Clinical evaluation of autologous gamma delta T cell-based immunotherapy for metastatic solid tumours

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BACKGROUND: Adoptive transfer of ex vivo expanded autologous Vγ9Vδ2 T cells may be of therapeutic benefit for cancer because of their potent direct cytotoxicity towards tumour cells, synergistic cytotoxicity when combined with aminobisphosphonates and enhancement of antibody-dependent cell-mediated cytotoxicity.

METHODS: To determine the feasibility and clinical safety of therapy with ex vivo expanded, activated Vγ9Vδ2 T cells in combination with zoledronate, we enrolled 18 subjects with advanced solid tumours into a phase I clinical study. Administered indium111-oxine-labelled Vγ9Vδ2 T cells were tracked in a cohort of patients.

RESULTS: Administered Vγ9Vδ2 T cells had an activated effector memory phenotype, expressed chemokine receptors predictive of homing to peripheral tissues and were cytotoxic in vitro against tumour targets. Adoptively transferred Vγ9Vδ2 T cells trafficked predominantly to the lungs, liver and spleen and, in some patients, to metastatic tumour sites outside these organs. No dose-limiting toxicity was observed, but most patients progressed on study therapy. However, three patients administered Vγ9Vδ2 T cells while continuing previously ineffective therapy had disease responses, suggesting an additive effect.

CONCLUSION: Therapy with aminobisphosphonate-activated Vγ9Vδ2 T cells is feasible and well tolerated, but therapeutic benefits appear only likely when used in combination with other therapies.

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The majority of gamma/delta (γδ) T cells in human peripheral blood are of the Vγ9Vδ2 phenotype and constitute 1–5% of circulating lymphocytes (Carding and Egan, 2002; Chen and Letvin, 2003). Many in vitro and in vivo studies have demonstrated anti-tumour activity of Vγ9Vδ2 T cells, including against renal, prostate, colon and pancreatic cancers and melanoma, myeloma and lymphoma (Kabelitz et al, 2004, 2007; Bonneville and Scotet, 2006; Dieli et al, 2007; Bennouna et al, 2008; Abe et al, 2009). Mechanisms of anti-tumour activity include direct MHC non-restricted killing of tumour cells, antibody-dependent cell-mediated cytotoxicity (ADCC) (Lanier et al, 1985; Braakman et al, 1992; Tokuyama et al, 2008) and indirectly through activation of other immune effectors. There is evidence that suggests that γδ T cell cytotoxicity against a range of tumour cell lines is greater than that achievable with alpha/beta (αβ) T cells, which have been the focus of most trials of cancer vaccines and adoptive immune therapy (Ensslin and Formby, 1991; Lopez et al, 2000; Guo et al, 2005; Liu et al, 2005).

Vγ9Vδ2 T cells have a unique capacity to recognise and be activated and expanded by non-peptide phosphoantigens, including aminobisphosphonate drugs, such as zoledronate and pamidronate. Zoledronate has potent activity in activating and expanding Vγ9Vδ2 T cells, especially in combination with IL-2 (Sato et al, 2005; Kondo et al, 2008), and also sensitises tumour cells to Vγ9Vδ2 T cell cytotoxicity in vitro (Gober et al, 2003; Sato et al, 2005; Marten et al, 2007; Mattarollo et al, 2007; Bouet-Toussaint et al, 2008; Todaro et al, 2009) and in vivo in macaque (Gertner-Dardenne et al, 2009), in addition to having potential direct and indirect anti-tumour effects, including apoptotic and anti-proliferative functions (Senaratne et al, 2006; Tassone et al, 2000; Marten et al, 2007). Zoledronate indirectly inhibits MMP-9 and VEGF that are associated with tumour progression and invasion (Santini et al, 2003; Giraudo et al, 2004). This occurs through inhibition of the enzyme farnesyl pyrophosphate (FPP) synthase of the cellular mevalonate pathway, causing accumulation of isoprenoids, such as isopentenyl pyrophosphate (IPP), which stimulates and activates γδ T cells (Gober et al, 2003; Green et al, 2004). Of the bisphosphonates tested in vitro, zoledronate is the most potent inhibitor of FPP synthase (Clezardin, 2003; Knight et al, 2005). These properties make zoledronate a particularly interesting candidate for use in therapy aiming to harness the anti-tumour activities of γδ T cells (Das et al, 2001; Kato et al, 2001; Gober et al, 2003; Sato et al, 2005; Kondo et al, 2008).
Adoptive transfer of ex vivo expanded γδ T cells (Kobayashi et al, 2007; Bennouna et al, 2008; Abe et al, 2009; Noguchi et al, 2011) and in vivo therapeutic manipulation of γδ T cells by phosphorytans and aminobisphosphonates with low-dose IL-2, have been reported and demonstrate potential anti-tumour activities of γδ T cells in patients with lymphoid malignancies (Wilhelm et al, 2003) and prostate cancer (Dieli et al, 2007). The combination of intravenous pamidronate or zoledronate and IL-2 for lymphoma, myeloma and prostate cancer was generally well tolerated but with side effects including fever, chills and transient flu-like symptoms (Wilhelm et al, 2003; Dieli et al, 2007).

