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*J Immunol* 2013; 191:1993-2000; Prepublished online 8 July 2013;
doi: 10.4049/jimmunol.1300255
http://www.jimmunol.org/content/191/4/1993

Supplementary Material  http://www.jimmunol.org/content/suppl/2013/07/08/jimmunol.1300255.DC1

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Repeated Systemic Administrations of Both Aminobisphosphonates and Human \( \gamma \delta \) T Cells Efficiently Control Tumor Development In Vivo

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Peripheral \( \gamma \delta \) T lymphocytes compose a major \( \gamma \delta \) T cell subset in primates with broad reactivity against tumor cells. \( \gamma \delta \) T cells are specifically activated by phosphorylated isoprenoid pathway metabolites called “phosphoagonists.” Accordingly, pharmacologic inhibitors of the mevalonate pathway, such as aminobisphosphonates (NBP) that upregulate the intracellular production of phosphoagonists, increase antitumor \( \gamma \delta \) T cell responses. Immunotherapeutic protocols exploiting GMP-grade agonist molecules targeting human \( \gamma \delta \) T lymphocytes have yielded promising, yet limited, signs of antitumor efficacy and therefore need to be improved for next-generation immunotherapies. In this study, we used a model of s.c. human tumor xenografts in severely immunodeficient mice to assess the antitumor efficacy of systemic NBP treatments when combined with the adoptive transfer of human \( \gamma \delta \) T cells. We show that infusion of \( \gamma \delta \) T cells, 24 h after systemic NBP treatment, efficiently delays tumor growth in mice. Importantly, our results indicate efficient but transient in vivo NBP-induced sensitization of tumor cells to human \( \gamma \delta \) T cells. The Journal of Immunology, 2013, 191: 1993–2000.

One of the best-studied \( \gamma \delta \) T cell subset in humans expresses a particular combination of TCR variable regions, \( \gamma \delta \) and \( \delta \). \( \gamma \delta \) T cells are mainly localized in the peripheral blood of human adults, where they represent several percent of the whole lymphoid pool (1–3). Although this \( \gamma \delta \) T cell subset is also found in the peripheral blood of most non-human primates, it has no counterpart in rodents. \( \gamma \delta \) T cells recognize in vitro a wide array of infected or transformed cells and are activated in vivo in various infectious and tumor contexts (4). The widespread reactivity of \( \gamma \delta \) T cells is contact-dependent and involves \( \gamma \delta \) TCR and nonclonal receptors (e.g., NKR, TLRs). Following antigenic activation, \( \gamma \delta \) T cells display rapid and broad functional responses, such as IL-2-dependent proliferation, cytotoxicity, and proinflammatory cytokine release.

Most human \( \gamma \delta \) T lymphocytes are strongly and specifically activated by small nonpeptidic phosphorylated intermediates, referred to as “phosphoagonists” (PAg), of the mammalian mevalonate or the microbial deoxysulpholose-phosphate pathways (5–7). Enhanced tumor cell recognition by \( \gamma \delta \) T cells correlates with upregulation of intracellular PAg levels resulting from increased cell metabolism and cholesterol biosynthesis (8). Accordingly, pharmacologic mevalonate pathway inhibitors acting upstream or downstream of PAg biosynthesis, respectively, decrease or increase \( \gamma \delta \) T cell activation. Among these compounds, GMP-grade aminobisphosphonates (NBPs), which have found widespread use in bone metabolism disorders, have been identified as potent inhibitors of the synthesis of both farnesyl and geranylgeranyl lipidic residues by blocking farnesyl pyrophosphate synthase (reviewed in Ref. 9). Treatment of human cells with high pinocytic activity (e.g., monocytes, macrophages, dendritic cells) or dysregulated metabolism (e.g., transformed tumor cells) by NBPs, like pamidronate and zoledronate, leads to endogenous PAg accumulation and sensitizes treated cells for recognition by human \( \gamma \delta \) T lymphocytes (10).

The absence of murine counterparts of \( \gamma \delta \) T cells and lack of tumor models in nonhuman primates have dramatically hampered assessment of the in vivo physiologic role and immunotherapeutic potential of this \( \gamma \delta \) T cell subset. Decreased numbers and hyporesponsiveness of peripheral \( \gamma \delta \) T cells, associated with low production of proinflammatory cytokines, have been reported in patients with cancer (11, 12). Such defects might result in impaired immune surveillance against tumors. These observations, as well as the high interindividual conservation of human

Received for publication January 25, 2013. Accepted for publication June 7, 2013.

