

## $\gamma\delta$ T cells for immune therapy of patients with lymphoid malignancies

Martin Wilhelm, Volker Kunzmann, Susanne Eckstein, Peter Reimer, Florian Weissinger, Thomas Ruediger, and Hans-Peter Tony

There is increasing evidence that  $\gamma\delta$  T cells have potent innate antitumor activity. We described previously that synthetic aminobisphosphonates are potent  $\gamma\delta$  T cell stimulatory compounds that induce cytokine secretion (ie, interferon  $\gamma$  [IFN- $\gamma$ ]) and cell-mediated cytotoxicity against lymphoma and myeloma cell lines in vitro. To evaluate the antitumor activity of  $\gamma\delta$  T cells in vivo, we initiated a pilot study of low-dose interleukin 2 (IL-2) in combination with pamidronate in 19 patients with relapsed/refractory low-grade non-Hodgkin lymphoma (NHL) or multiple myeloma (MM). The objectives of this trial were to determine toxicity, the most effective dose for in vivo activation/proliferation of  $\gamma\delta$  T cells, and antilym-

phoma efficacy of the combination of pamidronate and IL-2. The first 10 patients (cohort A) who entered the study received 90 mg pamidronate intravenously on day 1 followed by increasing dose levels of continuous 24-hour intravenous (IV) infusions of IL-2 (0.25 to  $3 \times 10^6$  IU/m<sup>2</sup>) from day 3 to day 8. Even at the highest IL-2 dose level in vivo,  $\gamma\delta$  T-cell activation/proliferation and response to treatment were disappointing with only 1 patient achieving stable disease. Therefore, the next 9 patients were selected by positive in vitro proliferation of  $\gamma\delta$  T cells in response to pamidronate/IL-2 and received a modified treatment schedule (6-hour bolus IV IL-2 infusions from day 1-6). In this patient group (cohort B), signifi-

cant in vivo activation/proliferation of  $\gamma\delta$  T cells was observed in 5 patients (55%), and objective responses (PR) were achieved in 3 patients (33%). Only patients with significant in vivo proliferation of  $\gamma\delta$  T cells responded to treatment, indicating that  $\gamma\delta$  T cells might contribute to this antilymphoma effect. Overall, administration of pamidronate and low-dose IL-2 was well tolerated. In conclusion, this clinical trial demonstrates, for the first time, that  $\gamma\delta$  T-cell-mediated immunotherapy is feasible and can induce objective tumor responses. (Blood. 2003;102:200-206)

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### Introduction

Despite significant improvement in the treatment of low-grade non-Hodgkin lymphoma (NHL) and multiple myeloma (MM), most patients relapse or become resistant to conventional treatment strategies such as chemotherapy or radiation. Therefore, there is need for alternative tumor therapies. One possibility is manipulating the immune system to target and eliminate neoplastic cells.

Most current immunotherapeutic approaches aim at inducing antitumor response via stimulation of the adaptive immune system, which is dependent on major histocompatibility complex (MHC)-restricted  $\alpha\beta$  T cells. Despite major advances in our understanding of the adaptive immunity toward tumors and the introduction of vaccine-based strategies, durable responses are rare, and active immunotherapy is still not an established treatment modality. Adaptive immunotherapeutic approaches have several disadvantages:  $\alpha\beta$  T cells need specific tumor-associated antigens (TAAs) and appropriate costimulatory molecules for activation. Failure or loss of TAAs, MHC molecules, and/or costimulatory molecules renders tumor cells resistant to  $\alpha\beta$  T-cell-mediated cytotoxicity or induces anergy of specific T cells.<sup>1</sup>

Mice deficient in innate effector cells such as natural killer (NK) cells, NK T cells, or  $\gamma\delta$  T cells show a significantly increased incidence of tumors and provide clear evidence for an immune

surveillance function of the innate immune system.<sup>2-4</sup> Recognition of transformed cells by the innate immune system seems to be dependent on expression of stress-induced ligands and/or loss of MHC class I molecules on tumor cells.<sup>5</sup> Several studies have demonstrated a role for human  $\gamma\delta$  T cells in recognition of transformed cells.<sup>6,7</sup>  $\gamma\delta$  T cells exhibit a potent MHC-unrestricted lytic activity against different tumor cells in vitro.<sup>8-10</sup> In addition,  $\gamma\delta$  T cells have been found with increased frequency in disease-free survivors of acute leukemia following allogeneic bone marrow transplantation.<sup>11</sup> Adoptive transfer of ex vivo-expanded human  $\gamma\delta$  T cells in a mouse tumor model further supports the in vivo antitumor effects of  $\gamma\delta$  T cells.<sup>12</sup>

V $\gamma$ 9V $\delta$ 2 T cells, which represent most of the human circulating  $\gamma\delta$  T cells, recognize small nonpeptide compounds with an essential phosphate residue (ie, microbial metabolites) or alkylamines.<sup>13-17</sup> As we have shown previously, also synthetic aminobisphosphonates such as pamidronate are potent  $\gamma\delta$  T-cell-stimulatory compounds.<sup>18</sup> In addition, we could demonstrate that pamidronate-activated  $\gamma\delta$  T cells produce cytokines (ie, interferon  $\gamma$  [IFN- $\gamma$ ]), exhibit specific cytotoxicity against lymphoma or myeloma cell lines, and lead to reduced survival of autologous myeloma cells.<sup>8</sup> It was confirmed that V $\gamma$ 9V $\delta$ 2 T cells recognize

From the Medizinische Poliklinik Wuerzburg, University of Wuerzburg, Germany; Lehrstuhl fuer Lebensmittelchemie, University of Wuerzburg, Germany; and Institute of Pathology, University of Wuerzburg, Germany.

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M.W. and V.K. contributed equally to this work.

**Reprints:** Volker Kunzmann, Medizinische Poliklinik Wuerzburg, Julius-Maximilians University Wuerzburg, Klinikstraße 6-8, 97070 Wuerzburg, Germany; e-mail: kunzmann\_v@medizin.uni-wuerzburg.de.

