD of experiments with lymphocytes from nine patients are shown [16].

Polyclonal or oligoclonal activation of CD4+ T cells?

Immunostimulatory effects of mistletoe extracts have been reported to modulate the non-specific cellular immune defence mechanisms (macrophages and natural killer cells). Therefore, we investigated whether the observed *in vitro* growth of CD4+ T cells after stimulation with vesicles was of polyclonal or oligoclonal origin. The stimulation of peripheral blood mononuclear cells with the polyclonal activator PHA did not significantly induce CD4+ T cells expressing one or several particular T-cell receptor Vβ elements, but rather stimulated all CD4+ T cells equally well. As expected, superantigen SEE (enterotoxin E from *Staphylococcus aureus*) stimulated the expansion of the same selected CD4+ subsets in all patients. In contrast, the vesicles of mistletoe extract revealed a different pattern, since they stimulated the selective growth of CD4+ T cells with 2–6 different T-cell receptor Vβ elements in patients. These results clearly indicated that the stimulation of peripheral blood mononuclear cells from mistletoe-treated patients with mistletoe vesicles induced an oligoclonal CD4+ T-cell activation *in vitro*. Comparing the distribution of reactive T-cell receptor Vβ families among different patients, the pattern has more similarity with an antigen-like than with a superantigen-like stimulation [15,16].

Our results demonstrate the *in vivo* sensitization of T cells during treatment with mistletoe extract, as revealed by the *in vitro* proliferation of peripheral blood mononuclear cells in the presence of autologous plasma. The strength and duration of sensitization depends on the duration of treatment and on the concentration of the injected extract. The most potent *in vitro* activators are the vesicles. There are also synergistic interactions between lectins and vesicles [18]. These results confirm that mistletoe components other than lectin(s) stimulate T-cell proliferation *in vitro*.

Stimulation of γδ T cells by heat-treated mistletoe extracts

The mistletoe extracts which stimulate T lymphocytes from mistletoe-treated cancer patients did not induce any proliferative response in T lymphocytes from healthy

untreated donors (data not shown) [18]. However, we observed that heat-treated mistletoe extracts of two different host trees (apple and pine) induced proliferation of peripheral blood mononuclear cells from healthy donors in concentrations between 50 and 500 μg/ml, as measured by ^{3}H-thymidine uptake (Fig. 2), which was augmented in the presence of exogenous recombinand interleukin-2 [19]. The phenotypic analysis of cells after 8 days of culture with heat-treated mistletoe extract and recombinand interleukin-2 revealed a dramatic expansion of Vγ/Vδ2 γδ T cells (Fig. 3). Whereas 0.9% Vγ9+ and 1.9% Vγ9– γδ+ T cells were present before culture (Fig. 3a), 68.3% Vγ9+ γδ+ T cells were measured after culture (Fig. 3c), all of them co-expressing Vδ2 (Fig. 3d).

The majority of CD3+ mature T lymphocytes express an αβ T-cell receptor heterodimer. A small fraction (1–10%) of CD3+ T cells, however, express the alternative γδ T-cell receptor. The usage of available Vγ/δ elements among peripheral blood γδ T cells is not randomly distributed. Thus, the major fraction of human peripheral

