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In vitro expansion of gamma delta T cells with anti-myeloma cell activity by Phosphostim and IL-2 in patients with multiple myeloma

Maka Burjanadzé¹, Maud Condomines¹⁻³, Thierry Reme^{1,2}, Philippe Quittet^{1,4}, Pascal Latry^{1,4}, Cécile Lugagne⁵, François Romagne⁶, Yanis Morel⁶, Jean François Rossi¹⁻⁴, Bernard Klein¹⁻⁵ and Zhao Yang Lu^{1,5}

1. INSERM, U847, MONTPELLIER, F-34197 FRANCE;
2. CHU Montpellier, Institute of Research in Biotherapy, MONTPELLIER, F-34285 FRANCE;
3. Université MONTPELLIER 1, F-34967 France;
4. CHU Montpellier, Department of Hematology and Clinical Oncology, MONTPELLIER, F-34285 FRANCE;
5. CHU Montpellier, Unit for Cellular Therapy, MONTPELLIER, F-34285 FRANCE;
6. Innate-Pharma, Marseille, FRANCE

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Running title: Anti-myeloma activity of amplified $\gamma\delta$ T cells

Authors' contribution. MB and MC performed the experiments and wrote the paper. BK, TR and LZ supervised the project and wrote the paper. PQ, GC and JFR provided blood or bone marrow plasma cells. CL provided technical assistance. YM and FR provided Phosphostim and some $\gamma\delta$ T cells.

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Corresponding author: Bernard KLEIN
Institute for Research in Biotherapy
Hopital St Eloi, Av Augustin Fliche
34285 MONTPELLIER Cedex 5
FRANCE
Phone: +33 4 67 33 78 88
Fax: + 33 4 67 33 79 05
email: klein@montp.inserm.fr

Summary

T cell mediated immunotherapy is a promising therapeutic option for multiple myeloma (MM). Gamma-delta T cells ($\gamma\delta$ T cells) recognize phosphoantigens and display strong anti-tumor cytotoxicity. The synthetic agonist Phosphostim (BrHPP) has been shown to selectively activate $V\gamma9V\delta2$ T cells. The aim of this study was to evaluate the expansion capacity and anti-myeloma cell cytotoxicity of circulating $\gamma\delta$ T cells from MM patients at different time points throughout the disease, using Phosphostim (BrHPP) and IL-2. Circulating $\gamma\delta$ T cell counts in patients with newly-diagnosed MM or in relapse did not differ from those in healthy donors. A 14-day culture of peripheral blood mononuclear cells with Phosphostim and IL-2 triggered a 100-fold expansion of $\gamma\delta$ T cells in 78% of newly-diagnosed patients. $\gamma\delta$ T cells harvested at the time of haematopoietic progenitor collection or in relapsing patients expanded less efficiently. Expanded $\gamma\delta$ T cells killed 13/14 myeloma cell lines as well as primary myeloma cells, but not normal CD34 cells. Their killing efficiency was not affected by 2-day IL-2 starvation. This study demonstrates the ability of Phosphostim and IL-2 to expand $\gamma\delta$ T cells from MM patients, and the efficient and stable killing of human myeloma cells by $\gamma\delta$ T cells.

Introduction

Despite recent therapeutic advances, multiple myeloma (MM) remains an incurable disease with conventional or high dose chemotherapy (HDC). HDC and autologous stem cell transplantation (ASCT) have improved the rate of complete remission, but some multiple myeloma cells (MMC) escape treatment and the majority of MM patients relapse (Attal, *et al* 1996). The development of immunotherapy designed to eliminate residual tumor cells may be one hopeful approach to improve MM treatment. One possibility is to trigger anti-MMC specific T cells. In particular, transduction of MMC with *CD80* (Tarte, *et al* 1999), *CD40LG* (Cignetti, *et al* 2005), or *TNFSF9* (also known as *4-1BBL*) (Lu, *et al* 2007) genes makes it possible to obtain anti-MMC $\alpha\beta$ T cell lines. MMC express a variety of tumor antigens such as cancer-testis antigens (Condomines, *et al* 2007, Pellat-Deceunynck, *et al* 2000, van Baren, *et al* 1999), or differentiation antigens such as Muc1 (Brossart, *et al* 2001) or HM-1.24 (Goto, *et al* 1994, Hundemer, *et al* 2006).

Human T cells bearing the $\gamma\delta$ T cell receptor (TCR) may be another source of anti-MMC T cells. They account for 2-5% of peripheral blood lymphocytes and have been shown to exert a major histocompatibility (MHC)-unrestricted natural cytotoxicity against infected and malignant cells (Hayday 2000, Kunzmann, *et al* 2000). Most peripheral $\gamma\delta$ T cells display a disulfide-linked $V\gamma9V\delta2$ TCR that recognizes small phosphorylated non-peptide antigens such as mycobacterial antigens (Constant, *et al* 1994) or isopentenyl-pyrophosphate (IPP), a natural metabolite produced through the mevalonate pathway in eukaryotic cells (Das, *et al* 2001, Tanaka, *et al* 1995). Increased IPP production in tumor cells leads to $\gamma\delta$ T cell activation (Gober, *et al* 2003). In particular, the Burkitt's lymphoma Daudi cell line is a good activator and target of $\gamma\delta$ T cells. Aminobiphosphonates, which are drugs commonly used in MM treatment to prevent osteolytic bone disease, share structural homology with natural $\gamma\delta$ T cell ligands and are able to activate $\gamma\delta$ T cells *in vitro* (Kunzmann, *et al* 2000, Mariani,