Adoptively transferred ex vivo expanded autologous Vγ9Vδ2 T cells with IL-2 also have limited toxicity, the most frequent adverse event being fever and general fatigue (Kobayashi et al, 2007; Bennouna et al, 2008; Abe et al, 2009). The toxicity of Vγ9Vδ2 T cells expanded with zoledronate and adoptively transferred in combination with a zoledronate infusion is yet to be reported in solid tumour patients.

Although healthy donor Vγ9Vδ2 T cells expand massively when stimulated in vitro by IL-2 in combination with phosphorytans or bisphosphonates (Wilhelm et al, 2003), the in vitro proliferative capacity of Vγ9Vδ2 T cells from patients with malignancy seems less reproducible. For example, in vitro expansion of Vγ9Vδ2 T cells was poor in 50% of lymphoma patients (Wilhelm et al, 2003) and in 25% of renal carcinoma patients (Viey et al, 2005). It is unknown whether this defect in Vγ9Vδ2 T cell proliferation is tumour specific or broadly associated with malignancy, and it is also unknown whether the differences are directly related to differences in Vγ9Vδ2 T cells or due to other cellular constituents in the expansion cultures evaluated.

Adoptive transfer of Vγ9Vδ2 T cells as a therapeutic modality has a number of distinct advantages over active immune therapy with vaccines and direct stimulation of Vγ9Vδ2 T cells in vivo with either pharmaceutical agents or vaccines, but can also be seen as an additional mode of therapy with its own unique set of roles, rather than simply as an alternative to active immune therapy. For example, Vγ9Vδ2 T cells can be adoptively transferred directly after chemotherapy or tumour-targeting monoclonal antibodies, allowing infused cells to provide potential additive or synergistic cytotoxicity timed to coincide with the cytotoxic effects of the pharmaceutical and, in the case of monoclonal antibodies, to provide the potential for enhanced ADCC through CD16/FcR expression of infused cytotoxic cells (Mattarollo et al, 2007; Tokuyama et al, 2008). In contrast, it is difficult to time the maximal activity of cells expanded with vaccines, and chemotherapy administered after vaccination may inhibit proliferation of the desired cell population. The use of IL-2 to expand Vγ9Vδ2 T cells in vivo has the disadvantage of substantial IL-2 toxicity and the potential for expansion of regulatory T cells (Tregs), which may inhibit anti-tumour immunity (although both of these latter advantages are negated if IL-2 is used after adoptive therapy of Vγ9Vδ2 T cells).

In order for adoptively transferred Vγ9Vδ2 T cells to have a therapeutic role, they must traffic to tumour sites. Tumour-infiltrating lymphocytes (TILs) are a type of T cells including colorectal, breast, prostate, ovarian and renal cell carcinomas, suggesting that these cells do have the capacity to infiltrate the tumour environment (Kabelitz et al, 2007). No clinical study has yet addressed the migration pattern of adoptively transferred Vγ9Vδ2 T cells in humans.

In the clinical study described here, the safety and feasibility of adoptive transfer of large numbers of ex vivo expanded autologous Vγ9Vδ2 T cells in combination with zoledronate infusion was investigated in patients with solid tumours. Evaluation of the destination of adoptively transferred Vγ9Vδ2 T cells is also reported. The in vivo effects of zoledronate on Vγ9Vδ2 T cells in peripheral blood were evaluated and localisation of adoptively transferred Vγ9Vδ2 T cells was assessed.

MATERIALS AND METHODS

Patients and healthy donors

Patients (n = 18) and healthy donors (for in vitro studies only, n = 10) were enrolled after providing informed consent. Patients with malignancy were enrolled for in vitro studies alone (n = 27) or for in vitro studies plus therapy with Vγ9Vδ2 T cells and zoledronate (n = 18). Patients enrolled had a range of metastatic solid tumours unresponsive to other therapies. Patient characteristics are summarised in Table 1. The study was approved by the Human Research Ethics Committee of the Greenslopes Private Hospital (Queensland, Australia).

Treatment protocols

Initially, six patients were treated with a dose-escalation protocol (group A). The planned dose-escalation range of Vγ9Vδ2 T cells was 0.5 × 10^7 to 500 × 10^7; however, the maximum dose achieved in this initial group was 280 × 10^7. Subsequently, nine patients received Vγ9Vδ2 T cells at an approximately fixed dose dependent on the proliferative capacity of their cells (group B). Each dose was generated from 1/8 of a single leukapheresis. Three additional patients received Vγ9Vδ2 T cells while continuing previous therapies that had not induced disease responses, but which were well tolerated (chemotherapy in two cases and hormonal therapy in one case) (group C).