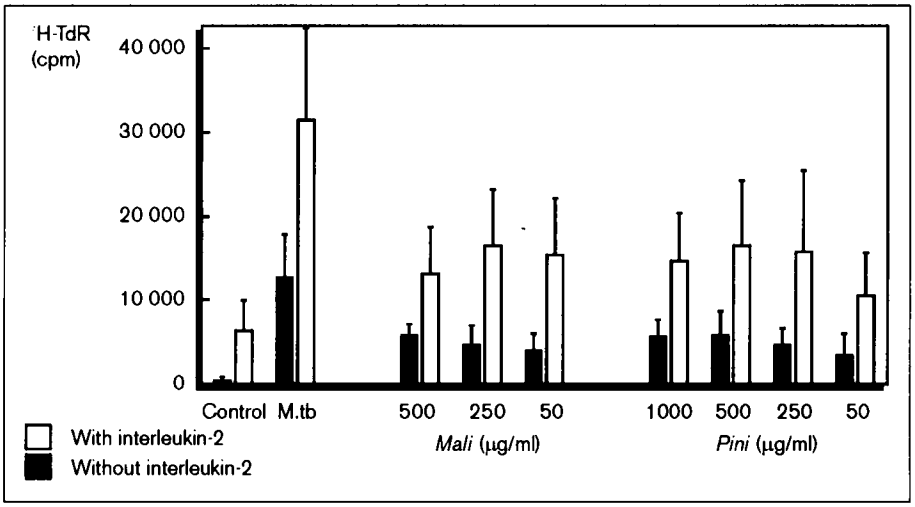

Figure 2. Induction of proliferation by heat-treated mistletoe extracts. Peripheral blood mononuclear cells from normal donors were stimulated with the indicated concentrations of heat-treated *mali* or *pini* extracts, or with 0.01% *Mycobacterium tuberculosis* (M.tb), in the absence or presence of 10 U/ml recombinand interleukin-2. ^{3}H-thymidine (^{3}H-TdR) uptake was measured after 7 days. Mean counts/min ± SD of 10 experiments are shown [19].

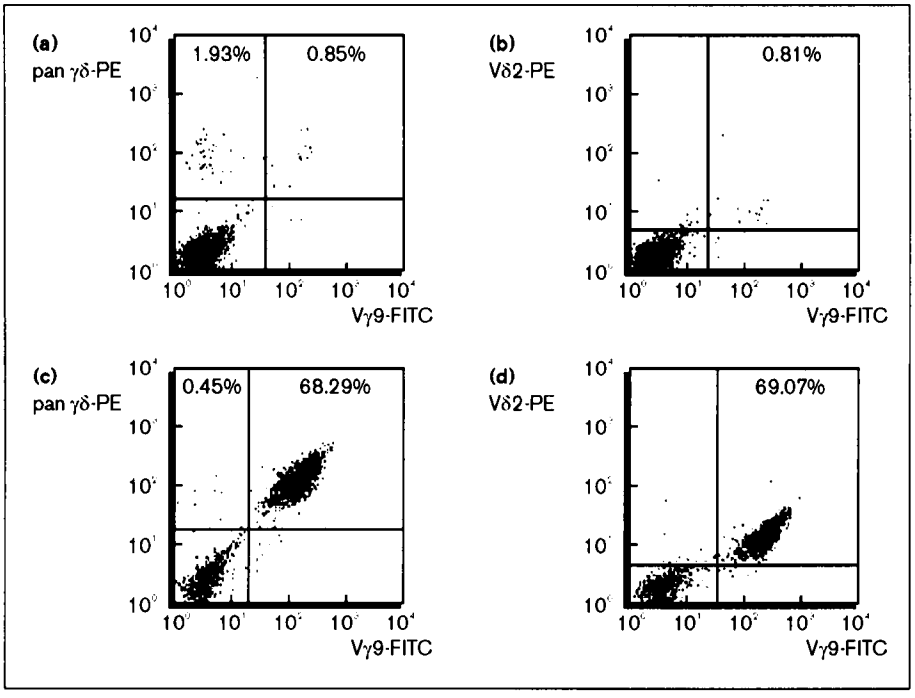

Figure 3. Phenotypic analysis of proliferating cells. Peripheral blood mononuclear cells were stained **(a,b)** before culture and **(c,d)** after 8 days of culture with 500 μg/ml heat-treated *mali* extract and 10 U/ml recomsinand interleukin-2. Two-colour fluorescence-activated cell sorter (FACS) analysis was performed after staining with monoclonal antibodies against Cγ, Vδ2, and Vγ9 as indicated [19]. FITC, fluorescein isothiocyanate.