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and kill a broad spectrum of B-cell lymphomas *in vitro*.<sup>9</sup> Furthermore, pamidronate enhances recognition of many other tumor cell lines by  $\gamma\delta$  T lymphocytes.<sup>10</sup> For an immunotherapeutic application it is important that  $\gamma\delta$  T cells have the potential for polyclonal expansion without prior priming.<sup>19</sup> An immunotherapeutic approach of inducing antitumor response via stimulation of  $\gamma\delta$  T cells *in vivo* has not been performed in humans so far.

The aim of this pilot study is to evaluate the feasibility of activation and/or expansion of  $\gamma\delta$  T cells *in vivo* using the combination of pamidronate and interleukin 2 (IL-2) in patients with refractory/relapsed lymphoma or myeloma, to determine the most effective IL-2 dose, to assess the toxicity of this regimen, and to evaluate its ability to exert antitumor effects.

## Patients, materials, and methods

### Patients

Adults suffering from low-grade B-cell lymphoma (NHL) or MM, refractory or relapsing after salvage therapy, were entered on the protocol. All patients signed an informed consent according to guidelines of the local ethics committee. Eligibility criteria required (1) an Eastern Cooperative Oncology Group (ECOG) score of less than 3 and (2) no severe impairment of cardiac, renal, or hepatic function. Pretreatment evaluation and follow-up studies included a history and physical examination, complete blood counts, extensive immunologic monitoring by fluorescence-activated cell sorter (FACS) analysis of peripheral blood lymphocytes, computed tomography (CT) scans, ultrasound, positron emission tomography (PET) scans, or bone marrow biopsy whichever was appropriate.

### Response criteria

Complete remission required disappearance of all lymphoma manifestations for at least 4 weeks. Partial remission was defined as 50% reduction or more of all measurable lymphoma manifestations for at least 4 weeks. In addition, no single manifestation should have shown enlargement of 25% or more in size, and no new lesions should have appeared during that period. Stable disease meant less than 50% reduction or no measurable change in lymphoma manifestations was present. Progressive disease (PD) was defined as increase of frequency and severity of disease-associated symptoms, occurrence of new nodal or extranodal lesions, increase of preexisting lymphoma manifestations by more than 25%, or any combination. Adverse events were assessed according to World Health Organization (WHO) criteria.

### $\gamma\delta$ T-cell proliferation assay

$\gamma\delta$  T-cell proliferation assay was described previously.<sup>8</sup> Briefly,  $5 \times 10^4$  peripheral blood mononuclear cells (PBMCs) were cultivated in triplicate in 100  $\mu$ L RPMI 1640 medium per well (Invitrogen, Karlsruhe, Germany), 10% pooled human AB serum, and 100 IU/mL IL-2 in round-bottom microtiter plates (Nunc, Wiesbaden, Germany). For determination of  $\gamma\delta$  T-cell activity, pamidronate (Novartis, Nuernberg, Germany) was added in concentrations between 1  $\mu$ M and 100  $\mu$ M on day 0. Cells were harvested on day 7 and were double or triple stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal CD69, HLA-DR, CD3, CD56, TCR pan  $\alpha\beta$ , TCR pan  $\gamma\delta$ , TCR V $\gamma$ 9, TCR V $\delta$ 2 (Coulter-Immunotech, Krefeld, Germany), or TCR V $\delta$ 1 (T Cell Diagnostics, Woburn, MA) antibodies. For NK cells (CD3<sup>-</sup>CD56<sup>+</sup>) triple staining was performed using allophycocyanin (APC)-labeled CD3 antibodies. Cells ( $5 \times 10^3$ ) from each sample were analyzed using a FACScan supported with Cellquest as acquisition and data analysis software (Becton Dickinson, Heidelberg, Germany). The lymphocytes were gated using forward/side scatter analysis.

Increase of  $\gamma\delta$  T cells was calculated by counting the number of viable cells per well and by cytofluorimetric identification of  $\gamma\delta$  T cells using FACS analysis on day 7 of culture. The stimulation index was determined

according to the following calculation: ( $\gamma\delta$  T-cell number in pamidronate/IL-2 culture) – ( $\gamma\delta$  T-cell number in medium/IL-2)/( $\gamma\delta$  T-cell number in medium/IL-2)  $\times$  100. A stimulation index greater than 2 was considered a significant increase of  $\gamma\delta$  T cells. More than 5% of  $\gamma\delta$  T cells in PBMC culture on day 7 were required to avoid nonproportional increases at lower numbers.

### FACS analysis of peripheral blood

Blood samples of patients were collected before each treatment course and on days 2 or 3, and 7 after start of treatment. PBMCs were stained with FITC-, PE-, or APC-conjugated antibodies and analyzed by flow cytometry as described earlier. For analysis of activation markers, CD69 was determined on day 2 or 3, HLA-DR on day 7 after infusion. Results represent percentage increase of antigen-expressing cells analyzed by double or triple staining (NK cells) according to the following calculation: (antigen expressing cells after pamidronate/IL-2 infusion) – (antigen-expressing cells before treatment)/(antigen-expressing cells before treatment)  $\times$  100. For a significant increase, more than 5% antigen-expressing cells were required to avoid unspecific staining and nonproportional increases at lower numbers.

For analysis of lymphocyte subset ( $\alpha\beta$  T cell, NK cell,  $\gamma\delta$  T cell) proliferation, absolute numbers of each lymphocyte subset before and on day 7 after treatment with pamidronate/IL-2 were counted. Results were shown as increase in percentage (%) according to the following calculation: (lymphocyte subset number on day 7 after pamidronate/IL-2 infusion) – (lymphocyte subset number before treatment)/(lymphocyte subset number before treatment)  $\times$  100. For significant increase, more than 1% (and  $> 10/\mu$ L) of cells of a lymphocyte subset on day 7 were required to avoid unspecific staining and nonproportional increases at lower numbers.

### Cytokine assays

IFN- $\gamma$ , tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and IL-4 concentrations in serum were determined, using commercial enzyme-linked immunosorbent assay (ELISA) systems from Pharmingen (San Diego, CA). Sera were collected before and on day 1 or 2 after therapy and stored at  $-80^\circ\text{C}$  until analysis.

### Statistical analysis

For statistical comparison Fisher exact test was performed.  $P < .05$  was considered statistically significant.