et al 2005). This activation is TCR and monocyte dependent and is mainly induced by targeting intracellular enzymes of the mevalonate pathway (Bukowski, *et al* 1995, Mariani, *et al* 2005, Miyagawa, *et al* 2001). Activated $\gamma\delta$ T cells can grow in the presence of IL-2 or IL-15, are potent IFN γ producers (Miyagawa, *et al* 2001), and kill tumor cell lines including myeloma cell lines (Kunzmann, *et al* 2000, Mariani, *et al* 2005, Viey, *et al* 2005) through mechanisms involving NKG2D and possibly other activating NK receptors binding MICA or MICB expressed by MMC (Girlanda, *et al* 2005, Halary, *et al* 1997, Rincon-Orozco, *et al* 2005). Aminobiphosphonates also stimulate anti-tumor $\gamma\delta$ T cells *in vivo*. A single pamidronate administration followed by low doses of IL-2 in relapsing patients with MM or non-Hodgkin lymphoma triggers the expansion of circulating $\gamma\delta$ T cells leading to tumor reduction and to some objective clinical responses (Wilhelm, *et al* 2003). However, a successful *ex vivo* amplification of $\gamma\delta$ T cells using pamidronate or zoledronate with IL-2 is reached in only about 50% of patients with MM (Kunzmann, *et al* 2000, Mariani, *et al* 2005, Wilhelm, *et al* 2003). Alternatively, $\gamma\delta$ T cells can be directly activated *in vitro* by pyrophosphomonoesters (Thompson, *et al* 2006) such as bromohydrin pyrophosphate (BrHPP/Phosphostim). Phosphostim is a synthetic V γ 9V δ 2 TCR agonist that mimics the biological properties of natural phosphoantigens found in hydrosoluble mycobacterial extracts (Espinosa, *et al* 2001). Starting from blood samples of patients with renal carcinoma, the use of Phosphostim and IL-2 enabled the selective outgrowth of $\gamma\delta$ T cells highly cytotoxic toward autologous primary tumor cells, in 70% of patients (Viey, *et al* 2005). In addition, Phosphostim and IL-2 infusions in non-human primates trigger a transient but large expansion of circulating V γ 9V δ 2 T cells, inducing the production of high levels of Th1 cytokines (Sicard, *et al* 2005).

The aim of this study was to compare the ability of Phosphostim and zoledronate to expand $\gamma\delta$ T cells from normal donors or patients with MM and to evaluate the *in vitro* anti-MMC cytotoxicity of Phosphostim-expanded $\gamma\delta$ T cells from patients with MM.

Materials and Methods

Patients and collection of peripheral blood and bone marrow samples

Buffy coats from 14 age-matched healthy donors (HD) were provided by the French Blood Center (Toulouse, France). Bone marrow and peripheral blood from 18 patients with newly-diagnosed MM (median age 61 years, 10 female patients and 8 male patients) were collected after written informed consent was given. According to Durie-Salmon classification, 5 patients were of stage IA, 5 of stage IIA, 4 of stage IIIA, and 4 of stage IIIB. Two patients had IgAk MM, 2 IgA λ MM, 8 IgGk MM, 4 IgG λ MM, 1 Bence-Jones κ MM, and 1 Bence-Jones λ MM. Of 13 MM patients relapsing after one treatment line (median age 66 years, 8 female patients and 5 male patients), 3 were of stage IA, 2 of stage IIA, 4 of stage IIIA, and 4 of stage IIIB. One patient had IgAk MM, 3 IgA λ MM, 2 IgGk MM, 3 IgG λ MM, 1 Bence-Jones κ MM, 1 IgA, and 1 IgD with undetermined light chain. One patient presented with plasma cell leukemia. Bone marrow mononuclear cells (BMMCs) and peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation on a Ficoll-Hypaque cushion (Cambrex BioScience, Walkersville, MD, USA). For patients treated by HDC and ASCT, circulating cells including haematopoietic stem cells (HSC) were collected by leucapheresis after mobilization by a single 4 g/m² cyclophosphamide (CTX) infusion followed by daily subcutaneous injections of G-CSF at 10 μ g/kg/day.

Immunophenotypic analysis

The phenotype of T cells was evaluated with the following monoclonal antibodies (mAbs): phycoerythrin (PE)-conjugated CD3, PE-pan $\gamma\delta$ TCR, PE-CD45RA, PE-CXCR4 (Becton Dickinson San Jose, CA), fluorescein isothiocyanate (FITC) pan- $\gamma\delta$ TCR, FITC-V γ 2TCR, FITC-V δ 9TCR (Beckman Coulter, Villepinte, France), FITC-CD45RA, FITC-pan CD45, and Cytochrome-conjugated CD27 (Beckman Coulter). Primary MMC were evaluated with the PE-conjugated B-B4 anti-syndecan-1 mAb (Wijdenes, *et al* 1996). Anti-CD138 mAb

labelled only viable MMC in the bone marrow of patients with MM (Costes, *et al* 1999, Jourdan, *et al* 1998). Corresponding irrelevant isotype-matched mouse mAbs were used as negative controls. Briefly, appropriate amounts of mAbs were added to 0.5×10^6 whole blood cells followed by a 30-min incubation at 4°C. Red cells were then lysed, cells were washed, and 30×10^4 total events or 10×10^4 events in the lymphocyte gate were acquired on a FACScan[®] cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed with the CellQuest software. Lymphocyte subsets were assessed by three-color immunofluorescence analysis.

Cell lines and primary myeloma cells

XG-1, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-13, XG-16, XG-19, and XG-20 human myeloma cell lines (HMCLs) were obtained and characterized in our laboratory (Gu, *et al* 2000, Rebouissou, *et al* 1998, Zhang, *et al* 1994). RPMI 8226, RAJI, Daudi, and U266 cell lines were purchased from ATTC (LGC Promochem, Molsheim, France). They were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 2 mM L-glutamine, and 2 ng/ml of human recombinant IL-6 (AbCys SA, Paris, France) for the IL-6-dependent HMCLs. Primary myeloma cells were purified from patients' tumor samples as described (Sun, *et al* 1997).