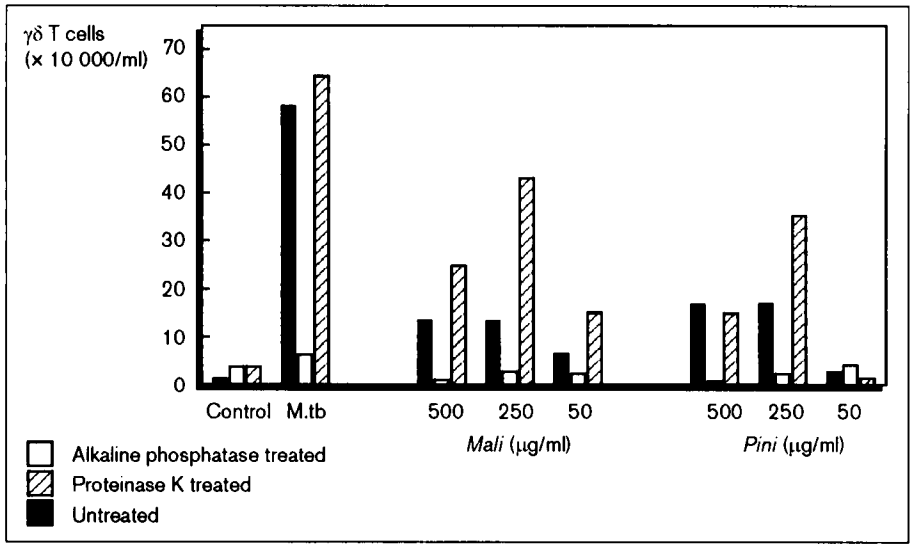

Figure 4. Sensitivity of heat-treated mistletoe extracts to enzyme treatment. Heat-treated *mali* or *pini* extracts were treated with alkaline phosphatase (50U) or proteinase K (three incubation circles with 25 μg). Peripheral blood mononuclear cells were stimulated with the various preparations in the presence of 10 U/ml recombinand interleukin-2. The cellular expansion of Vγ9 cells was determined by standard cell dilution assay after 8 days of culture [19].

blood γδ T-cells expresses Vγ9 paired with Vδ2 [20]. Vγ9/Vδ2+ γδ T-cells readily respond to certain micro-organisms, including *Mycobacterium tuberculosis* and some other gram-positive or gram-negative bacteria [21]. The γδ-stimulating ligands from *M. tuberculosis* have been defined as non-proteinaceous, low molecular weight compounds containing thymidine-phosphate conjugates [22–24]. A second group of γδ-stimulating ligands includes mycobacteria-derived, as well as synthetic, pyrophosphates [25].

The mycobacterial ligands stimulating Vγ9/Vδ2 T cells are sensitive to treatment with alkaline phosphatase but are resistant to treatment with proteinase K. As illustrated in Fig. 4, the γδ-stimulating ligands present in heat-treated mistletoe extracts share these features with mycobacterial ligands. Treatment with proteinase K did not destroy the γδ T-cell stimulation. Thus, the ligand is a protein, but it must be a phosphorylated molecule since treatment with alkaline phosphatase prevented the γδ T-cell stimulation of the heat-treated mistletoe extracts.

Heat-treatment apparently destroys the toxic lectin component of the mistletoe extracts. Investigations have to be performed on the effect of these activated γδ T cells. Activated γδ T cells also have a high cytotoxic activity against tumour cells [26–29]. Vδ1+ and Vδ2+ γδ T cells were found in tumour-infiltrating lymphocytes from different tumours [30]. The presence of γδ T cells in tumour-infiltrating lymphocytes suggest that they have a function in tumour defence. In SCID mice (mice without functional B and T lymphocytes), experiments have been performed with Daudi tumour cells and Vγ9+Vδ2+ T cells. Mice with human peripheral blood cells and Daudi tumour cells survived for a longer time than mice without human peripheral blood cells. Also the Vγ9+Vδ2+ γδ T cells expanded in these mice [31,32].