## Results

Nineteen patients, with a median age of 61 years (range, 36-83 years), were enrolled in this pilot study (Table 1). Eleven patients (58%) had a diagnosis of low-grade B-NHL (4 follicle center lymphoma [FCL], 4 chronic lymphocytic leukemia [CLL], 2 mantle zone lymphoma [MZL], 1 immunocytoma [IC]), and 8 patients had a diagnosis of MM (42%). The majority of patients had advanced disease and received pamidronate/IL-2 as their second or subsequent salvage therapy (74%). Only patients with progressive disease were eligible for inclusion in the study.

### Treatment schedule

The treatment schedule was adopted from our *in vitro* experience, where up to 50-fold expansion of  $\gamma\delta$  T cells was achieved in the presence of pamidronate and IL-2. The consequences of a selective activation of  $\gamma\delta$  T cells *in vivo* were not known at the beginning of the study, and pamidronate alone could have induced a cytokine-mediated acute phase reaction. Therefore, the first 10 patients (cohort A, Table 1) received increasing dose levels of continuous 24-hour IV infusions of low-dose IL-2 ( $0.25\text{-}3 \times 10^6$  IU/m<sup>2</sup>) from

**Table 1. Patient characteristics and response to therapy**

Patient	Age, y/sex	Diagnosis/ stage*	Prior therapy (no. of cycles)†	Off therapy, mo‡	IL-2 dose level (no. of cycles)	Side effects§	γδ T-cell Proliferation		Response¶
							In vitro	In vivo	
Cohort A									
1A	83/M	MM/III	MP (15)	2	0.25-0.5 × 10 <sup>6</sup> IU/m <sup>2</sup> (2)	–	–	–	PD
2A	79/M	CLL/IV	CLB (10)	11	0.25-3 × 10 <sup>6</sup> IU/m <sup>2</sup> (6)	F(1), T(3)	+ #	–	SD
3A	67/F	CLL/IV	PM (10), CLB (5)	2	0.25-0.5 × 10 <sup>6</sup> IU/m <sup>2</sup> (2)	I(1), F(1), T(2)	–	–	PD
4A	57/M	IC/IV	CLB/P (24), LR (30 Gy)	14	0.5 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	T(2)	–	–	PD
5A	76/F	MM/III	MP (9)	10	0.5 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	I(2), F(2), T(2)	–	ND	NE
6A	63/F	CLL/IV	CLB/P (25), MCP (6), F (12), R (4)	5	0.5 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	F(2), I(2)	ND	–	PD
7A	68/M	CLL/IV	CLB/P (4), COP (6), CLB (12)	2	1-2 × 10 <sup>6</sup> IU/m <sup>2</sup> (2)	I(2), F(2), T(3)	ND	–	PD
8A	66/M	MM/III	MP (15), VID (6)	2	1 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	–	ND	–	PD
9A	58/M	MZL/III	MCP (6), TBI/CY + PBSCT (1)	29	1 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	F(2), T(2)	ND	–	PD
10A	79/F	MM/III	CHOP (2), LR (50 Gy)	10	2-3 × 10 <sup>6</sup> IU/m <sup>2</sup> (2)	F(1), T(2)	ND	–	PD
Cohort B									
1B	73/M	MM/III	MP (2), VID (6)	3	0.25 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	–	+	–	PD
2B	59/M	MM/III	MP (16), VID (6), HD-M + PBSCT (1), IFN	2	0.25 × 10 <sup>6</sup> IU/m <sup>2</sup> (2)	T(2)	+	–	PD
3B	52/M	FCL/IV	MCP (4), CHOP (2), Dexa-BEAM (2), IFN, LR (44 Gy), I <sup>131</sup> -R	4	0.25 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	F(1), T(2)	++	–	PD
4B	43/F	FCL/III	TNI (49 Gy)	24	0.5-1 × 10 <sup>6</sup> IU/m <sup>2</sup> (4)	F(2), T(2)	+++	+++	SD
5B	57/M	MM/II	VID (4)	2	0.5-2 × 10 <sup>6</sup> IU/m <sup>2</sup> (9)	F(1), T(2)	+++	+	PR
6B	36/F	MM/II	LR (30 Gy)	3	0.5 × 10 <sup>6</sup> IU/m <sup>2</sup> (1)	F(1), S(1)	+	++	PD
7B	46/M	MZL/IV	TNI (44 Gy)	29	1 × 10 <sup>6</sup> IU/m <sup>2</sup> (4)	F(2), T(2)	+++	–	SD
8B	51/F	FCL/IV	COP (10), LR (30 Gy), MCP (4)	48	2 × 10 <sup>6</sup> IU/m <sup>2</sup> (4)	F(1), T(2), S(2)	+++	+	PR
9B	55/M	FCL/III	CHOP (4), Dexa-BEAM (2), TBI/CY + PBSCT (1), LR (30 Gy)	6	1-2 × 10 <sup>6</sup> IU/m <sup>2</sup> (8)	F(1)	+	+	PR

\*MM indicates multiple myeloma; CLL, chronic lymphocytic leukemia; IC, immunocytoma; MZL, mantle zone lymphoma. Staging according to Durie and Salmon (MM), Rai (CLL), Ann-Arbor (IC; MZL).

†MP indicates melphalan/prednisone; CLB, chlorambucil; PM, prednimustine; COP, cyclophosphamide/vincristine/prednisone; VID, vincristine/idarubicin/dexamethasone; MCP, mitoxantrone/chlorambucil/prednisone; CHOP, cyclophosphamide/vincristine/prednisone; F, fludarabine; R, rituximab; TBI/CY, total body irradiation/high-dose cyclophosphamide followed by peripheral blood stem cell transplantation (PBSCT); LR, local radiotherapy (dose); I<sup>131</sup>-R, radioimmunotherapy with iodine<sup>131</sup>-rituximab; Dexa-BEAM, dexamethasone/carmustine/etoposide/cytarabine/melphalan; HD-M, high-dose melphalan followed by PBSCT; TNI, total nodal irradiation (dose); and IFN, INF-α (maintenance therapy).

‡Months between last chemotherapy/radiotherapy and first pamidronate/IL-2 treatment.