Expansion of $\gamma\delta$ T cells

PBMCs from healthy donor buffy-coats (n=14) and from patients with MM were seeded at 10^6 /mL in 24-well culture plates at 37°C in 5% CO₂ in RPMI 1640 medium and 10% fetal calf serum (FCS). Polyclonal V γ 9V δ 2 T cells were specifically expanded in the presence of 3 μ M of Phosphostim (BrHPP molecule, Innate Pharma, Marseille, France) or 1 μ M of zoledronate (Novartis, Basel, Switzerland) and 150 U/ml IL-2 (Proleukin, Chiron, Basel, Switzerland) for 14 days. Phosphostim or zoledronate was added once at the onset of the culture. Every 3 days, one half of the culture medium volume was replaced with fresh medium containing 150 U/ml IL-2.

Cytotoxic assays

⁵¹Cr release assay. Expanded $\gamma\delta$ T cells were tested for cytotoxicity against allogeneic HMCLs (RPMI 1640, U266, XG-1, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-13, XG-16, XG-19, XG-20) or the Burkitt's lymphoma cell lines - Daudi and Raji - in a 4 h ⁵¹Cr release assay. Target cells were labelled with 100 μ Ci ⁵¹Cr for 60 minutes. The effector:target (E:T) ratios ranged from 30:1 to 0.1:1. Specific lysis (expressed as percentage) was calculated using the standard formula $\{(\text{experimental} - \text{spontaneous release} / \text{total} - \text{spontaneous release}) \times 100\}$ and is expressed as the mean of triplicate assays. In some experiments, target HMCL cells (10^6 /mL) were pre-incubated with 50 μ M zoledronate at 37°C for 16 hours and then added to effector cells.

Flow cytometric cytotoxic T lymphocyte assay. Expanded $\gamma\delta$ T cells were tested for cytotoxicity against primary myeloma cells or XG-6 cells using the CyToxiLux^RPlus! Kit Easy (OncoImmunit, Gaithersburg, MD, USA). Target cells were labelled with a fluorescent dye and then co-incubated with effector cells in the presence of a fluorogenic caspase substrate according to the manufacturer's recommendations (*Standard Protocol*). The E:T ratios ranged from 30:1 to 3:1. After washes, samples were analyzed by flow cytometry.

GM-CFU assay

Leucapheresis-derived purified CD34 haematopoietic stem cells from 5 patients with MM were assayed for their ability to generate granulocyte and/or macrophage colonies in a semi-solid culture medium with haematopoietic cytokines (GF H4434; StemCell Technologies, Vancouver, BC, Canada). Cells were pre-incubated or not (control) with expanded $\gamma\delta$ T cells at 1:30 and 1:5 ratios for 2 hours before semi-solid cultures. The number of granulocyte-macrophage colonies was counted on day 14 of culture.

Statistical analysis

Amplification rates percentages between the different groups were compared with a chi-square test. All the other statistical analyses were done using a Mann-Whitney test. *P*-values <0.05 were considered significant.

Results

$\gamma\delta$ T cell and CD3 cell counts in the peripheral blood and bone marrow of patients

$\gamma\delta$ T cells were evaluated in the peripheral blood (PB) and in the bone marrow (BM) of 18 patients with newly-diagnosed MM and in PB of 11 age-matched HD. A median $\gamma\delta$ T cell percentage of 0.35 % (range 0.04 - 2.4 %) was found in PB leucocytes of patients with MM (Table I). This percentage was not statistically different from that found in 11 healthy donors' PB leucocytes (0.62 %, range 0.4 - 1.8%, $P = .19$, results not shown). A median $\gamma\delta$ T cell percentage of 0.38 % (range 0.01 – 1.11 %) was found in the bone marrow of these 18 patients. It was not significantly different from that found in the PB of the same patients ($P = .27$). The median $\gamma\delta$ T cell percentages in CD3 cells in PB and BM were 2.1 % and 2.8 %, respectively. Again, these percentages were not statistically different from those found in HD (3.9 %, range 2.4 – 11.1 %, $P = .28$, results not shown). To look for a $\gamma\delta$ T cell source for clinical application, $\gamma\delta$ T cell counts were evaluated in PB at diagnosis and in the leucapheresis products harvested at the time of HSC mobilization with high dose CTX and G-CSF of seven patients with MM (Table I). The median $\gamma\delta$ T cell percentages in CD3 cells at diagnosis (2.3 %, 0.01 – 5.9 %) and at the time of mobilization (1.6 %, 0.02 – 6.5 %) were not statistically different. However, CTX and G-CSF treatment induced a 3-fold depletion in $\gamma\delta$ T cell and CD3 cell counts at the time of leucapheresis collection (results not shown) in agreement with our previous study (Condomines, *et al* 2006). The leucapheresis products contained a median number of 68×10^6 $\gamma\delta$ T cells (range 0.2 – 394), *i.e.*, 13-fold less than the median CD34 count.