Conclusion

The mistletoe extract ABNOBAviscum did not induce proliferation of lymphocytes from untreated donors, whereas treated donors responded with an oligoclonal expansion of CD4+ T cells *in vitro*. The T cells were sensitized *in vivo*. The vesicles are a very potent stimulator of the specific immune system, namely the CD4+ T cells. Lymphocytes of untreated donors only react to heat-treated mistletoe extracts with a γδ T-cell expansion. The clinical relevance of these findings has to be investigated.

Acknowledgement

This work forms part of the Ph.D. thesis of S. Fischer.

References

1. Franz H: Compounds of mistletoe (*Viscum album* L.) as potential drugs [in German]. *Pharmazie* 1985, **40**: 97–104.
2. Franz H. Mistletoe lectins and their A and B chains. *Oncology* 1986, **43**: 23–34.
3. Jordan E, Wagner H: Structure and properties of polysaccharides from *Viscum album* L. *Oncology* 1986, **43**: 8–15.
4. Lee RT, Gabius HJ, Lee YC: The sugar-combining area of the galactoside-specific toxic lectin of mistletoe extends beyond the terminal sugar residue: comparison with a homologous toxic lectin, ricin. *Carbohydrate Res* 1994, **254**: 269–276.
5. Schrader G, Apel K: Isolation and characterization of cDNAs encoding viscotoxins of mistletoe (*Viscum album*). *Eur J Biochem* 1991, **198**: 549–553.
6. Sweenwaey EC, Palmer RA, Pfüller U: Crystallization of the ribosome inactivating protein ML1 from *Viscum album* (mistletoe) complexed with β-D-galactose. *J Mol Biol* 1993, **234**: 1279–1281.
7. Hamprecht K, Anderer FA: Autolytic generation of dialysable components in extracts of *Viscum album* exhibiting different