§T indicates thrombophlebitis; F, fever; S, skin-erythema; I, infection; (2) WHO grade 2.

||In vitro results represent percentages of control culture according to the following calculation: (γδ T-cell number in pamidronate/IL-2 culture) – (γδ T-cell number in medium/IL-2)/(γδ T-cell number in medium/IL-2) × 100. In vivo results represent percentage of increase according to the following calculation: (V<sub>y9δ2</sub> T-cell number on day 8 after pamidronate/IL-2 infusion) – (V<sub>y9δ2</sub> T-cell number before treatment)/(V<sub>y9δ2</sub> T-cell number before treatment) × 100. – Indicates <20%; +, 20% to 100%; ++, >100% to 200%; + + +, >200% increase of γδ T-cell number; ND, not done.

¶SD indicates stable disease; PR, partial remission; PD, progressive disease; NE, not evaluable.

#No absolute counts are available; however, percentage of γδ T cells increased from 7% to 19.5%.

day 3 to day 8 after an initial pamidronate infusion on day 1 (90 mg/3 h). The subsequent 9 patients (cohort B, Table 1) received IL-2 from day 1 to day 6 directly after the pamidronate infusion (90 mg/3 h) in the form of increasing dose levels (0.25-2 × 10<sup>6</sup> IU/m<sup>2</sup>) of a 6-hour IV bolus infusion.

A minimum of 3 patients were included at each dose level and observed for at least 3 weeks prior to starting additional patients at an increased dose. Dose escalations in subsequent patients were in 100% increments until toxicity greater than grade 2 based on the National Cancer Institute (NCI) criteria was reached. Patients who showed no toxicity greater than grade 2 were allowed to continue receiving the next dose level. Maximal tolerated dose (MTD) was defined as the dose that caused grade 3 toxicity in 2 of the first 3 to 6 patients at a particular dose level. Maximal effective dose was defined as the dose that was able to induce significant γδ T-cell proliferation and/or activation without significant concomitant αβ T-cell or NK-cell stimulation.

## Toxicity

Both treatment schedules of pamidronate/IL-2 were generally well tolerated. Fourteen patients (74%) developed low-grade fever and/or chills (grade 1-2) during IL-2 therapy, which peaked on day 2 and 3 in cohort A and on day 3 and 4 in cohort B, respectively. These side effects were transient and easily controlled by oral paracetamol. Thirteen patients (68%) developed postinfusional thrombophlebitis (grade 2). Two patients (10%) developed a local erythema (grade 1-2) at the infusion site. Three patients (16%) experienced mild infections (grade 1-2) that were not considered to be related to the study medication. Only 2 patients (10%) experienced grade 3 toxicity (10%): a catheter-associated jugular vein thrombosis and a recurrent femoral vein thrombosis that occurred at an IL-2 dose level of 2 × 10<sup>6</sup> IU/m<sup>2</sup> and 3 × 10<sup>6</sup> IU/m<sup>2</sup>, respectively. Because only one toxicity greater than grade 2 was observed on each particular IL-2 dose level, IL-2 was escalated

**Table 2. In vitro proliferation of  $\gamma\delta$  T cells in response to pamidronate/IL-2**

	Healthy donors (%)	All patients (%)	MM (%)	NHL (%)	B-CLL (%)
Positive	14/16 (88)	20/41 (49)	12/26 (46)	8/10 (80)	1/5 (20)
Negative	2/16 (12)	21/41 (51)	14/26 (54)	2/10 (20)	4/5 (80)

up to  $3 \times 10^6$  IU/m<sup>2</sup> per day. No dose-limiting toxicity for the combination of pamidronate and IL-2 could be defined in this study.

**Activation and proliferation of  $\gamma\delta$  T cells**

None of the first 10 patients (cohort A, patients 1A-10A) showed a measurable  $\gamma\delta$  T-cell response during pamidronate/IL-2 treatment in vivo, even at the highest IL-2 dose level (Table 1). Although in vitro proliferation of  $\gamma\delta$  T cells in response to pamidronate/IL-2 was not regularly examined in this patient group, the majority of tested patients (4 of 5) had negative in vitro proliferation assays (Table 1). Therefore, in vitro testing of  $\gamma\delta$  T cells in response to pamidronate/IL-2 was performed for all further eligible patients, and only patients with significant in vitro proliferation qualified for study entry. Compared with an age-matched group of healthy donors in which 88% exhibited an in vitro response to pamidronate/IL-2, only 49% of patients with lymphoid malignancies (n = 41) showed significant in vitro  $\gamma\delta$  T-cell proliferation (Table 2). In accordance with the data observed in vivo, the proportion of patients with B-CLL showing  $\gamma\delta$  T-cell proliferation to pamidronate/IL-2 in vitro was quite low. Thus, the underlying disease (eg, B-CLL) seems to have an effect on  $\gamma\delta$  T-cell reactivity. Furthermore, in vitro testing revealed that addition of IL-2 on day 1 instead of day 3 significantly increased proliferation of  $\gamma\delta$  T cells in response to pamidronate (data not shown).

On the basis of these results, we changed the treatment schedule (IL-2 start on day 1), and only patients with significant in vitro proliferation of  $\gamma\delta$  T cells to pamidronate/IL-2 were defined to be eligible. In this patient group (cohort B, patients 1B-9B) significant in vivo proliferation of  $\gamma\delta$  T cells could be achieved in 5 of 9 patients (55%) (Table 1). The pronounced effect of the pamidronate/IL-2 combination on  $\gamma\delta$  T lymphocytes in vivo becomes even more evident when expression of activation markers was analyzed (Table 3). In contrast to  $\alpha\beta$  T cells and NK cells, we found a significant increase of CD69 and/or HLA-DR activation antigens on  $\gamma\delta$  T cells. Concomitant expression of early (CD69) and late (HLA-DR) activation markers demonstrates a more specific stimulation. Both antigens were up-regulated on  $\gamma\delta$  T cells in a dose-dependent manner, whereas on  $\alpha\beta$  T cells and NK cells this effect was achieved only at the highest dose level of IL-2 in a significant proportion of patients (Table 3).