Expansion of $\gamma\delta$ T cells with Phosphostim

Activation of $\gamma\delta$ T cells by Phosphostim and IL-2 made it possible to expand $\gamma\delta$ T cells from PB at least 100-fold in 14 out of 18 (78%) newly-diagnosed patients. The median expansion was 297-fold, ranging from 10- to 4406-fold (Table II). The expanded $\gamma\delta$ T cells

expressed V γ 9V δ 2 ($\geq 80\%$, data not shown). Based on our previous experience, we chose this 100-fold amplification cut-off to get enough cells for an adoptive T cell transfer, starting with one leucapheresis product, in the prospect for a clinical trial (infusion of at least 3×10^9 $\gamma\delta$ T cells, Salot *et al*, submitted). We also assayed $\gamma\delta$ T cell amplification using zoledronate and IL-2 for 10 newly-diagnosed patients. The rate of successful amplification (≥ 100 fold) was 70% and was not different from that obtained with Phosphostim and IL-2 (78%, $P = .54$). In addition, a strong correlation of amplification rates with Phosphostim and zoledronate was found ($r = 0.9$, $P = .001$). An efficient amplification of $\gamma\delta$ T cells failed in four out of 18 patients (< 100 fold; *i.e.* 10-, 28-, 39-, and 61-fold). No statistical differences in $\gamma\delta$ T cell counts and percentages of $\gamma\delta$ T cells in CD3 cells were found in PB cells of patients with a successful or unsuccessful amplification rate. Increasing IL-2 concentrations up to 1000 U/ml instead of 100 U/ml improved the magnitude of amplification without reaching a 100-fold amplification (results not shown).

We then investigated whether $\gamma\delta$ T cell expansion could vary throughout disease. We considered 3 groups of MM patients - patients with newly-diagnosed MM ($n=18$), patients at the time of HSC mobilization with CTX and G-CSF ($n=18$), and relapsing patients ($n=13$). The successful $\gamma\delta$ T cell expansion rate (≥ 100 fold) in newly-diagnosed patients (78%) was not significantly different from that obtained in HD (70%, $P = .98$) and from that obtained in relapsing patients (69%, $P = .86$). However, the median purity of $\gamma\delta$ T cells in the relapsing patient group (21%) was lower than that obtained in the newly-diagnosed patient group (80%) (Table II, $P = .008$). The percentage of patients with a successful $\gamma\delta$ T cell amplification rate (≥ 100 fold) at the time of HSC mobilization was 50%. It was not statistically lower than those obtained in newly-diagnosed or relapsing patients ($P = .17$ and $P = .28$, respectively). Again, the median purity of $\gamma\delta$ T cells in this group (11%) was lower than that of the newly-diagnosed patient group (Table II, $P < .001$).

It was reported that the ability of $\gamma\delta$ T cells to be expanded by aminobisphosphonates depends on their naïve/memory phenotype (Mariani, *et al* 2005). In this study, the memory phenotype was predominant in $\gamma\delta$ T cells of HD that could be expanded with zoledronate and IL-2, whereas the effector phenotype was predominant in cases of poorly expanding cells. We determined the four subsets of $\gamma\delta$ T cells, *i.e.* naïve (CD45RA⁺ CD27⁺), central memory (CD45RA⁻ CD27⁺), effector memory (CD45RA⁻ CD27⁻), and terminally differentiated (CD45RA⁺ CD27⁻), in 10 MM patients and 10 HD (Table III). We found no correlation between the capacity of $\gamma\delta$ T cells to be expanded with Phosphostim and IL-2 and their naïve/memory phenotype.

Myeloma cell lines are efficiently killed by Phosphostim-expanded $\gamma\delta$ T cells

$\gamma\delta$ T cells expanded from HD display strong cytotoxic activity against some HMCLs *in vitro* (Kunzmann, *et al* 2000). In order to determine the potential lytic capacity of expanded $\gamma\delta$ T cells against a panel of HMCLs, we first used $\gamma\delta$ T cells amplified from two HD by Phosphostim stimulation as described above ($\geq 90\%$ $\gamma\delta$ purity). The Phosphostim-expanded $\gamma\delta$ T cells efficiently killed the Burkitt's lymphoma Daudi cells, unlike Burkitt's lymphoma Raji cells known to be resistant to $\gamma\delta$ T cells. Of interest, 13 out of 14 HMCLs were killed by these expanded $\gamma\delta$ T cells. At an E:T ratio of 30:1, the percentage of lysed cells ranged from 10% to 60% (Fig 1A). Only the XG-3 cells could not be killed by the various expanded $\gamma\delta$ T cells. Pre-incubation of XG-3 cells with zoledronate did not abrogate their lysis resistance (data not shown).

In addition, expanded $\gamma\delta$ T cells from 7 patients with MM killed 5 cell lines (Raji, RPMI 8226, XG-5, XG-6, and XG-19) as efficiently as those from 6 HD (results not shown). Fig 1B shows two representative experiments using XG-6 and XG-19 HMCLs as targets.

Expanded $\gamma\delta$ T cells from patients with MM are able to kill primary MMC *in vitro*

As ^{51}Cr could not be efficiently incorporated in primary MMC, the Cytotoxicity assay kit was used to determine the cytotoxicity of $\gamma\delta$ T cells toward purified primary MMC at different E:T ratios. Purified primary MMC were obtained from the PB of one patient with plasma cell leukemia (CD138⁺: 86%) and from the bone marrow of a second patient using FACS sorting (CD138⁺ > 90%). The XG-6 HMCL was used as a control target. $\gamma\delta$ T cells were expanded from the PB of one MM patient by Phosphostim. The lysis of primary MMC by $\gamma\delta$ T cells was > 40% at a 30:1 E:T ratio (Fig 1C).

Survival of expanded $\gamma\delta$ T cells in medium without IL-2 and their effect on CD34 cells

We speculated that Phosphostim and IL-2-expanded $\gamma\delta$ T cells would be starved of IL-2, at least transiently, after injection *in vivo*. To evaluate whether such starvation could affect their cytotoxic potential, $\gamma\delta$ T cells were cultured with or without IL-2 for 48 hours and their survival and capacity to kill XG-6 cells were determined at 24h (results not shown) and at 48h. As shown in Fig. 2A, the lytic capacities of $\gamma\delta$ T cells cultured for 48h with or without IL-2 were not significantly different ($P = .465$). Thus, deprivation of IL-2 did not significantly affect the survival and killing efficiency of expanded $\gamma\delta$ T cells.