This work was supported by INSERM, Université de Nantes, Association for the Recherche sur le Cancer (R10139NN), Institut National du Cancer (V9V2THER), Investissements d'Avenir (Agence Nationale de la Recherche - Programme Laboratoires d’Excellence Immunotherapy Graft Oncology), and Cancéropôle Grand-Ouest.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ApppI, triphosphoric acid 1-adenosin-5’-yl ester 3-(3-methylbut-3-enyl) ester; IPP, isopentenyl pyrophosphate; NBP, aminobisphosphonate; NSG, NOD-SCid-IL2rgtm1Wjl/SzJ; PAg, phosphoagonist; rhIL-2, recombinant human IL-2.

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peripheral Vγ9Vδ2 T cells, their lack of MHC restriction, and their ability to both directly lyse a broad array of tumor cells in vitro and to secrete adjuvant cytokines (e.g., IFN-γ), provide a strong rationale for Vγ9Vδ2 T cell–based cancer immunotherapies (reviewed in Ref. 13). In this regard, adoptive transfer of autologous human Vγ9Vδ2 T cells, previously activated and expanded ex vivo with PAg and recombinant human IL-2 (rhIL-2), has been performed in tumor-xenografted immunodeficient mouse models (14) and in cancer patients with solid tumors (15–17). Repeated infusions of Vγ9Vδ2 T cells, either alone or with rhIL-2, were well tolerated and yielded promising signs of antitumor efficacy. Active immunotherapies relying on in vivo activation and expansion of peripheral Vγ9Vδ2 T cells by GMP-grade agonists (either synthetic PAg [BrHPP/IPH101 Phosphomid]) (18) or NBPs (e.g., zoledronic acid [Zometa]) have also been evaluated in non-human primates and phase I/II trials in patients with hematopoietic or solid tumors (19–21). Specific expansions and maturation to effecter phenotype of peripheral Vγ9Vδ2 T cells have been achieved after injection of either soluble PAgS or NBPs and rhIL-2, with limited toxicity and promising antitumor efficacy. However, systemic Vγ9Vδ2 T cell expansions induced by these protocols were transient, and exhaustion of these proliferative responses was observed after repeated treatments (22). Therefore, despite encouraging results in terms of feasibility, tolerance, and antitumor efficacy, strategies targeting peripheral human Vγ9Vδ2 T cells need to be optimized. In this study, we show that systemic injection of NBP enhances the antitumor efficiency of adoptively transferred Vγ9Vδ2 T cells in xenografted immunodeficient mice. We also provide evidence that repeated treatment with both NBP and γδ T cells is required to achieve efficient control of tumor growth in this in vivo model. Altogether, these results provide new insights into the design of more efficient next-generation Vγ9Vδ2 T cell–based immunotherapies.

Materials and Methods
Abs and flow cytometry
The following mAbs were obtained from BD Biosciences (Le Pont de Claix, France): FITC–anti-CD107a (clone HA43) and PE–anti-TCRVγ9 (clone 7B6). The following mAbs were obtained from Beckman Coulter (Villepinte, France): FITC–anti-TCRVδ2 (clone IMMU389), PE–cytin 3–anti-CD3ε (clone UCHT1) and purified anti-human TCR pan γδ (clone IMMUS10). Allophycocyanin–anti-IFN-γ (clone B27) was obtained from BD Biosciences and used for intracellular stainings. Flow cytometry data were collected on a FACSCalibur cytometer (BD Biosciences) and analyzed with CellQuest Pro (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) software.