In addition, significant expansion of V $\gamma$ 9 $\delta$ 2 T cells, which are the target population of pamidronate stimulation, was observed in vivo (Table 4). Although at the first IL-2 dose level there was no

significant proliferation of V $\gamma$ 9 $\delta$ 2 T cells, absolute numbers of this  $\gamma\delta$  T-cell subset increased at a dose level of  $0.5 \times 10^6$  IU/m<sup>2</sup> IL-2 and reached a maximum increase of 128% compared with before treatment. However, at the highest IL-2 dose level, absolute increase of V $\gamma$ 9 $\delta$ 2 T cells was less pronounced, which might be a secondary effect because of activation of bystander cells. We also found a similar dose-response curve in vitro with an inferior capacity of  $\gamma\delta$  T cells to proliferate at higher IL-2 concentrations. The absolute numbers of V $\gamma$ 9V $\delta$ 2 T cells in patients who showed positive in vivo proliferation after the first cycle of pamidronate/IL-2 continued to increase after subsequent cycles (data not shown).

A remarkable in vivo response from patient 4B is depicted in Figure 1: after infusion of pamidronate/IL-2,  $\gamma\delta$  T cells disappeared from peripheral blood presumably because of activation-induced transmigration through the endothelial layer and reappeared several days later in a highly activated status and increased in number. Simultaneously, the patient developed fever and showed significant increase of serum IFN- $\gamma$ . Similar results were observed in other patients from cohort B who showed significant activation and proliferation of  $\gamma\delta$  T cells in vivo. Measurement of Th1 and Th2 cytokines revealed that IFN- $\gamma$  concentrations (but not IL-4 or TNF- $\alpha$  levels) were significantly increased in the serum of 7 of 9 patients of cohort B (data not shown).

**Clinical response to therapy**

None of the 9 analyzable patients of cohort A (patients 1A-10A) achieved an objective tumor response (Table 1). In one patient (patient 2A) stable disease was observed which lasted 6 months.

In contrast, 3 of 9 patients in cohort B (patients 5B, 8B, 9B) achieved a partial remission (PR), giving an objective response rate of 33% (Table 1). Two additional patients achieved stable disease, still ongoing after 7 months (patient 7B) and lasting 13 months in another patient (patient 4B). Responding patients received IL-2 at a dose level of  $1 \times 10^6$  IU/m<sup>2</sup> to  $2 \times 10^6$  IU/m<sup>2</sup>. The response profile of these 5 patients revealed that the time interval from start of therapy until maximum response was quite long, ranging from 4 months to 23 months (Figure 2).

A more detailed description of the patients achieving an objective tumor response gives an impression of how  $\gamma\delta$  T-cell-mediated immunotherapy might operate: patient 9B with a follicle center lymphoma had relapsed despite high-dose chemotherapy. After 3 cycles of pamidronate/IL-2, CT scan revealed insignificant

**Table 3. Expression of activation antigens after pamidronate/IL-2 infusion of patients from cohort B**

Dose level, IL-2 IU/m <sup>2</sup>	Patient, n	$\alpha\beta$ T cells			NK cells			$\gamma\delta$ T cells		
		CD69, % mean (range)	HLA-DR, % mean (range)	CD69 <sup>+</sup> HLA-DR, n/patients (%)	CD69, % mean (range)	HLA-DR, % mean (range)	CD69 <sup>+</sup> HLA-DR, n/patients (%)	CD69, % mean (range)	HLA-DR, % mean (range)	CD69 <sup>+</sup> HLA-DR, n/patients (%)
$0.25 \times 10^6$	3	3 (-56-71)	10 (0-15)	0/3 (0)	24 (-17-43)	0 (0-123)	0/3 (0)	0 (-23-101)	163 (-7-375)	1/3 (33)
$0.5 \times 10^6$	3	33 (-23-95)	37 (27-86)	1/3 (33)	0 (-26-81)	0 (0-67)	0/3 (0)	53 (38-167)	400 (0-831)	2/3 (66)
$1 \times 10^6$	4	62 (3-153)	45 (0-122)	0/4 (0)	23 (18-27)	8 (-38-54)	0/2 (0)	183 (82-426)	322 (0-1253)	3/4 (75)
$2 \times 10^6$	3	65 (52-98)	60 (19-121)	2/3 (66)	81 (-37-103)	93 (79-291)	2/3 (66)	186 (62-230)	124 (122-571)	3/3 (100)

Results represent means of increase of the percentages of positive cells analyzed by double or triple staining (NK cells). CD69 was determined on day 2 or 3 and HLA-DR on day 7 after infusion. For analysis of patients expressing CD69 and HLA-DR, a threshold of 50% positive cells on day 8 was defined.

**Table 4. Change of cell number of lymphocyte subpopulations after pamidronate/IL-2 infusion of patients from cohort B**

Dose level, IL-2 IU/m <sup>2</sup>	Patients, n	Increase $\alpha\beta$ T cells, % mean (range)	Increase NK cells, % mean (range)	Increase $\gamma\delta$ T cells, % mean (range)	
				V $\delta$ 1 (range)	V $\gamma$ 9 $\delta$ 2 (range)
0.25 $\times$ 10 <sup>6</sup>	3	47 (-3-82)	44 (42-152)	64 (0-114)	0 (0)
0.5 $\times$ 10 <sup>6</sup>	3	19 (11-23)	122 (19-136)	0 (-3-0)	128 (24-257)
1 $\times$ 10 <sup>6</sup>	4	35 (7-70)	30 (0-98)	0 (0-36)	89 (-15-210)
2 $\times$ 10 <sup>6</sup>	3	12 (-5-14)	37 (26-71)	0 (0-8)	57 (21-95)

Results represent means of increase in absolute cell numbers on day 7.

changes of most lymph nodes, except one that had increased in size. Biopsy of this lymph node revealed a predominant fibrosis with only small numbers of lymphoma cells left. Thereafter, tumor mass steadily declined and, after 19 months of treatment, a PR has been reached (Figure 3).

Patient 8B had relapsed with a follicle center lymphoma and developed multiple subcutaneous manifestations in addition to multiple lymph node involvement. After the start of pamidronate/IL-2 treatment, the subcutaneous nodules rapidly disappeared (Figure 3), whereas nodal manifestations regressed slowly. Two months later, treatment was stopped because of noncompliance of the patient.

Patient 5B suffered from an IgA $\kappa$  (immunoglobulin A $\kappa$ ) multiple myeloma (stage IIA) refractory to conventional chemotherapy. After 9 cycles of immunotherapy the patient's bone marrow was cleared from tumor cells and IgA level decreased from 3400 mg/dL to 1740 mg/dL.