Initial findings from the first three patients in the dose-escalation phase of the study indicated that zoledronate pretreatment substantially reduced Vγ9Vδ2 T cell number and expansion capacity. Therefore, the following 15 subjects underwent leukapheresis procedures before zoledronate administration and this initial dose was omitted. The zoledronate dose was split to administer a first dose (1 mg per treatment) 24 h before cell administration and a second 1 mg dose immediately before cell administration. This was based on our in vitro time course studies showing that zoledronate gives rapid but transient tumour sensitisation to Vγ9Vδ2 T cell killing in some cases, but that 24 h was required before maximal tumour sensitisation develops in other cases (Mattarollo et al, 2007).

Clinical responses

Computed tomography (CT) scanning was used to evaluate treatment response. Complete remission (CR), partial remission (PR), stable disease (SD) or progressive disease (PD) were determined based on the RECIST criteria (Response Evaluation Criteria In Solid Tumors) (Therasse et al, 2000). During treatment, symptoms, clinical evaluation and haematological and biochemical evaluation of blood were used to monitor adverse events.

Immunological monitoring

The following monoclonal antibodies for evaluating cell phenotypes using flow cytometry were obtained from Beckman Coulter (Fullerton, CA, USA): CD3 (UCHT1), CD4 (13B8.2), CD8 (SFCl21Thy2D3), CD69 (TP1.55.3), CD56 (N901), CD27 (1A4CD27), CD45RA (2H4DH11LDB9), CD45 (J33), TCR-γδ (IMMU360), TCR-Vδ2 (IMMU389) and TCR-pan γδ (IMMU510). Monoclonal chemokine receptor antibodies CCR5 (CTC5),CCR7 (150.503), CXCR3 (49.801) and CXCR5 (51.505.111) were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Cell number was assessed by addition of flow-count beads (Beckman Coulter), and cell viability was determined by exclusion with 7-AAD (BD Biosciences, San Jose, CA, USA). Cells were stained according to the manufacturers’ recommendations. All flow-cytometric analyses were performed using the Coulter Cytomics FC500 five-colour flow cytometer (Beckman Coulter).
Proliferation and preparation of Vγ9Vδ2 T cells

Cells for adoptive transfer were generated under good manufacturing practice (GMP) conditions in purpose-built GMP laboratories within the University of Queensland laboratories at the Greenslopes Private Hospital. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) and Vγ9Vδ2 T cells selectively proliferated by culture of PBMCs in RPMI 1640 media (Lonza, Walkersville, MD, USA) supplemented with 10% human AB plasma (Lonza),L-glutamine (2 mM; Lonza) and human AB plasma (Lonza),L-glutamine (2 mM; Lonza) and recombinant human IL-2 (350 IU ml−1; Novartis, Basel, Switzerland) and zoledronate (1 μM; Novartis) were added on day 0 and additional IL-2 (350 IU ml−1) was added every 2–3 days during the culture period. After 7–14 days culture, purified effector cell populations containing 70–95% Vγ9Vδ2 T cells were obtained for in vitro functional assessment by depletion of CD4+, CD8+ and CD56+ cells using miniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Cell populations for adoptive transfer were not purified, but were enriched by the culture procedure. The percentage of Vγ9Vδ2 T cells and treatment clinical outcomes

Table 1: Characteristics of patients, ex vivo expansion of patients’ Vγ9Vδ2 T cells and treatment outcomes

| Patient | Age (years)/sex | Primary cancer | Metastasis | Previous therapy | Previous Zol. treatment | After ex vivo expansion | Expansion fold | Max. dose/ treatment (×10^6 cells) | Total dose (×10^6 cells) | Toxicity | Clinical response | Comment |
|---------|----------------|----------------|------------|------------------|-------------------------|------------------------|---------------|-----------------------------------|--------------------------|----------|-------------------|---------|
| Group A (GDT dose escalation/Zol. treatment) |
| A1 | 58/F | Melanoma | Lung | | Yes | 0.4 (2.0) | 8.9 (2.8) | 28 (13) | 8 | 0.04 | 0.1 | Yes | PD |
| A2 | 59/M | Melanoma | Lung | | Yes | 2.4 (3.0) | 23.5 (4.0) | 8 (2) | 8 | 0.2 | 0.5 | No | SD |
| A3 | 66/F | Melanoma | Lung, liver | I | Yes | 0.5 (0.7) | 20.3 (4.8) | 95 (24) | 8 | 0.6 | 2.0 | No | PD |
| A4 | 65/F | Ovarian cancer | Peritoneum | C | No | 5.7 (0.3) | 62.3 (5.0) | 34 (7) | 8 | 1.5 | 3.5 | No | SD |
| A5 | 67/F | Melanoma | Abdomen | | No | 1.3 (0.7) | 55.7 (4.3) | 262 (81) | 8 | 2.3 | 5.0 | No | PD |
| A6 | 56/F | Colon cancer | Lung, liver | | No | 11.1 (2.8) | 85.8 (4.5) | 47 (11) | 8 | 2.8 | 5.5 | Yes | PD |