Reagents
t-glutamine, leukoagglutinin, streptomyacin, penicillin, monensin A, PMMA, imonycin, collagenase type IV, DMEM, and RPMI 1640 medium were purchased from Sigma-Aldrich (St. Louis, MO). rhIL-2 (ProLeukin) was obtained from Chiron (Emeryville, CA). Pamidronate was obtained from Novartis (Basel, Switzerland) and supplied with tumor cells at 37˚C in complete RPMI 1640 medium. As positive controls, nonspecific activations of Vγ9Vδ2 T cells were induced by a combination of PMA and ionomycin used, respectively, at 1 μM and 0.5 μg/ml. The cytolytic activity and cytokine production of activated Vγ9Vδ2 T cells were measured by flow cytometry. Briefly, Vγ9Vδ2 T cells were cocultured with tumors at 37˚C in the presence of 10 μM monensin and fluorochrome-labeled anti-CD107a mAb. After 4 h, γδ T cells were harvested and stained with fluorochrome-labeled anti-Vγ9 TCR mAb. When indicated, intracellular stainings of IFN-γ were performed within the same samples.

Tumor xenographs and adoptive transfers of γδ T cells in immunodeficient mice
All experiments involving animals, including their housing and care in pathogen-free conditions and experimental protocols, were conducted in accordance with the relevant laws and institutional guidelines, and were approved by the local ethics committee. Sex-matched NOD.Cg-PkdcsidcR2gndm/WiSl/SJ (NSG) mice were purchased from Charles River Laboratories (Wilmington, MA) and used at 6–10 wk of age. For tumor transplantation assays, the indicated numbers of human PC3 cells (1 to 10 ×10^6 cells) were reseeded in sterile PBS and injected s.c. in NSG mice. Tumor size was weekly calculated by external measurement of the diameter of s.c. tumor xenographs using a Vernier caliper. At day 14 after inoculation of tumor cells, when tumor diameter had reached a diameter >5 mm, mice were assigned randomly to four treatment groups (n = 6 mice/group): placebo (sterile PBS), pamidronate (50 μg/kg i.v. at day 14), Vγ9Vδ2 T cells (1 ×10^6 i.v. at day 15), and pamidronate plus Vγ9Vδ2 T cells (50 μg/kg pamidronate i.v. at day 14 and 1 ×10^6 Vγ9Vδ2 T cells i.v. at day 15). When indicated, pamidronate and Vγ9Vδ2 T cells were administered every week for 4 wk. Mice with tumors exceeding 25 mm in diameter were sacrificed.

Digestion of tumors into single-cell suspensions and histologic analysis
For ex vivo experiments, solid tumors were excised carefully from killed NSG mice under sterile conditions at the indicated times (1–7 d) after injection of pamidronate in vivo. Collected tumors were dissociated after a 4 h-digestion with collagenase type IV (200 U/ml). Cells were filtered through sterile 40-μm nylon filters and used for cytometric analysis and functional assays after assessment of cell yield and viability. For immunohistologic analysis, serial tissue sections (4 μm thick) were prepared from frozen tumor samples by using a cryostat (Leica CM1950, Leica Biosystems, Nanterre, France) and mounted on glass slides. After rehydration, sections were stained with mAb for human TCR pan γδ and 3,3′-diaminobenzidine (DAB) incubation. Tumors were counterstained with hematoxylin, according to standard histologic procedures. Images were acquired using a NanoZoomer 2.0-HT digital slide scanner and analyzed with the NDP software (Hamamatsu Photonics, Massy, France).

Statistical analysis
All data were analyzed using GraphPad Prism software (version 5; GraphPad Software). The statistical significance of differences between populations was assessed with two-tailed Student t tests. For all experiments, p < 0.05 was considered significant.

Results
Human PC3 prostate tumor s.c. xenographs in immunodeficient NSG mice
Owing to the restricted reactivity of Vγ9Vδ2 T cells toward human tumors and the lack of natural counterparts in rodents, our study first aimed at designing a robust model of human tumor xenographs in immunodeficient mice, which could allow assessment of the antitumor efficacy of adoptively transferred human Vγ9Vδ2 T cells. NSG mice represented a relevant candidate for the recipient mouse strain because they lack mature T and B cells and functional NK cells, and they are deficient in γ-chain cytokine receptor signaling. In this regard, NSG mice support a better engraftment of cells of human origin (e.g., solid or circulating tumor
cells, hematopoietic stem cells, PBMCs, T lymphocytes) than any other previously published immunocompromised mouse strain (23, 24).