The *in vivo* expansion of  $\gamma\delta$  T cells of all analyzable patients from cohorts A and B correlated with the response to therapy, confirming a  $\gamma\delta$  T-cell-mediated effect. It seems that expansion of  $\gamma\delta$  T cells *in vivo* is a necessary prerequisite for tumor regression (3 of 5 patients responded;  $P = .015$ ; Table 5). Because none of the patients without  $\gamma\delta$  T-cell stimulation achieved an objective tumor response, the negative predictive value was 100%.

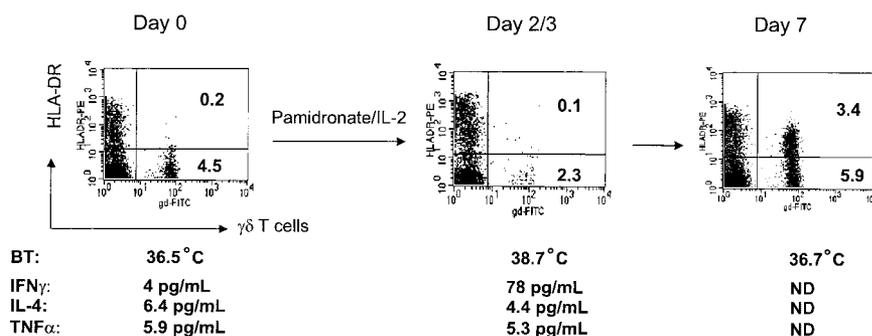
## Discussion

There has been no study published so far on *in vivo* stimulation of  $\gamma\delta$  T cells in humans, and the consequences of a selective activation of  $\gamma\delta$  T cells *in vivo* were not known. Therefore, evaluation of toxicity was one major end point of this study. We started with a low IL-2 dose of 0.25  $\times$  10<sup>6</sup> IU IL-2/m<sup>2</sup> and subsequently increased the IL-2 dose to 3  $\times$  10<sup>6</sup> IU IL-2/m<sup>2</sup> in cohort A and to 2  $\times$  10<sup>6</sup> IU IL-2/m<sup>2</sup> in cohort B. Overall, the combination of pamidronate and IL-2 was well tolerated, and no dose-limiting toxicity was observed. Most of the patients developed self-limiting fever and thrombophlebitis at the infusion site. Local thrombophlebitis has been described as a rare side effect in

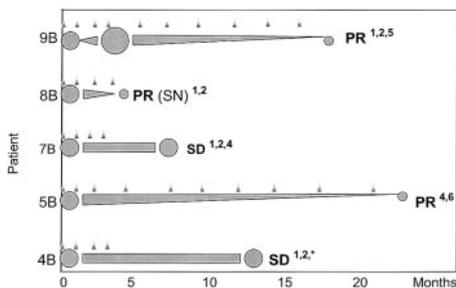
patients receiving pamidronate alone.<sup>20,21</sup> The high frequency of local thrombophlebitis in patients receiving pamidronate in combination with IL-2 might reflect immune-mediated effects on endothelial cells. It has also been recently shown that aminobisphosphonates have dose-dependent effects on proliferation-inhibition and apoptosis-induction of human endothelial cells *in vitro*.<sup>22</sup>

Next we asked whether the combination of pamidronate and IL-2 induces activation and proliferation of  $\gamma\delta$  T cells *in vivo*. None of the first 10 patients included in this pilot study (cohort A, Table 1) developed a measurable  $\gamma\delta$  T-cell response *in vivo*. The inability to induce  $\gamma\delta$  T-cell proliferative response *in vivo* correlated with the negative *in vitro* proliferation of  $\gamma\delta$  T cells in response to pamidronate/IL-2 in 4 of 5 analyzable patients. Therefore, extensive prior *in vitro* testing was initiated for all further eligible patients. Using this strategy, we found that a much lower proportion of patients with hematologic malignancies showed positive *in vitro* proliferation of  $\gamma\delta$  T cells in response to pamidronate/IL-2 compared with a control group of healthy donors (49% versus 88%). Although the exact mechanisms of this defect are currently under investigation, a severe immunodeficiency caused by extensive prior chemotherapy in these relapsed/refractory patients and/or the underlying disease itself may account for this observation. Indeed, the type of underlying disease seems to influence the *in vitro* proliferative response to pamidronate/IL-2 (Table 2). The failure of patients with B-CLL to develop a measurable  $\gamma\delta$  T-cell proliferative response may be a result of the very small number of  $\gamma\delta$  T cells in peripheral blood, which were often below the detection limit in our series. However, a larger number of patients with distinct disease entities and at different disease stages (eg, untreated versus treated) need to be evaluated to support this observation and to identify additional clinical parameters influencing  $\gamma\delta$  T-cell reactivity. Furthermore, extensive prior *in vitro* testing in eligible patients revealed that  $\gamma\delta$  T-cell proliferation in response to pamidronate can be significantly enhanced by concomitant addition of IL-2 to PBMC cultures on day 1 instead of day 3 (as previously done).

Thus, for all further patients the treatment schedule was changed (concomitant administration of IL-2 on day 1), and only patients with significant *in vitro* proliferation of  $\gamma\delta$  T cells in the



**Figure 1. Representative example demonstrating activation and expansion of  $\gamma\delta$  T cells during pamidronate/IL-2 treatment.** Peripheral blood lymphocytes (PBLs) of patient 4B were stained with FITC- and PE-labeled antibodies and analyzed by flow cytometry before and after infusion of pamidronate and IL-2. Percentages of cells are given per quadrant. Body temperature (BT) was measured 2 times daily. Cytokine concentrations were determined in serum using ELISA systems. ND indicates not done.