Group B (GDT non-dose escalation/Zol. treatment) |
| B1 | 67/M | Melanoma | Adrenal gland, heart | | No | 0.3 (0.1) | 15.3 (2.2) | 728 (111) | 6 | 0.3 | 1.0 | No | SD |
| B2 | 48/F | Adenocarcinoma | Bone | R | No | 2.1 (0.5) | 53.6 (9.9) | 144 (72) | 8 | 0.5 | 1.1 | Yes | PD |
| B3 | 47/M | Cholangiocarcinoma | Local advanced disease | | No | 1.8 (0.1) | 59.5 (4.8) | 17 (2) | 8 | 0.4 | 1.4 | No | PD |
| B4 | 65/F | Melanoma | Lung, abdominal mass | | No | 0.5 (0.1) | 12.3 (1.9) | 159 (84) | 8 | 0.5 | 1.4 | No | NE |

Group C (GDT/Zol. treatment with other therapy) |
| C1 | 58/F | Breast cancer | | | No | 0.8 (0.0) | 71.4 (6.6) | 586 (273) | 7 | 1.0 | 1.7 | No | PD |
| C2 | 61/F | Ovarian carcinoma | Peritoneum | | No | 5.1 (0.7) | 86.6 (2.0) | 43 (7) | 8 | 1.0 | 3.0 | No | PD |
| C3 | 51/F | Colon cancer | Lung, liver | C, R, I | No | 2.6 (0.3) | 70.0 (3.8) | 86 (14) | 8 | 0.8 | 3.3 | Yes | PD |

Abbreviations: C = chemotherapy; CR = complete remission; γδ T = Vγ9Vδ2 T cell; H = hormonal therapy; I = immunotherapy; inj. = injection; NE = not evaluable; PD = progressive disease; PR = partial remission; R = radiotherapy; S = surgery; SD = stable disease; Zol = Zoledronate; *Represents the mean (s.e.) from 6–8 vaccines. 1Fever after infusion, A1 also had vomiting. 2Large bulk of disease but stable. 3No new lesions. 4With chemotherapy. 5With hormonal therapy.
17 MBq. Patients received an infusion of $5 \times 10^7$-labelled $\gamma$Vd2 T cells through a peripheral intravenous (IV) line. Full-body $\gamma$-imaging was performed using a dual-headed $\gamma$-camera (20% energy windows $\sim$173 and $247$ keV) to monitor the location of labelled $\gamma$Vd2 T cells serially for 48 h starting within 30 min of injection ($t = 0$ h) and then at 1, 4, 8, 24 and 48 h after injection. Cell accumulation in different organs was scored on a scale from 0 to 4: 0 = no accumulation, 1 = minimal accumulation, 2 = low accumulation, 3 = medium accumulation and 4 = large accumulation.

Statistical analysis

All statistical analyses were performed using Student’s $t$-test and results were considered significant if $P < 0.05$.

RESULTS

Phenotype and cytotoxic activity of in vitro expanded $\gamma$Vd2 T cells

The characteristics of $\gamma$Vd2 T cells in peripheral blood and after in vitro expansion for healthy donors ($n = 10$) and patients with active, metastatic cancer ($n = 45$) are summarised in Figure 1. Additional details for the subset of cancer patients ($n = 18$) involved in the treatment phase of this study are summarised in Table 1.

In comparison with healthy donors, patients with active cancer, as a group, had significantly lower $\gamma$Vd2 T cells as a percentage of T cells ($P < 0.01$; Figure 1A), lower percentages of $\gamma$Vd2 T cells in a potential therapeutic product after culture ($P < 0.01$; Figure 1B) and lower numbers of $\gamma$Vd2 T cells that could be generated from a starting point of $1 \times 10^6$ PBMCs ($P < 0.05$; Figure 1C). Although the expansion capacity of $\gamma$Vd2 T cells was similar for cancer patients as a group compared with healthy individuals (Figure 1D), the cancer patient population was highly variable with respect to the expansion potential of $\gamma$Vd2 T cell numbers, and this is reflected in substantial variation in the purity of the potential $\gamma$Vd2 T cell therapeutic product (Figure 1B) and the total number of $\gamma$Vd2 T cells that could be generated (Figure 1C). The percentage of T cells that were $\gamma$Vd2 T cells before expansion culture predicted for both the purity of the final product (Figure 1E) and the total number of $\gamma$Vd2 T cells that could be generated (Figure 1F). The relative number of $\gamma$Vd2 T cells from different subsets before culture was predictive for $\gamma$Vd2 T cell expansion capacity, with high numbers of Tn + Temra predicting for poor expansion (Figure 1G) and the patient subgroups, defined as high and low responders, as shown in Figure 1B and D, differing with respect to their $\gamma$Vd2 T cell subset profile.