We defined two key criteria for selecting human cell lines as tumor xenografts and assessing the efficacy of NBP- and γδ T cell–based approaches in NSG mice. First, tumor cells should not be recognized readily by Vγ9Vδ2 T cells, but should induce broad and sustained functional responses by allogeneic ex vivo–expanded human Vγ9Vδ2 T cells after NBP treatment. Next, following their engraftment in NSG mice, these cells should rapidly yield clearly discernible solid tumors. To allow tumor growth monitoring without sacrificing mice, we privileged s.c. engraftment of human tumor cells. Adherent tumor cells from the human PC3 tumor prostate cell line fulfilled both criteria. Indeed, untreated PC3 cells were modestly recognized in vitro by Vγ9Vδ2 T cells, but they triggered strong, specific, and dose-dependent IFN-γ cytokine response and CD107a upregulation by Vγ9Vδ2 T cells after pamidronate treatment (Fig. 1A and Supplemental Fig. 1).

Moreover, analysis of the kinetics of tumor growth in vivo indicated that PC3 cells could form, in a cell number–dependent manner, easily measurable tumors in less than 6 wk following s.c. grafting in NSG mice (Fig. 1B).

Adoptive transfer of ex vivo–activated human Vγ9Vδ2 T cells, together with a single systemic NBP administration, slows the growth of s.c. PC3 tumors in NSG mice

Antitumor reactivity of ex vivo–expanded human Vγ9Vδ2 T cells toward PC3 tumor xenografts was next evaluated in vivo. As described in Fig. 2A, PC3 cells (1 × 10^7) implanted s.c. in mice were allowed to form discernible tumor masses (~5 mm in diameter) for 2 wk before starting immunotherapy. To test the antitumor efficacy of a strategy combining NBP and γδ T cells, pamidronate was injected i.v. (50 μg/kg) at day 14 in xenografted NSG mice. After 24 h, human Vγ9Vδ2 T cells (1 × 10^6), previously activated and expanded (NBP + rhIL-2) in vitro for 2 wk from fresh PBMCs of healthy donors, were injected i.v. in mice.

When administered alone, NBP or Vγ9Vδ2 T cells had no detectable effects on PC3 tumor growth kinetics in NSG mice compared with untreated controls (Fig. 2B). However, the growth of PC3 tumors was significantly decreased in mice receiving both systemic NBP injection and then human Vγ9Vδ2 T cells 1 day later, when compared with other conditions (Fig. 2B, right panel; 10 versus 20 mm in average diameter at week 5; p < 0.0005).

FIGURE 1. Ability of human PC3 prostate tumor cells to induce strong Vγ9Vδ2 T cell activation after NBP treatment in vitro and to rapidly form s.c. tumors in NSG mice. (A) Human PC3 tumor cells were treated overnight by grading doses of pamidronate (NBP). CD107a surface mobilization and IFN-γ production were measured by flow cytometry within activated Vγ9Vδ2 T cells following incubation with NBP-treated PC3 tumor cells (effector-to-target cell ratio, 1:1). Values for the percentage of IFN-γ+ and CD107a+ γδ T cells are indicated. Results are representative of three independent experiments. (B) Tumor growth in NSG mice receiving s.c. injections of grading doses of human PC3 tumor cells (1 × 10^6, 5 × 10^6, and 10 × 10^6 cells). The tumor size was measured weekly for 6 wk after cell inoculation. As indicated on the graph, mice with tumors exceeding 25 mm in diameter (dotted line) were sacrificed. Results are mean ± SD of five mice per group.