We next looked for the effect of expanded $\gamma\delta$ T cells on the survival of CD34 cells that were purified from leucapheresis products. CD34 cells were pre-incubated with expanded $\gamma\delta$ T cells at 30:1 and 5:1 ratios for 4 hours, and then seeded in methylcellulose semi-solid medium to evaluate their haematopoietic colony forming potential. The killing capacity of $\gamma\delta$ T cells in this experiment was checked in the meantime using the XG-6 HMCL as described above (data not shown). As shown in Fig 2B, the ability of CD34 cells to form GM-CFU was unaffected by a pre-incubation with expanded $\gamma\delta$ T cells ($P = .873$).

All the expanded $\gamma\delta$ T cell lines expressed the chemokine (C-X-C motif) receptor 4 (CXCR4). Fig.2C depicts the expression of CXCR4 by four representative $\gamma\delta$ T cell lines.

Discussion

Our results show that peripheral blood $\gamma\delta$ T cells from patients with MM can be efficiently expanded in a short-time culture using Phosphostim and IL-2. These cells are highly cytotoxic against all but one HMCL and efficiently kill primary MMC.

The frequency of circulating $\gamma\delta$ T cells has been reported to be reduced in cancer diseases (Katsuta, *et al* 2006, Re, *et al* 2005). We show here that the $\gamma\delta$ T cell counts in the peripheral blood of patients with newly-diagnosed MM were similar to those of age-related HD. In addition, $\gamma\delta$ T cell counts were not significantly affected in patients with MM relapsing from chemotherapy.

Although several authors showed a lower successful amplification (about 50%) of $\gamma\delta$ T cell expansion in MM patients using pamidronate (Kunzmann, *et al* 2000, Wilhelm, *et al* 2003) or zoledronate (Mariani, *et al* 2005), we could not confirm this observation. For more than 70% of the patients with MM or for HD, a 14-day expansion rate greater than 100-fold was obtained both with Phosphostim or zoledronate. This may be explained by the fact that in the previous studies, the rate of successful amplification was evaluated on day 7 of culture with different criteria.

The function of $\gamma\delta$ T cells from MM patients was also comparable to those from HD. Phosphostim-amplified $\gamma\delta$ T cells efficiently killed 13/14 HMCLs and primary myeloma cells. In humans, it was reported that many molecules, such as LFA-1, CD2 (Kato, *et al* 2003, Wang and Malkovsky 2000), and MICA-MICB/NKG2D (Bauer, *et al* 1999, Rincon-Orozco, *et al* 2005) are involved in tumor cell recognition and lysis by $\gamma\delta$ T cells. A recent study showed that the natural cytotoxicity receptor NKp44, present on activated polyclonal $\gamma\delta$ T cells, could play a role in their cytotoxic activity against MM cells (von Lilienfeld-Toal, *et al* 2006). Another work demonstrated that MICA/MICB expressed by MMC are costimulators of V γ 9V δ 2 T cells (Girlanda, *et al* 2005). The mechanisms of activation of

V γ 9V δ 2 cells by phosphoantigens remain unclear. F1-ATPase and apolipoprotein A-I are membrane proteins which represent other $\gamma\delta$ TCR ligands (Scotet, *et al* 2005). Thirteen out of the 14 HMCLs were killed by expanded $\gamma\delta$ T cells. Noteworthy, the XG-3 HMCL was fully resistant even after zoledronate treatment, which is known to increase the sensitivity of target cells to $\gamma\delta$ T cells (results not shown). Using Affymetrix™ microarrays, no difference in the level of expression of *MICA*, *MICB*, *FDPS* nor *HMGCR* was found between XG-3 and other HMCLs. Future investigations are necessary for understanding the mechanisms of MMC recognition by $\gamma\delta$ T cells.

In addition to their killing of HMCLs, we show here that Phosphostim and IL-2 expanded $\gamma\delta$ T cells could also efficiently kill patients' primary MMC, emphasizing the attraction of these cells for immunotherapy.

To foresee a clinical trial of adoptive therapy with $\gamma\delta$ T cells, we have answered three requirements: 1) the feasibility of expanding enough $\gamma\delta$ T cells. Starting from one leucapheresis harvested at diagnosis or at relapse after various chemotherapy treatments, a ≥ 100 -fold $\gamma\delta$ T cell expansion can be achieved in about 70% of patients with a 78% purity in $\gamma\delta$ T cells, insuring the obtaining of 2×10^9 $\gamma\delta$ T cells, which represent an adequate amount of cells to treat patients. However, our current data show that at least 2×10^9 $\gamma\delta$ T cells will be hardly obtained after a CTX + G-CSF mobilization regimen because of T cell (including $\gamma\delta$ T cell) depletion induced by CTX (Condomines, *et al* 2006). In addition, in patients with a successful $\gamma\delta$ T cell amplification rate (≥ 100 fold) at the time of HSC mobilization, the purity of $\gamma\delta$ T cells was found to be about 11%. The remaining 89% cells comprised mainly $\alpha\beta$ T cells, poorly characterizing this source of expanding cells for a clinical use. A pilot clinical trial of cell therapy conducted in patients with renal carcinoma showed low toxicity of several infusions of 10^9 $\gamma\delta$ T cells (Kobayashi, *et al* 2007). Few objective clinical responses were observed. However, in that study, $\gamma\delta$ T cells were

activated by a natural phosphoantigen which has been shown to be less effective than BrHPP/Phosphostim (Espinosa, *et al* 2001). We found no predictive marker for a successful expansion, particularly according to the $\gamma\delta$ T cell effector/memory phenotype. Thus, we suggest using a pre-screening expansion assay before performing a large volume leucapheresis. 2) We looked for the ability of $\gamma\delta$ T cells to keep their cytotoxic capacity upon deprivation of IL-2. Noteworthy, no detectable IL-2 could be detected in the aplasia window 5-10 days post high dose melphalan (unpublished data). We found here that a 2-day IL-2 deprivation did not abrogate the cytotoxic potential of $\gamma\delta$ T cells, suggesting that the injected $\gamma\delta$ T cells will keep their cytotoxic ability *in vivo*. 3) We demonstrated that expanded $\gamma\delta$ T cells had no cytotoxic activity toward haematopoietic stem cells, with regard to their ability to generate 14 day-haematopoietic colonies.