Patient clinical factors found to predict for the capacity to generate large numbers of $\gamma$Vd2 T cells included whether there had been previous treatment with zoledronate (Figure 2A) and tumour type (melanoma vs non-melanoma, Figure 2B). Patients who had received zoledronate at any time before collection of blood samples had lower initial $\gamma$Vd2 T cell numbers, less $\gamma$Vd2 T cells as a fraction of all lymphocytes, lower proliferative potential and a lower final number of $\gamma$Vd2 T cells achievable (Figure 2A). A group, patients with melanoma had lower initial $\gamma$Vd2 T cells and lower final numbers of $\gamma$Vd2 T cells generated, despite no patients having been exposed to zoledronate (Figure 2B). The lack of exposure to zoledronate may explain the greater proliferative capacity of melanoma patients, as a group, than patients with other malignancies of whom many had received zoledronate.

Our method of in vitro culture generated $\gamma$Vd2 T cells with purity of up to 90% after 14 days (without an additional purification step, Figure 1B and 3A). CD69 expression on $\gamma$Vd2 T cells increased significantly after in vitro expansion, indicating an activated phenotype (Figure 3B). The majority of the expanded
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Figure 2 Proliferation of V9Vδ2 T cells cultured in vitro with zoledronate and IL-2 for 14 days. Results of total cell numbers obtained are derived from a fixed starting number of PBMCs. (A) Previous treatment with zoledronate (n = 6) vs no previous treatment with zoledronate (n = 12). (B) Melanoma patients (n = 7) vs other cancer patients (n = 11) (mean ± s.e.m., **P < 0.01, *P < 0.05) (γδ T = V9Vδ2 T cells).

Figure 3 Phenotype and functional activity of ex vivo expanded patient V9Vδ2 T cells. (A) Representative flow cytometry dot plot showing selective expansion of V9Vδ2 T cells after 14 days culture in zoledronate and IL-2. (B) CD69 expression on V9Vδ2 T cells (MFI value) after in vitro culture (mean ± s.e.m.; n = 10). (C) Relative proportions of V9Vδ2 T cell subsets during the in vitro culture period (mean ± s.e.m.; n = 7). (D) Cytotoxicity of expanded V9Vδ2 T cells against various solid tumour cell lines (mean ± s.e.m.). Means are derived from separate killing assays using V9Vδ2 T cells from 3 to 5 subjects. (E) Chemokine receptor profiles (peripheral-homing CCR5 and CXCR3; lymph node-homing CCR7 and CXCR5) of V9Vδ2 T cells during in vitro culture (mean ± s.e.m.; n = 4) (γδ T = V9Vδ2 T cells).

V9Vδ2 T cell population from normal donors and patients in the high-responder group were of the effector memory (Tem) and of the central memory (Tcm) phenotype (Figure 3C). Correlating with the effector phenotype observed using surface marker analysis, cultured V9Vδ2 T cells were cytotoxic against a range of solid tumour cell lines in vitro, including HT29, DLD-1, NCI-H358 and TSU-Pr1 (Figure 3D). In vitro expanded V9Vδ2 T cells had upregulated expression of peripheral tissue-homing chemokine receptors, CCR5 and CXCR3. In contrast, expression of lymphoid-homing receptors, CCR7 and CXCR5, decreased to undetectable levels (Figure 3E). These results show that V9Vδ2 T cells expanded in vitro from cancer patients have effector cell characteristics including the capacity to effectively kill tumour targets and chemokine receptor expression profiles, suggesting the...
potential to migrate to peripheral tumour sites, although potentially not to disease-involved lymph nodes.

The population of cells infused to patients was predominantly V9Vd2 T cells as shown in Table 1. The other cells infused (expressed as a mean, s.d. and range) included NK cells (18 ± 22%, range 0.5–75%) CD4+ T cells (6 ± 6%, range 0.5–24%) and CD8+ T cells (17 ± 16%, range 2–64%). We did not directly evaluate for co-expression of the V9Vd2 T cell receptor and CD56 (as separate analysis tubes were used), but V9Vd2 T cells are distinguishable in the context of these cultured cells from most αβ T cells (either CD4+ or CD8+) as they are CD3 positive but double negative for CD4 and CD8. The majority of cells administered were both V9Vd2 TCR positive and CD4CD8 double negative. With these caveats, our results indicate that a substantial proportion (about half) of the administered V9Vd2 T cells are CD56+ (49 ± 23%, mean ± s.d., range 27–73%).