FIGURE 2. Adoptive transfer of ex vivo–activated human Vγ9Vδ2 T cells together with a single systemic NBP treatment delays the growth of s.c. human PC3 tumors in NSG mice. (A) Mice were inoculated s.c. with 1 × 10^7 PC3 cells. Two weeks (day 14) after tumor cells inoculation, mice were treated i.v. with pamidronate (NBP, 50 μg/kg). After 24 h of treatment (day 15), mice were inoculated i.v. with 1 × 10^6 ex vivo activated human Vγ9Vδ2 T cells. Tumor size was measured weekly for 6 wk after cell inoculation. Mice with tumors exceeding 25 mm in diameter were sacrificed. (B, left panel) Mean tumor size (mm) ± SD of five mice per group. Arrows indicate inoculations of pamidronate (gray) and γδ T cells (black). Right panel, Tumor size measured at week 5 in individual mice within in each group. Bars represent mean values. γδ, Adoptive transfer of Vγ9Vδ2 T cells alone; Ctrl, no treatment; NBP, pamidronate treatment alone; NBP + γδ, pamidronate treatment followed by adoptive transfer of Vγ9Vδ2 T cells; ns, not significant. ***p < 0.0005 compared with no treatment.
Similar results were obtained by directly injecting pamidronate within s.c. tumor xenografts (data not shown). Analysis of Vγ9Vδ2 T cell distribution within secondary lymphoid organs (lymph nodes, spleen) and tumor xenografts shows that while γδ T cells preferentially located in secondary lymphoid organs immediately after infusion (peaking at week 1), they reached tumor xenografts thereafter, with a frequency peak at week 2 after γδ T cell infusion (Fig. 3A). Accordingly, immunohistochemical analysis revealed the presence of infiltrating human γδ T cells only within tumors of NSG mice that received both NBP and Vγ9Vδ2 T cells (Fig. 3B). Therefore, combined systemic NBP treatment and Vγ9Vδ2 T cells infusion yielded significant antitumor responses associated with γδ T cell infiltration within tumors in vivo.

**Systemic NBP treatment in NSG mice efficiently, but transiently, sensitizes PC3 xenografted tumors to Vγ9Vδ2 T cell responses**

To gain further insight into the optimal timeframe for infusion of Vγ9Vδ2 T cells, we next defined the strength and kinetics of PC3 tumor cell sensitization induced by in vivo NBP treatment. To this end, tumor cells were collected from 2-wk s.c. tumor xenografts at different time points after systemic in vivo NBP treatment in NSG mice, and readily tested for their ability to induce Vγ9Vδ2 T cell activation in vitro. At day 1 after NBP treatment, tumor cells collected from s.c. xenografts induced strong in vitro activation of Vγ9Vδ2 T cells (~60% CD107a+ γδ T cells; Fig. 4). However, NBP-induced sensitization of tumor cells to γδ T cell recognition rapidly declined, reaching baseline level 3 d after treatment. Efficient recognition of tumor cells collected at late time points (e.g., from day 3 to 7) could be induced by NBP treatment in vitro, thus arguing against an escape mechanism associated with decreased susceptibility to Vγ9Vδ2 T cell recognition. Therefore transient γδ T cell recognition of PC3 tumor cells after systemic NBP treatment is probably due to a transient increase of endogenous PAg levels (e.g., isopentenyl pyrophosphate [IPP]) within s.c. tumors.

These results indicate that, although a single systemic NBP treatment of NSG mice efficiently sensitized s.c. PC3 tumor cells to Vγ9Vδ2 T cell recognition, this effect was only transient (24–72 h). However, they also suggested that prolonged sensitization of tumor cells to Vγ9Vδ2 T cell recognition in vivo could be achievable by repeated NBP treatments.

**Combination of both repeated systemic administrations of NBP and Vγ9Vδ2 T cells infusions strongly improves antitumor efficacy of γδ T cell therapy in vivo**

In line with the aforementioned kinetics of PC3 tumor cell sensitization in vivo, we next investigated whether repeated systemic NBP treatments, when combined to Vγ9Vδ2 T cells infusions, could improve antitumor efficacy of NBP/γδ therapies in vivo (Fig. 5A).

We first analyzed the antitumor effects of four cycles of weekly systemic NBP administrations in NSG mice carrying PC3 s.c. xenografts, combined with a single injection of Vγ9Vδ2 T cells performed at day 1 after the first NBP injection. As shown in Fig. 5B, this protocol did not improve tumor control by adoptively transferred γδ T cells, compared with mice receiving a single NBP injection (Fig. 1). Similarly, four cycles of weekly human Vγ9Vδ2 T cell infusions, combined with a single systemic administration of NBP, did not lead to any improvement of γδ T cell–mediated antitumor response in vivo (Fig. 5C). However, when both systemic NBP administrations and Vγ9Vδ2 T cells injections were repeated weekly for four cycles (Fig. 6A), a strong and long-term control of PC3 tumor growth was achieved (Fig. 6B).