**Figure 2. Time course of responding patients.** Response confirmed by (1) CT scan, (2) ultrasound, (3) PET scan, (4) blood tests, (5) lymph node, or (6) bone marrow biopsy.  $\Delta$  indicates treatment cycle;  $\circ$ , relative tumor mass; SN, subcutaneous nodules; \* lost to follow up.

presence of pamidronate and IL-2 were included (cohort B, Table 1). After these modifications, significant *in vivo* expansion of  $\gamma\delta$  T cells could be observed in 5 of 9 patients (55%) (Table 1). *In vivo* proliferation of  $\gamma\delta$  T cells was associated with a robust up-regulation of early (CD69) and late (HLA-DR) activation markers, whereas pamidronate and IL-2 failed to induce comparable effects on  $\alpha\beta$  T cells and NK cells (Table 3). These data support *in vitro* findings that the action of pamidronate is highly specific and, except for V $\gamma$ 9V $\delta$ 2 T cells, it does not activate other immune effector cells.<sup>8,23,24</sup> However, at higher IL-2 doses unspecific stimulation effects of IL-2 became more evident because a proportion of patients showed a moderate up-regulation of activation markers on  $\alpha\beta$  T cells and NK cells at the highest dose level of IL-2 tested in this study. On the basis of the analysis of activation marker expression and proliferation we conclude that  $1 \times 10^6$  IU IL-2/m<sup>2</sup> IL-2 per day seems to be the most effective dose with respect to specific and effective  $\gamma\delta$  T-cell stimulation *in vivo*.

Another aim of our study was to assess the clinical response. None of the 9 analyzable patients of cohort A (Table 1) achieved an objective tumor response. After change of protocol and inclusion criteria (cohort B, Table 1) 3 of 9 patients (33%) achieved an objective tumor response (3 PR). Clinical response could be associated with  $\gamma\delta$  T-cell proliferation *in vivo*, because all 4 patients from cohort B without  $\gamma\delta$  T-cell proliferation *in vivo* did

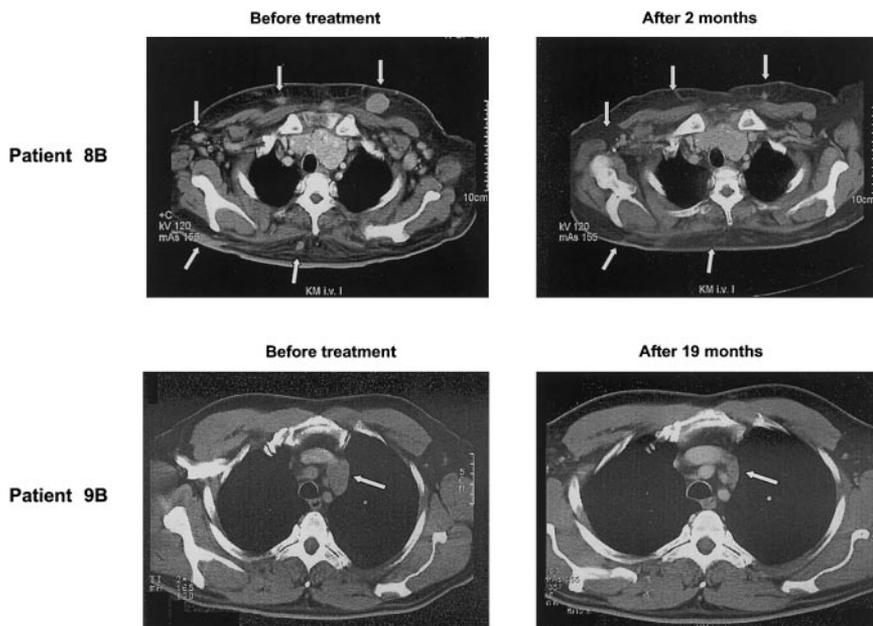
**Table 5. *In vivo* proliferation of  $\gamma\delta$  T cells and response to treatment with pamidronate/IL-2\***

Objective response	<i>In vivo</i> $\gamma\delta$ T-cell proliferation		$\Sigma$
	+	-	
+	3	0	3
-	2	13	15
$\Sigma$	5	13	18

All analyzable patients (n = 18) according to Table 1 were included. \*P = .015, Fisher exact test.

not experience an objective tumor response, and 4 of 5 patients with  $\gamma\delta$  T-cell proliferation *in vivo* responded (3 PR, 1 stable disease [SD]). These results suggest that the observed tumor regression in our patients is dependent on  $\gamma\delta$  T-cell activation and proliferation. The relevance of this correlation is underlined by the fact that pamidronate-stimulated  $\gamma\delta$  T cells possess an increased capacity for killing tumor cells *in vitro*.<sup>8,10</sup> It is still open which mechanisms may have been responsible for the clinical responses. Several other antitumor effects have been attributed to aminobisphosphonates. However, at pharmacologically achievable concentrations *in vivo*, only the specific stimulation of V $\gamma$ 9V $\delta$ 2 T cells can be observed.<sup>8</sup> Alternatively, the occurrence of clinical remissions may be attributed to an IL-2-mediated effect on other immune effector cells. However, our immunologic monitoring indicates that the combination of pamidronate and low-dose IL-2 does not induce specific activation and expansion of  $\alpha\beta$  T cells or NK cells compared with the effect on  $\gamma\delta$  T cells. In addition, the concentrations of IL-2 used here are much lower than the doses required in other immunotherapeutic approaches for these malignancies.<sup>25-27</sup>

The important question of what precise mechanisms are involved in tumor recognition and eradication by  $\gamma\delta$  T cells is out of the scope of this study and will require further *in vitro* and *in vivo* studies. However, tumor cell recognition by  $\gamma\delta$  T cells seems to be modulated by a balance of positive and negative signals.<sup>28</sup> Although killer inhibitory receptors (KIRs) are obviously involved in the mediation of negative signals, the positive signals are only incompletely understood. One example of such a positive signal is



**Figure 3. Clinical response to treatment.** CT scans of patients 8B and 9B before and after several cycles of pamidronate/IL-2 therapy show regression of skin metastases and mediastinal lymph node (arrows), respectively.

the NKG2D-DAP10 receptor complex, which is known to interact with stress-induced ligands on tumor cells such as MICA and Rae-1.<sup>29</sup> The very slow response profiles of most of the patients in our series strongly argue for an indirect influence on lymphoma cells rather than a sole cytotoxic effect. One possible mechanism may be secretion of cytokines, which influence tumor cells or their microenvironment.<sup>30</sup> We have already shown that IFN- $\gamma$  is the major cytokine secreted by pamidronate-activated  $\gamma\delta$  T cells.<sup>8,31</sup> IFN- $\gamma$  has multiple antitumor effects such as direct inhibition of tumor growth, blocking angiogenesis, or stimulation of macrophages.<sup>32</sup> Recently, a significant negative correlation between angiogenic factors (ie, VEGF) and IFN- $\gamma$  serum levels was described in patients treated with pamidronate.<sup>33</sup> Therefore, IFN- $\gamma$  might be one of the key cytokines involved in the  $\gamma\delta$  T-cell-mediated antitumor response.