Clinical outcomes of V9Vd2 T cell administration

Details of administered V9Vd2 T cells, including cell numbers, purity, possible treatment-related adverse effects and clinical outcomes are summarised in Table 1. For preliminary assessment of safety and maximal tolerated doses of intravenous infusion of in vitro expanded V9Vd2 T cells, six patients (four melanoma, one ovarian cancer, one colon cancer) were treated with escalating doses of V9Vd2 T cells. In this group, the maximum V9Vd2 T cell dose per injection ranged from 1.0 × 10^6 to 2.8 × 10^7. The total accumulated dose of V9Vd2 T cells over the treatment period ranged from 0.1 × 10^7 to 5.5 × 10^7 with a mean of 2.8 × 10^7. Maximal single dose and total dose were much lower than those predicted from the preclinical evaluation (before zolendronate administration) in the first three subjects because of markedly reduced expansion of V9Vd2 T cells from harvests undertaken after zolendronate administration.

The maximum dose of V9Vd2 T cells per treatment in the subsequent non-dose-escalation protocol (n = 12) ranged from 0.3 × 10^7 to 2.2 × 10^7. The total doses of V9Vd2 T cells administered ranged from 1.0 × 10^8 to 7.2 × 10^8 with a mean of 2.8 × 10^8, from a total of 6–8 treatments. Three patients (two breast cancer and one cervical) were treated with concurrent use of other therapy (two with chemotherapy and one with hormone therapy).

Of the 18 patients treated, 7 had fevers above 38 ºC believed to be related to study therapy. Overall, side effects were manageable, tolerated by patients, did not interfere with their treatment and resolved within 24 h.

Treatment outcome

Clinical response to treatment is summarised in Table 1. In the group of 15 patients with advanced cancer treated only with V9Vd2 T cells plus zolendronate, 3 had SD while 12 patients had PD during the study period. Although difficult to assess with their generally poor outcomes, there seemed no correlation between maximum or total dose of V9Vd2 T cells and clinical outcome. For example, patient A2 had large bulky disease, progressing before enrolment, but was stable during treatment and for a prolonged period after therapy was completed.

Three additional patients were assessed for treatment outcomes although their V9Vd2 T cells were administered in parallel with other therapies. All three of these patients experienced at least a partial response and one (breast cancer) had a complete response. Although it is not possible to know whether V9Vd2 T cells contributed to these responses, inclusion criteria dictated that subjects enrolled in our study were considered unlikely to respond to standard therapy. For example, patient C2 with breast cancer was non-responsive to hormonal therapy alone but had CR when hormonal therapy was combined with V9Vd2 T cell/zolendronate infusions. Patient C1 had PD when treated with chemotherapy alone before enrolment but had a PR and substantial symptomatic improvement when V9Vd2 T cell/zolendronate infusions were added to the same chemotherapy protocol. Patient C3, who presented with a rapid and florid relapse soon after cessation of previous chemotherapy, had a partial response to the combination of further chemotherapy and V9Vd2 T cell/zolendronate infusions.

In vivo distribution and tumour localisation of adoptively transferred V9Vd2 T cells

To establish the migratory pattern of V9Vd2 T cells in vivo, trafficking of in vitro expanded V9Vd2 T cells was investigated in a cohort of patients (n = 3).

In all patients administered In111-labelled V9Vd2 T cells (patients A2, B1, B7, 5 × 10^7 V9Vd2 T cells per dose), rapid migration to the lungs occurred, where cells remained for 4–7 h. During this period, cell numbers (according to γ-activity) in the lungs then slowly decreased with gradual migration into the liver and spleen. Our previous studies have shown that this pattern does not relate to movement of free indium that might be released from cells after administration (Nieda et al, 2004). After 24 h, almost all cells were located in the liver and spleen and virtually no activity remained in the lungs. Although the timing of cell migration varied slightly, this pattern was consistent for all patients. Predominant accumulation of radioactivity in the lung, liver and spleen was consistent with a previous observation investigating initial localisation of tumour-specific αβ T cells (Meidenbauer et al, 2003) and there was no blood pooling (as indicated by the absence of significant radioactivity within the cardiac shadow), indicating egress of V9Vd2 T cells from the circulating blood pool. Repeat treatments with labelled V9Vd2 T cells gave the same pattern in all patients assessed more than once, including subjects B1 and A2 described below.

Assessment of the number of peripheral blood V9Vd2 T cells at multiple time points in the 48 h after V9Vd2 T cell infusion, mirroring the time points at which we evaluated V9Vd2 T cell by indium labelling, showed no substantial change in the number of peripheral blood V9Vd2 T cells compared with pre-infusion levels. These data are consistent with the nuclear medicine data indicating that few of the V9Vd2 T cells remain in the bloodstream.