**FIGURE 3.** Adoptively transferred ex vivo–activated human Vγ9Vδ2 T cells infiltrate tumor xenografts in NSG mice. (A) Kinetics of γδ T cell migration to secondary lymphoid organs and s.c. tumor xenograft sites. Lymph nodes, spleens, and tumor xenografts were collected weekly following adoptive transfer of γδ T cells. The values for the percentage of pan-human γδ TCR+ cells were determined by flow cytometry after tissue dissociation and are indicated on the y-axis. Data are mean ± SD of two to three mice for each time point. (B) Immunohistochemical analysis of serial frozen tumor tissue sections from tumor xenografts. Tissue sections were stained using mAb specific for pan-human γδ TCR. Arrows indicate infiltrated Vγ9Vδ2 T cells (brown). Nuclei are counterstained with hematoxylin (blue). γδ, adoptive transfer of Vγ9Vδ2 T cells alone; NBP, pamidronate treatment alone; NBP + γδ, pamidronate treatment followed by adoptive transfer of Vγ9Vδ2 T cells. Original magnification ×20. Scale bar, 100 μm.

**FIGURE 4.** Systemic NBP treatment in NSG mice efficiently but transiently sensitizes PC3 xenografted tumors to human Vγ9Vδ2 T cell recognition. Two weeks after PC3 tumor cell inoculation (1 × 10⁶ cells), NSG mice were treated i.v. with pamidronate (NBP, 50 μg/kg). Tumor xenografts were explanted from mice at the indicated time points after NBP treatment. Dissociated tumor cells were pretreated (□) or not (■) in vitro with pamidronate (100 μM) and cocultured with Vγ9Vδ2 T cells (effector-to-target cell ratio, 1:1). CD107a surface mobilization was measured by flow cytometry within Vγ9Vδ2 T cells. Mean values for the percentage ± SD (two mice for each time point) of CD107a+ γδ T cells are indicated.
The clinical benefit of anticancer immunotherapies using GMP-grade Vγ9Vδ2 T cell agonists is supported by disease stabilization and by partial and complete clinical responses observed in patients with solid or hematologic malignancies after treatment with both IL-2 and NBP (reviewed in Ref. 13). However, larger clinical trials performed in patients with renal cancer receiving both IL-2 and PAg did not yield significant clinical responses, although a trend for prolonged progression-free survival has been reported in the patient arm showing on average the largest Vγ9Vδ2 T cell expansions. Several strategies have been proposed to enhance antitumor efficacy of such active immunotherapies targeting Vγδ T cells. Both the enhanced expression of CD16 on Vγ9Vδ2 PBL, compared with αβ PBL, and the ability of activated Vγ9Vδ2 T cells to mediate Ab-dependent cellular cytotoxicity toward solid and hematopoietic tumors, either in vitro or in vivo in xenografted mice, provide a strong rationale for combination approaches involving Vγδ agonist molecules and antitumor mAb (25–28). In this regard, treatment with anti-CD20 mAb (Rituximab), PAg, and IL-2 for patients with follicular lymphoma and tumor relapse or resistance after first line of rituximab treatment resulted in an increased rate of complete responses, when compared with historical groups treated with a second line of rituximab alone or rituximab and IL-2 (13).

The limited efficacy of active immunotherapies that aim at triggering in vivo–specific expansion and activation of Vγ9Vδ2 T cells might be linked to the progressive exhaustion of the targeted T cell population after repeated treatments, and Vγ9Vδ2 T cell hyporesponsiveness observed in many patients before cancer treatment. Such limitations could be circumvented by adoptive T cell transfer approaches, because large numbers of Vγ9Vδ2 T cells can be expanded and activated in vitro using clinical-grade protocols, even in patients with cancer (29). Thus far, disease stabilization or partial clinical response, or both, have been observed in patients with solid tumor after repeated adoptive Vγ9Vδ2 T cell transfer (15–17, 30). The possibility to expand this T lymphocyte subset efficiently upon short-term culture of PBMCs from healthy donors with GMP-grade Vγ9Vδ2-agonist molecules and rhIL-2, as well as the lack of alloreactivity of Vγδ T cells (31–34), could justify immunotherapies relying on the adoptive transfer of allogeneic Vγ9Vδ2 T cells in patients with cancer and impaired autologous Vγ9Vδ2 T cell expansion capacities. This is feasible based on previous studies showing limited rejection of transferred allogeneic human αβ T cells carrying common HLA haplotypes, such as A1, B8, or DR3, in Caucasian patients. Importantly, this perspective must consider an efficient antitumor reactivity of allogeneic human Vγδ T cells. In this regard, we and others have reported a strong in vitro lytic activity of Vγ9Vδ2 T cells against a wide set of tumor targets.
In this setting, the enhanced clinical benefit of combined therapies that repeated and combined administrations of NBP and NBP, delays tumor growth in mice. Importantly, our data indicate that autologous and allogeneic human tumor cells (35). Accordingly, we obtained evidence that Vγ9Vδ2 T cells, expanded ex vivo from PBMCs of healthy donors, achieved robust cytolytic responses against low-passage tumor cells isolated from ovarian cancer female patients, when compared with autologous Vγ9Vδ2 T cell responses (Supplemental Fig. 2).