Thus, we would recommend injecting $\gamma\delta$ T cells in the lymphoid and haematopoietic depletion window occurring 5-10 days after high dose melphalan and graft of haematopoietic stem cells. In this window, the leukocyte count in the bone marrow is minimum (10^5 cells/L) and comprises mainly MMC that have escaped melphalan. The expanded $\gamma\delta$ T cells expressed CXCR4 (**Fig. 2C**), the receptor for SDF-1, which controls homing of haematopoietic stem cells and myeloma cells into bone marrow (Alsayed, *et al* 2006, Chute 2006, Gazitt and Akay 2004). Thus, we may expect that these $\gamma\delta$ T cells will home to the bone marrow, have easy access to the chemoresistant myeloma cells, and provide a second cytotoxic hit to fully kill them, without killing the haematopoietic stem cells that are necessary to repair haematopoietic tissue.

In addition, it was shown recently that $\gamma\delta$ T cells were also professional antigen-presenting cells (Brandes, *et al* 2005). A large panel of adhesion and costimulatory molecules like CD40, CD54, CD80, and CD86 were expressed in our expanded $\gamma\delta$ T cells from 7 HD and 13 patients with MM (results not shown). Taking these above results together, adoptive

immunotherapy using $\gamma\delta$ T cells probably has two different roles, one to act as effector cells directly against tumour cells and the other to act as APC for a tumour vaccine.

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Legends to figures

Figure 1. Killing of myeloma cells by $\gamma\delta$ T cells stimulated with Phosphostim and IL-2.

(A) $\gamma\delta$ T cells from 2 healthy donors (HD) were expanded with Phosphostim and IL-2 and their cytotoxic activity to HMCLs (4-h ^{51}Cr release assay) was measured at the indicated E:T ratios. $\gamma\delta$ T cells were tested against the Raji cell line as a resistant target, Daudi cell line as a sensible target, and a panel of 14 HMCLs. Data are those obtained with one $\gamma\delta$ T cell expansion and are representative of 6 experiments. **(B)** The cytotoxic activity of expanded $\gamma\delta$ T cells from 7 patients with MM (P1-P7) against XG-6 (left panel) and XG-19 (right panel) HMCLs was measured in a 4-h ^{51}Cr release assay at the indicated E:T ratios. **(C)**. Cytotoxicity of expanded $\gamma\delta$ T cells against primary myeloma cells was measured using the Cytoxilux assay kit at E:T ratios of 30:1, 10:1, and 3:1 as described in material and methods. Expanded $\gamma\delta$ T cells from one patient with MM were tested against purified primary tumour cells either from a patient with a PCL (CD138⁺, PB) or from a patient with intramedullary myeloma (CD138⁺, BM) or against the XG-6 HMCL.

Figure 2. Killing properties of $\gamma\delta$ T cells

(A). The killing activity of $\gamma\delta$ T cells is not affected by IL-2 starvation. $\gamma\delta$ T cells from 3 HD (D1-3) were expanded with Phosphostim and IL-2 for 14 days, washed and cultured for 48 hours with or without IL-2 (150 U/ml). The cytotoxicity against the XG-6 HMCL was measured using the Cytoxilux assay kit for the E:T ratios 30, 10, and 3:1 as described previously. Results are expressed as the percentage of lysis at an E:T ratio of 30:1 after 48 hours of culture. **(B)**. No killing activity of expanded $\gamma\delta$ T cells to haematopoietic progenitors. CD34⁺ haematopoietic progenitors were purified from leucapheresis products of 5 patients mobilized by CTX and G-CSF. They were pre-incubated with Phosphostim and IL-2 expanded $\gamma\delta$ T cells at different E:T ratios for 4 hours and then grown in a semi-solid culture medium with haematopoietic growth

factors. The number of haematopoietic colonies (GM-CFU) was counted on day 14 of culture. Results are expressed using the following formula: number of GM-CFU with CD34 cells preincubated with $\gamma\delta$ T cells / number of GM-CFU with CD34 cells without $\gamma\delta$ T cell pre-incubation x 100. $\gamma\delta$ T cells efficiently killed XG-6 cells used in this experiment as a control (not shown). (C). Phosphostim-expanded $\gamma\delta$ T cells were stained with an anti-CXCR4-PE mAb (open histograms). The corresponding IgG2a-PE isotype-matched murine Ab was used as a negative control (filled histograms). Numbers in FACS plots indicate the percentages of CXCR4 positive $\gamma\delta$ T cells. Data are those obtained with $\gamma\delta$ T cells expanded from 4 patients with MM.

Table I. $\gamma\delta$ T cells in the peripheral blood or in the bone marrow of patients with newly-diagnosed MM or in the leucapheresis product at time of haematopoietic stem collection.

	PB at diagnosis		Bone Marrow	Leucapheresis
Number of patients	18	7	18	7
Leucocytes ($10^9/L$)	5 (4-14)	5 (4-11)	11 (7-50)	
CD3 cells (%)	15.1 (4-67.7)	25.1 (10.2-52.7)	8.4 (3.2-24.5)	5.22 (1.7-3.1)
$\gamma\delta$ T cells (%)	0.35 (0.04-2.4)	0.6 (0.01-2.4)	0.38 (0.01-1.1)	0.21 (0.01-1.7)
$\gamma\delta$ T cells in CD3 cells (%)	2.1 (0.38-15.6)	2.3 (0.01-5.9)	2.8 (0.03-17.6)	1.6 (0.02-6.5)
$\gamma\delta$ T cells ($10^6/L$)	24.4 (2.97-118.1)	21.3 (0.1-144)	36 (0.3-142)	
$\gamma\delta$ T cells ($10^6/product$)				68 (0.2-394)
CD34 cells (%)				2.8 (0.4-5.4)
CD34 cells ($10^6/product$)				910 (77-3156)

Results are the median values and ranges of data obtained with the patients' samples.