mechanisms of enhancement of human NK-cytotoxicity against tumor cells. *Int J Immunopharmacol* 1990, **3**: 63–73.
8. Zhu HG, Zollner TM, Klein-Franke A, Anderer FA: Enhancement of MHC-unrestricted cytotoxic activity of human CD56+CD3– natural killer (NK) cells and CD4+ T cells by rhamnogalacturonan: target cell specificity and activity against NK-insensitive targets. *Cancer Res Clin Oncol* 1994, **120**: 383–388.
9. Hajto T, Hostanska K, Frei K, Rordorf C, Gabius HJ: Increased secretion of tumor necrosis factors alpha, interleukin 1, and interleukin 6 by human mononuclear cells exposed to beta-galactoside-specific lectin from clinically applied mistletoe extract. *Cancer Res* 1990, **50**: 3322–3326.
10. Männel DN, Becker H, Gundt A, Kist A, Franz H: Induction of tumor necrosis factor expression by a lectin from *Viscum album*. *Cancer Immunol Immunother* 1991, **33**: 177–182.
11. Scheer R, Metelmann R, Errenst M, Scheffler A: Influence of the host tree on the content and the pattern of active principles of mistletoe preparations and consequences for therapy [abstract FC57]. *Eur J Pharm Sci* 1994, **2**: 115.
12. Scheer R: Standardized processes: basis for an appropriate safety trial of mistletoe drugs [in German]. *Erfahrungsheilkunde* 1994, **43**: 305–310.
13. Schultze JL, Stettin A, Berg PA: Demonstration of specifically sensitized lymphocytes in patients treated with an aqueous mistletoe extract (*Viscum album* L.). *Klin Wochenschr* 1991, **69**: 397–403.
14. Stein G, Berg PA: Non-lectin component in a fermented extract from *Viscum album* L. grown on pines induces proliferation of lymphocytes from healthy and allergic individuals *in vitro*. *Eur J Clin Pharmacol* 1994, **47**: 33–38.
15. Fischer S: *Stimulation of the Immune Defence with Mistletoe Compounds*. In Vitro *Experiments on T-Cell Reactivity* [in German]. Stuttgart: Hippokrates Verlag, 1996.
16. Fischer S, Scheffler A, Necker A, Kabelitz D: Oligoclonal *in vitro* response of CD4 T-cells fraction of mistletoe extracts in mistletoe-treated cancer patients. *Cancer Immunol Immunother* 1997, in press.
17. Pechhold K, Pohl T, Kabelitz D: Rapid quantification of lymphocyte subsets in heterogeneous cell populations by flow cytometry. *Cytometry* 1994, **16**: 152–159.
18. Scheffler A, Musielski H, Scheer R: Synergisms between lectins and vesicles of *Viscum album* L. [in German]. *Dtsch Ztschr Onkol* 1995, **27**: 72–75
19. Fischer S, Scheffler A, Kabelitz D: Activation of human γδ T-cells by heat-treated mistletoe plant extracts. *Immunol Lett* 1996, **52**: 69–72.
20. Kabelitz D: Function and specificity of human γδ-positive T cells. *Crit Rev Immunol* 1992, **11**: 281–303.
21. Kabelitz D, Bender A, Prospero T, Wesselborg S, Janssen O, Pechhold K: The primary response of human γδ T cells to *Mycobacterium tuberculosis* is restricted to Vγ9 bearing cells. *J Exp Med* 1991, **173**: 1331–1338.
22. Constant P, Davodeau F, Peyrat A, *et al.*: Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands. *Science* 1994, **264**: 267–270.
23. Constant P, Poquet Y, Peyrat MA, Davodeau F, Bonneville M, Fournie JJ: The antituberculous mycobacterium bovis BCG vaccine is an attenuated mycobacterial producer of phosphorylated nonpeptidic antigens for human gamma delta T cells. *Infect Immun* 1995, **63**: 4628–4633.
24. Schoel B, Sprengler S, Kaufmann SHE: Phosphate is essential for stimulation of Vγ9Vδ2 T lymphocytes by mycobacterial low molecular weight ligand. *Eur J Immunol* 1994, **24**: 1886–1892.
25. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR: Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* 1995, **375**: 155–158.
26. DiFabrizio L, Kimura Y, Ware R, Rogozinski L, Chess L: Specific triggering of γδ T-cells by K562 activates the γδ T-cell receptor and may regulate natural killer-like function. *J Immunol* 1991, **146**: 2495–2503.
27. Zocchi MR, Ferarrini M, Rufarli C: Selective lysis of the autologous tumor by dTCS1+ γδ tumor-infiltrating lymphocytes from human lung carcinomas. *Eur J Immunol* 1990, **20**: 2685–2689.
28. Bensussan A, Lagabrielle JF, Degos L: TCR γδ bearing lymphocyte clones with lymphokine-activated killer activity against autologous leukemic cells. *Blood* 1989, **73**: 2077–2080.
29. Ensslin AS, Formby B: Comparison of cytolytic and proliferative activities of human γδ and αβ T cells from peripheral blood against various human tumor cell lines. *J Natl Cancer Inst* 1991, **83**: 1564–1569.
30. Kabelitz D: Role of γδ T cells in the immune response against tumor cells. *Cancer J* 1995, **8**: 190–194.
31. Malkovska V, Cigel F, Storer BE, Hong R: Antilymphoma activity of human γδ T cells in mice with severe combined immune deficiency. *Cancer Res* 1992, **52**: 5610–5616.
32. Malkovska V, Cigel F, Storer BE: Human T cells in hu-PBL-SCID mice proliferate in response to Daudi lymphoma and confer antitumor immunity. *Clin Exp Immunol* 1994; **12**: 158–165.