An attractive way to improve the efficacy of adoptive γδ T cell transfer approaches might be to combine them with NBP treatments, which should sensitize tumor cells to γδ T cell recognition. In this setting, the enhanced clinical benefit of combined therapies would rely on the ability of γδ T cells activated ex vivo, when transfused soon after a first NBP administration, to eradicate NBP-sensitized tumor cells more efficiently. Interestingly, the rationale and feasibility for such a combined approach has been supported by a recent phase I trial conducted in advanced renal carcinoma patients, which reported occurrence of a complete response and high rate of stable disease in treated patients (36). However in this latter study, the mechanisms underlying tumor stabilization or regression have remained unclear, because the ability of NBP to enhance antitumor efficacy of adoptively transferred Vγ9Vδ2 T cells has not been proved formally. To address this shortfall, we designed a preclinical in vivo model of s.c. human tumor xenografts in immunodeficient NSG mice. This robust mouse model carrying human tumor xenografts was exploited to assess the effects of combined systemic administrations of both NBP and ex vivo expanded activated human Vγ9Vδ2 T cells. Our results show that adoptive transfer of Vγ9Vδ2 T cells, performed 24 h after systemic injection of NBP, delays tumor growth in mice. Importantly, our data indicate that in vivo NBP-induced sensitization of tumor cells to human Vγ9Vδ2–T cell recognition lasts for a few days, which implies that repeated and combined administrations of NBP and γδ T cells are both required to improve significantly the antitumor efficacy of such a combination approach. These results extend observations made recently, in a different setting by Benzaïd et al. (37), who assessed the antitumor efficacy of a single line of NBP and IL-2 treatment in NOD/scid mice with i.p. injection of human PBMCs. In this study, the potency of antitumor Vγ9Vδ2 T cell responses in vitro and in vivo correlated with IPF/App1 (triphosphonic acid 1-adenosin-5′-yl ester 3-[3-methylbut-3-enyl] ester) levels in zoledronate-treated breast cancer cells. Interestingly, although the intracellular level of IPP is assumed to be the primary correlate of tumor cell susceptibility to Vγ9Vδ2 T cell recognition, high levels of intracellular IPP were detected in explanted tumor xenografts 1 d after systemic injection of zoledronate, but dropped rapidly at days 2 and 3, at time points when explanted tumor from pamidronate-treated mice still showed enhanced susceptibility to Vγ9Vδ2 T cell responses. This apparent discrepancy between the present and the former studies could be explained by the fact that duration of the Vγ9Vδ2 sensitizing effect of NBP depends on at least three distinct parameters. First, Vγ9Vδ2 T cells respond not only to NBP but also to App1, the intracellular levels of which peak at days 2–4 after zoledronate treatment, and thus could contribute to enhanced γδ T cell responses at later time points. Second, responsiveness to NBP treatment greatly varies from one tumor cell line to another, and it might differ between the tumors used in these studies. Third, γδ T cell responses induced by pamidronate-treated tumor cells, although weaker than those induced by zoledronate-treated cells, tend to last longer, as suggested by our in vitro studies (E. Scotet and M. Bonneville, unpublished observations).