Table II. Expansion of $\gamma\delta$ T cells with Phosphostim or zoledronate in patients with MM, at different times throughout the disease.

	PBMC at diagnosis		PBMC at time of HSC mobilisation		PBMC at relapse
	stimulated				
	with Phosphostim	with zoledronate	with Phosphostim	with zoledronate	with Phosphostim
Number of patients	18	10	18	5	13
CD3 cell number on day 0 of culture (10^6)	1.7 (0.9-2.3)	1.6 (0.9-2.2)	1.1 (0.1-2.4)	0.60 (0.1-1.5)	1.1 (0.2-13.2)
$\gamma\delta$ T cells on day 0 (%)	2 (0.2-12.9)	1.7 (0.4-4.7)	0.5 (0.01-15.3)	0.4 (0.01-3.9)	0.5 (0.001-3.1)
$\gamma\delta$ T cell number in culture on day 0 (10^3)	59.1 (6.9-387)	51.9 (12-141)	14.7 (0.3-458)	6.0 (3-117)	42 (1.6-551)
$\gamma\delta$ T cells on day 14 of culture (%)	88.7 (5.9-98.1)	88.2 (38.6-95.2)	11.4 (0.15-83.5)	36.6 (3-61.5)	20.7 (3.4-89)
$\gamma\delta$ T cell number on day 14 of culture (10^3)	2301 (42-11807)	2541 (67-7089)	141 (30-2816)	178 (47-2253)	1890 (12.2-6640)
Fold amplification	297 (10-4406)	301 (56-2499)	107 (0.1-3516)	807 (17-1976)	276 (1-4176)
Successful amplification rate (> 100 fold, %)	78	70	50	80	69
% of patients with $\gamma\delta$ T cell number $\geq 2 \times 10^6$ on day 14	89	80	50	40	62

PBMCs from patients with MM were collected at diagnosis, at the time of HSC mobilization by high dose CTX and G-CSF, or at relapse. They were cultured with Phosphostim or zoledronate and IL-2 as described in Materials and Methods. After 14 days of culture, the absolute number of $\gamma\delta$ T cells was evaluated. The amplification fold was calculated by dividing the absolute number of $\gamma\delta$ T cells obtained at the end of the culture by the absolute number of $\gamma\delta$ T cells at the culture start. **Results are the median values of data obtained with the patients' samples.**

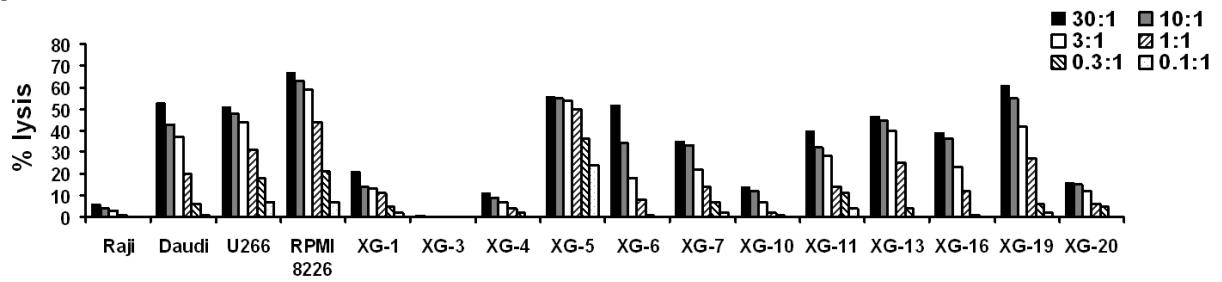
Table III. Expansion of $\gamma\delta$ T cells in response to Phosphostim did not correlate with their phenotype $\gamma\delta T_N / \gamma\delta T_{CM} / \gamma\delta T_{EM} / \gamma\delta T_{TD}$

Patients	% $\gamma\delta$ T cells				Amplification fold	$\gamma\delta$ T cell phenotype (%) according to their proliferative capacity	
	$\gamma\delta T_N$	$\gamma\delta T_{CM}$	$\gamma\delta T_{EM}$	$\gamma\delta T_{TD}$		$\gamma\delta T_N + \gamma\delta T_{CM}$	$\gamma\delta T_{EM} + \gamma\delta T_{TD}$
1	32.3	13.2	1.5	53.1	302.7	45.5	54.5
2	27.5	37.9	31.1	3.5	156.3	65.4	34.6
3	35.4	24.6	20.7	19.3	276.3	60.0	40.0
4	34.4	11.4	11.7	42.5	2.2	45.8	54.2
5	84.6	1.1	1.1	13.3	3.6	85.6	14.4
6	28.9	45.2	9.5	16.4	563.5	74.2	25.8
7	37.8	19.9	10.1	32.2	3530.7	57.7	42.3
8	15.4	13.3	0.2	71.1	20.8	28.7	71.3
9	41.0	29.5	3.6	26.0	162.0	70.5	29.5
10	0.6	0.9	96.6	1.9	822.8	1.4	98.6
Healthy donors							
1	1.0	1.6	26.8	70.7	1057.0	2.6	97.4
2	1.2	1.2	70.4	27.2	136.0	2.4	97.6
3	1.1	1.2	36.5	61.2	249.8	2.3	97.7
4	8.8	47.7	36.8	6.7	1383.0	56.4	43.6
5	3.3	2.5	23.8	70.4	224.0	5.8	94.2
6	59.5	0.5	36.2	3.9	35.4	60.0	40.0
7	12.5	36.0	37.8	13.7	2.4	48.5	51.5
8	14.1	13.2	10.2	62.6	3.4	27.2	72.8
9	0.8	1.2	62.2	35.8	459.5	2.0	98.0
10	4.7	0.6	19.3	75.5	853.8	5.3	94.7