In patient B1, who presented with an 84 × 57 × 75 mm³ metastatic mass on the left adrenal gland, a proportion of In111-labelled V9Vd2 T cells appear to have migrated to the tumour by 1 h after infusion. Maximal activity was seen in the tumour area at 4 h and tracer (and presumably intact cells) remained in the metastatic tumour site for the remainder of the 48 h observation period (Figure 4A–C). Accumulation of labelled cells in the tumour followed a different time course to that in other organs (Figure 4D). In patient A2, who presented with 89 mm and 26 mm tumours in the left lung, accumulation of labelled cells at the 89 mm tumour site was also observed at 4 h, but this activity was more subtle than for patient B1 (data not shown) suggesting lower numbers of cells within the tumour site.

DISCUSSION

The phase I trial described here indicates that combination therapy involving V9Vd2 T cells with zolendronate is feasible in patients with advanced solid tumours and is well tolerated. Our results highlight a number of practical issues that need addressing in future studies, including ways of increasing the proportion of patients who may benefit from this treatment, the need for more studies evaluating the destination of infused cells and the need to evaluate V9Vd2 T cells in combination with other therapies. Despite the technical demands of generating V9Vd2 T cells for adoptive therapy, adoptive therapy is ideal for evaluating combinations of V9Vd2 T cells with other therapeutic modalities.
as administration of Vγ9Vδ2 T cells can be appropriately timed to ensure maximal synergy and avoidance of chemotherapeutic damage to Vγ9Vδ2 T cells.

Our study confirms that in vitro generation of Vγ9Vδ2 T cells for adoptive therapy is achievable in many cancer patients, despite advanced disease or previous chemotherapy. However, to maximise the potential for a therapeutic benefit from Vγ9Vδ2 T cell adoptive immune therapy higher cell doses may be required. Minimal therapeutic benefits were observed at the doses used in our study. To evaluate potential benefits of higher cell doses, factors inhibiting Vγ9Vδ2 T cell number and in vitro expansion capacity in cancer patients need to be addressed. Furthermore, ways to ensure Vγ9Vδ2 T cell survival (and possibly additional in vivo expansion), trafficking to tumour sites and retention of cytotoxic activity after infusion need to be explored.

In the initial stages of our study, depletion of circulating Vγ9Vδ2 T cell numbers and in vitro expansion capacity were repeatedly observed after a single dose of zoledronate (data not shown). The changes were so marked that a protocol change was necessitated, avoiding zoledronate administration before cell harvesting. Similar aminobisphosphonate-induced decreases in Vγ9Vδ2 T cell numbers have been reported in patients with prostate cancer (Dieli et al, 2007) and in primates (Cendron et al, 2007). In our patient samples, low baseline percentages of Vγ9Vδ2 T cells and increased proportions of Tn+Temra subsets (as a fraction of total preculture T cells) predicted for low numbers of Vγ9Vδ2 T cells achieved after culture (data not shown).

Regulatory T cells may also negatively regulate Vγ9Vδ2 T cell proliferation. Regulatory T cells dampen T cell immunity (Zou, 2006) and are known to inhibit immune responsiveness in patients with advanced malignancy (Gallimore and Godkin, 2008). The percentage of Tregs in the blood of melanoma patients is significantly higher than that for healthy donors (Cesana et al, 2006), and we observed poor Vγ9Vδ2 T cell expansion in melanoma patients in our study. Our preliminary data (unpublished observations) and previous reports (Kabelitz et al, 2007; Kunzmann et al, 2009) indicate that depletion of Tregs from patient mononuclear cells leads to greater Vγ9Vδ2 T cell expansion in vitro.

Depletion of Tregs, either in vivo before harvest or in vitro and cryopreservation of large numbers of PBMCs before aminobisphosphonates, may be a pre-requisite for successful utilisation of Vγ9Vδ2 T cells for adoptive cell therapy in subjects who may receive aminobisphosphonates as part of their standard therapy for bone disease or as part of an immunotherapeutic strategy.

Efficient migration into tumour sites of actively cytotoxic subsets is a prerequisite for adoptively transferred Vγ9Vδ2 T cells to be therapeutically useful in cancer treatment. We demonstrated that Vγ9Vδ2 T cells we infused, which contained a range of subsets, had cytotoxic activity in vitro and included a substantial proportion of Vγ9Vδ2 T cells predicted to have cytotoxicity by virtue of CD56 expression (Thedrez et al, 2009; Urban et al, 2009). However, as we did not select subsets for our trafficking studies or our cytotoxicity assays, we do not know whether those cells eliciting in vitro cytotoxicity were those that migrated to the tumour sites.