As reported previously, IL-2, which is mainly produced by Th lymphocyte subsets and, to a much lesser extent, by activated Vγ9Vδ2 T cells, is an obligatory cytokine required for the expansion of activated human Vγ9Vδ2 T cells (38, 39). Our study aimed at optimizing passive Vγ9Vδ2 immunotherapies without exogenous rhIL-2 administrations, which could lead to deleterious side effects in recipients. However, it is curious why, in treated NSG mice, the frequencies of human Vγ9Vδ2 T cells, following their injection without exogenous rhIL-2, peaked at week 1 within secondary lymphoid organs and at week 2 within s.c. tumor xenografts. The absolute numbers of Vγ9Vδ2 T cells within these sites, at different time points after injection, were not determined, which did not allow us to state the effective growth or survival of injected human γδ T cells. Interestingly, a recent study has shown that, in patients with advanced colorectal cancer who received weekly injections of autologous Vγ9Vδ2 T cells, the frequency and absolute number of peripheral γδ T cells gradually increased during the course of treatment (40). Surprisingly, functionally active Vγ9Vδ2 T cells persisted for a prolonged period after the last infusions, even in the absence of exogenous rhIL-2. These results,
and the observation that NBP-activated Vγ9Vδ2 T cells, in contrast to the resting ones, lack IL-7Rα expression and express only IL-2Rβ and γc, suggest the implication of IL-2-related endogenous factors (e.g., transpresentation of IL-15) for the mechanism maintaining adaptively transferred Vγ9Vδ2 T cells. Because murine IL-2 is active on human T lymphocytes, although at a very low efficiency (41), we cannot exclude that host murine factors contribute to human Vγ9Vδ2 T cell growth and survival within xenografted NSG mice.

In conclusion, our study provides a strong preclinical rationale for repeated NBP injections combined with adoptive Vγ9Vδ2 T cell transfer. Importantly, our results indicate that such an approach prevents tumor expansion, but it does not eradicate preexisting tumors. This partial efficacy cannot be explained merely by impaired tumor accessibility, because infiltrating γδ T cells were detected even within the tumor bed. Nevertheless, the hypoxic environment within the tumor might enhance resistance to γδ T cell–mediated immune attack. In this regard, one method of circumventing this escape mechanism and further improve efficacy of the present combination approach might be to use low-dose chemotherapies, which are known to enhance γδ T cell susceptibility of zoloderanate-sensitized tumor cells (42); cytokines known to boost antitumor cytotoxic and proinflammatory responses of γδ T cells, such as IL-21 (43) and IL-27 (44); or TLR ligands (45).

Acknowledgments

We thank M.J. Lorient (Etablissement Français du Sang, Nantes, France) for providing blood samples, F. Paris (INSERM, Nantes, France) for providing PC3 tumor cells, S. Nedellec (Cellular and Tissular Imaging Core Facility of Nantes University, Structure Fédérative de Recherche François Bonamy) for technical help, and M. Capone (Centre National de la Recherche Scientifique, Bordeaux, France) and P. Clénard (INSERM, Lyon, France) for help and discussions.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure S1. Vγ9Vδ2 T cells were expanded ex vivo from fresh human PBMCs, following incubation with NBP (pamidronate) and rhIL-2. After 2 weeks of culture, resting PBLs (CD3+ cells > 99%) were cocultured together with human PC3 prostate tumor cells (effector to target cell ratio 1/1) that have been either left untreated (PC3 untreated) or treated for 2 h by NBP (PC3 NBP-treated). CD107a surface mobilization was measured by flow cytometry within Vδ2+ (Vδ2pos) or Vδ2− (Vδ2neg) PBL subsets. Values for the percentage of CD107a+ cells are indicated in the quadrants. One representative experiment, performed with an ex vivo-expanded PBL population with a significant frequency (30%) of Vδ2− T cells, is shown.
**Supplemental Figure S2.** $\gamma\delta$ T cells were expanded *ex vivo* from fresh human PBMCs isolated from either an healthy donor (*black circles*) or an ovarian carcinoma patient (*white circles*), following incubation with BrHPP and rhIL-2. After 2 weeks of culture, resting $\gamma\delta$ T cells were co-cultured together with low-passage (<50) ovarian tumor cells from patient #O370 (effector to target cell ratio 1/1) that have been pretreated for 2 h by grading doses of NBP (zoledronate, *Zol*). CD107a surface mobilization was measured by flow cytometry within $\delta^2$+ PBLs. Controls: resting (*No stim.*) and BrHPP (3 mM)-activated $\gamma\delta$ T cells alone. Values for the percentage of CD107a$^+$ $\gamma\delta$ T cells are indicated.