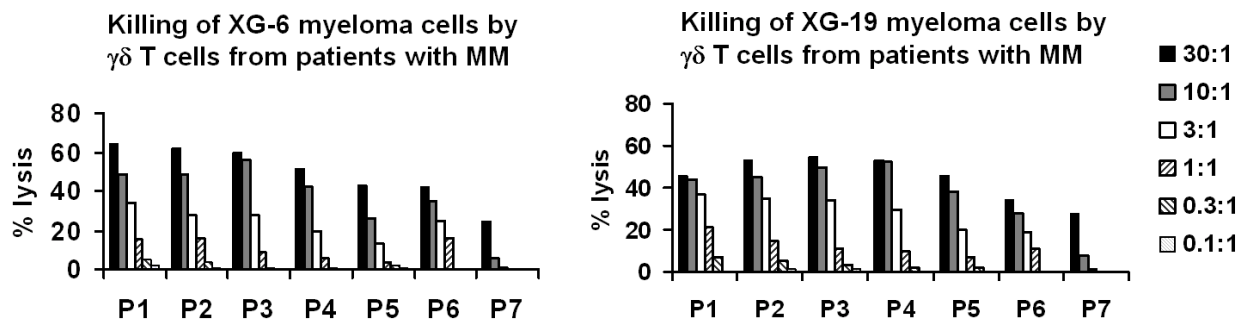
$\gamma\delta$ T cell phenotype was determined by flow cytometry in PBMC from 10 patients with MM and 10 healthy donors. $\gamma\delta T_N$ – naïve $\gamma\delta$ T cells (CD45RA⁺ CD27⁺), $\gamma\delta T_{CM}$ - central memory $\gamma\delta$ T cells (CD45RA⁻ CD27⁺), $\gamma\delta T_{EM}$ – effector memory $\gamma\delta$ T cells (CD45RA⁻ CD27⁻) and $\gamma\delta T_{TD}$ - terminally differentiated $\gamma\delta$ T cells (CD45RA⁺ CD27⁻). In a previous study (Dieli, *et al* 2003), $\gamma\delta T_N$ and $\gamma\delta T_{CM}$ were shown to display a high proliferative capacity whereas $\gamma\delta T_{EM} + \gamma\delta T_{TD}$ displayed effector functions but a low proliferative capacity.

Figure 1

A



B



C

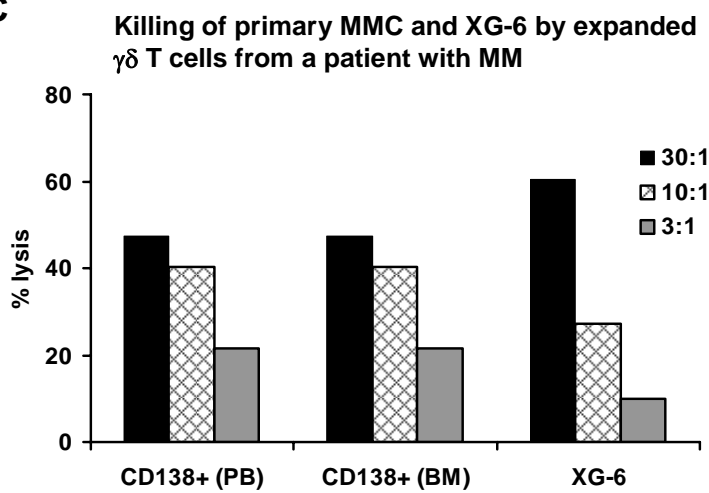
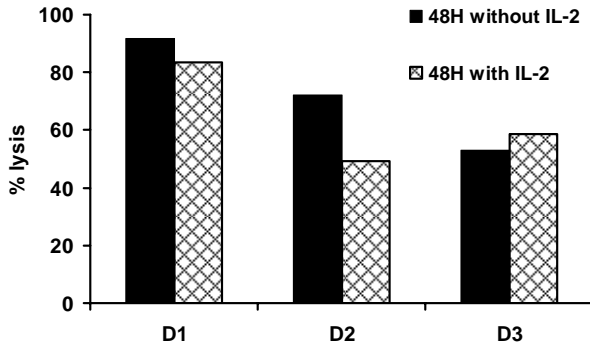


Figure 2

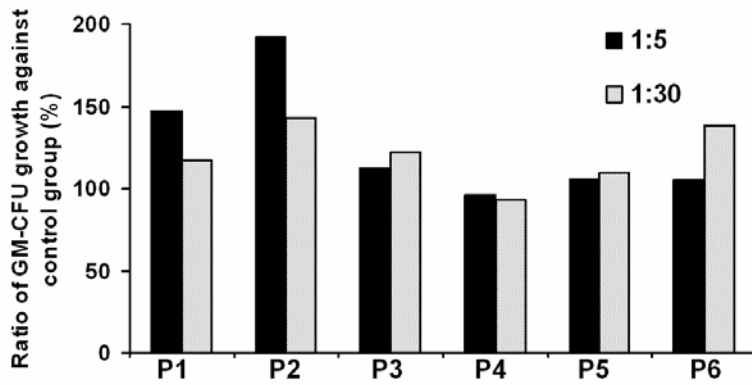
A.

Killing efficiency of $\gamma\delta$ T cells with or without IL-2



B.

Growth of hematopoietic progenitors



C.