In conclusion, this study indicates for the first time that in vivo  $\gamma\delta$  T-cell stimulation by pamidronate and low-dose IL-2 is a safe and promising immunotherapy approach in the treatment of

patients with low-grade B-NHL and MM. Further studies are necessary to confirm the clinical efficacy of this novel strategy. Our immunologic and clinical monitoring data provide further insight into the capacity of  $\gamma\delta$  T cells to induce an antitumor immune response. However, this study also reveals that the function of  $\gamma\delta$  T cells can be impaired in some patients with lymphoid malignancies. Therefore, the results of this study provide principles relevant to the design of future trials, including appropriate prior in vitro testing.

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## References

- Schultze JL, Nadler LM. T cell mediated immunotherapy for B cell lymphoma. *J Mol Med*. 1999;77:322-331.
- Kim S, Iizuka K, Aguila HL, Weissman IL, Yokoyama WM. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci U S A*. 2000;97:2731-2736.
- Smyth MJ, Thia KY, Street SE, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med*. 2000;191:661-668.
- Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by  $\gamma\delta$  T cells. *Science*. 2001;294:605-609.
- Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol*. 2001;2:293-299.
- Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial  $\gamma\delta$  T cells. *Science*. 1998;279:1737-1740.
- Fisch P, Meuer E, Pende D, et al. Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human V $\gamma$ 9/V $\delta$ 2 T cells in tumor immunity. *Eur J Immunol*. 1997;27:3368-3379.
- Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of  $\gamma\delta$  T cells by aminobisphosphonates and induction of anti-plasma cell activity in multiple myeloma. *Blood*. 2000;96:384-392.
- Sicard H, Al Saati T, Delsol G, Fournie JJ. Synthetic phosphoantigens enhance human V $\gamma$ 9V $\delta$ 2 T lymphocytes killing of non-Hodgkin's B lymphoma. *Mol Med*. 2001;7:711-722.
- Kato Y, Tanaka Y, Miyagawa F, Yamashita S, Minato N. Targeting of tumor cells for human  $\gamma\delta$  T cells by nonpeptide antigens. *J Immunol*. 2001;167:5092-5098.
- Lamb LS Jr, Henslee-Downey PJ, Parrish RS, et al. Increased frequency of TCR  $\gamma\delta^+$  T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. *J Hematother*. 1996;5:503-509.
- Zheng BJ, Chan KW, Im S, et al. Anti-tumor effects of human peripheral  $\gamma\delta$  T cells in a mouse tumor model. *Int J Cancer*. 2001;92:421-425.
- Tanaka Y, Morita CT, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human  $\gamma\delta$  T cells. *Nature*. 1995;375:155-158.
- Constant P, Davodeau F, Peyrat MA, et al. Stimulation of human  $\gamma\delta$  T cells by nonpeptidic mycobacterial ligands. *Science*. 1994;264:267-270.
- Jomaa H, Feurle J, Luhs K, et al. V $\gamma$ 9/V $\delta$ 2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis. *FEMS Immunol Med Microbiol*. 1999;25:371-378.
- Feurle J, Eckstein S, Espinosa E, et al. E. coli phosphoantigens activating  $\gamma\delta$  T cells. *J Biol Chem*. 2001;277:148-154.
- Bukowski JF, Morita CT, Brenner MB. Human  $\gamma\delta$  T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity*. 1999;11:57-65.
- Kunzmann V, Bauer E, Wilhelm M.  $\gamma\delta$  T-cell stimulation by pamidronate [letter]. *N Engl J Med*. 1999;340:737-738.
- Morita CT, Beckman EM, Bukowski JF, et al. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human  $\gamma\delta$  T cells. *Immunity*. 1995;3:495-507.
- Gucalp R, Ritch P, Wiernik PH, et al. Comparative study of pamidronate disodium and etidronate disodium in the treatment of cancer-related hypercalcemia. *J Clin Oncol*. 1992;10:134-142.
- Glover D, Lipton A, Keller A, et al. Intravenous pamidronate disodium treatment of bone metastases in patients with breast cancer: a dose-seeking study. *Cancer*. 1994;74:2949-2955.
- Wood J, Bonjean K, Ruetz S, et al. Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. *J Pharmacol Exp Ther*. 2002;302:1055-1061.
- Das H, Wang L, Kamath A, Bukowski JF. V $\gamma$ 2V $\delta$ 2 T-cell receptor-mediated recognition of aminobisphosphonates. *Blood*. 2001;98:1616-1618.
- Miyagawa F, Tanaka Y, Yamashita S, et al. Essential contribution of germline-encoded lysine residues in J $\gamma$ 1.2 segment to the recognition of non-peptide antigens by human  $\gamma\delta$  T cells. *J Immunol*. 2001;167:6773-6779.
- Whittington R, Faulds D. Interleukin-2: a review of its pharmacological properties and therapeutic use in patients with cancer. *Drugs*. 1993;46:446-514.
- Weber JS, Yang JC, Topalian SL, Schwartzentruber DJ, White DE, Rosenberg SA. The use of interleukin-2 and lymphokine-activated killer cells for the treatment of patients with non-Hodgkin's lymphoma. *J Clin Oncol*. 1992;10:33-40.
- Peest D, Leo R, Bloche S, et al. Low-dose recombinant interleukin-2 therapy in advanced multiple myeloma. *Br J Haematol*. 1995;89:328-337.
- Moris A, Rothenfusser S, Meuer E, Hangretinger R, Fisch P. Role of  $\gamma\delta$  T cells in tumor immunity and their control by NK receptors. *Microbes Infect*. 1999;1:227-234.
- Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature*. 2001;413:165-171.
- Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature*. 2001;411:375-379.
- Miyagawa F, Tanaka Y, Yamashita S, Minato N. Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human  $\gamma\delta$  T cells by aminobisphosphonate antigen. *J Immunol*. 2001;166:5508-5514.
- Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon- $\gamma$ . *Annu Rev Immunol*. 1997;15:749-795.
- Santini D, Vincenzi B, Avvisati G, et al. Pamidronate induces modifications of circulating angiogenic factors in cancer patients. *Clin Cancer Res*. 2002;8:1080-1084.