Figure 4 Localisation of adoptively transferred in vitro expanded Vγ9Vδ2 T cells. (A) Anterior γ-camera images of the chest and abdomen at various time points after In111-labelled Vγ9Vδ2 T cell transfer (5 × 10⁶ cells). Arrows point to location of tumour. (B and C) Abdominal CT scan (B) showing the tumour mass, the outline of which is superimposed onto the 4 h γ-image (C) to show Vγ9Vδ2 T cell activity at the tumour site. (D) Accumulation of Vγ9Vδ2 T cells in different organs over time, scored on a scale of 0 to 4, as described in the ‘Materials and Methods’ section (γd T = Vγ9Vδ2 T cells).
Increased effector memory γδ T cells are reported to correlate with objective clinical outcomes in patients treated with zoledronate and IL-2 (Diel et al., 2007). The culture conditions we used generated large numbers of effector memory Vγ9Vδ2 T cells from the blood of cancer patients. In addition, as a surrogate assessment to predict the capacity for in vitro activated Vγ9Vδ2 T cells to migrate to tumour tissue, chemokine receptor expression was investigated. Expression of CCR5 and CXCR3 by effector T cells is reported to be important for trafficking to tumour sites as these receptors respond to a range of chemokines such as I-TAC, MIG, IP-10 (ligands for CXCR3) and RANTES, MIP-1α, MIP-1β, MCP-2 (ligands for CCR5) released by tumours and inflammatory tissue (Moser and Loetscher, 2001). Vγ9Vδ2 T cells from healthy donors are reported to express high constitutive levels of CCR5, but this is downregulated within 24–48 h of IFN stimulation in parallel with upregulation of the lymphoid-homing chemokine receptor, CCR7 (Glatzel et al., 2002; Brandes et al., 2003). Although a similar pattern of initial CCR5 downregulation and CCR7 upregulation was observed with our zoledronate- and IL-2-containing cultures (data not shown), after further Vγ9Vδ2 T cell expansion, there were large increases in both CCR5 and CXCR3 expression, peaking at days 7–10. Minimal changes in CCR7 and CXCR3 expression were observed, with levels remaining low at the end of the culture period.

The results of the trafficking studies with activated Vγ9Vδ2 T cells reported here indicate localisation of Vγ9Vδ2 T cells at a large tumour site from as early as 1 h after infusion, with maximal activity at 4 h and persistence for at least 48 h. Although only a minority of the administered cells localised to the tumour, this is the first direct demonstration of adoptively transferred Vγ9Vδ2 T cell localisation at a tumour site with concurrent use of zoledronate. Although these results are encouraging, further work is required to characterise the properties of Vγ9Vδ2 T cells that migrate to the tumour and to increase the proportion of Vγ9Vδ2 T cells that migrate to tumour sites. The overall migration pattern we observed, in which initial, transient retention in the lungs is followed by movement predominantly to the liver and spleen, has been observed previously for other lymphocyte populations and is not specific to Vγ9Vδ2 T cells (Fisher et al., 1989; Nieda et al., 2004). It is unknown to what extent accumulation of activity in the lungs, liver and spleen indicates active homing of viable Vγ9Vδ2 T cells rather than passive ‘trapping’. It is also unclear whether Vγ9Vδ2 T cells infiltrate the parenchyma (providing an opportunity for therapeutic benefits for tumours in these organs) or remain within the lumen of blood vessels. More sensitive imaging technology is required to answer these questions.

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Evaluation of clinical responses was not a major end point of the trial but clearly no disease responses (other than stabilisation) were observed in the 15 patients treated with only the Vγ9Vδ2 T cell/zoledronate combination. However, of potential interest, was the observation of clinical responses in three of three patients in whom conventional therapy was used in parallel with Vγ9Vδ2 T cell/zoledronate therapy (one CR in combination with ongoing hormone therapy after failure of hormone therapy alone and two PR in combination with chemotherapy, including one patient with chemorefractory disease and another patient with very early relapse after previous chemotherapy). These clinical observations are consistent with in vitro studies indicating that the combination of Vγ9Vδ2 T cells with chemotherapy produces significantly greater tumour cell death than when either modality of treatment is used alone (Matarollo et al., 2007). Of added interest are the previous in vitro observations of synergistic cytotoxic effects of CD16 expressing Vγ9Vδ2 T cells with therapeutic monoclonal antibodies (Tokuyama et al., 2008).

Previously, successful studies of adoptive immune therapy have included the use of IL-2 after cell therapy administration. The withdrawal of exposure to high concentrations of IL-2 used in the laboratory may limit the survival and function of Vγ9Vδ2 T cells after their infusion. We did not administer IL-2 to patients in our study to evaluate the toxicity profile of Vγ9Vδ2 T cells but this may have limited the potential for therapeutic benefits.

In summary, the combination of zoledronate and IL-2 generates large numbers of Vγ9Vδ2 T cells in vitro with cytotoxic activities against a range of tumour types, even in heavily pretreated patients with advanced malignancy. Administration of these cells is safe. Administered cells have a phenotype suggesting the potential to migrate to tumour tissues and we provide preliminary clinical evidence for migration of Vγ9Vδ2 T cells to tumour sites. As cells similar to those administered in this study have previously been shown to enhance the cytotoxic effects of chemotherapeutic agents and monoclonal antibodies, we propose further studies of zoledronate-activated Vγ9Vδ2 T cells in combination with chemotherapy and monoclonal antibodies.

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