γδ T Cell Modulation in Anticancer Treatment

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Abstract: The broad antimicrobial and antitumoral reactivity of Vγ9Vδ2 T cells, their ability to produce inflammatory cytokines involved in protective immunity against intracellular pathogens and tumors and their strong cytolytic and bactericidal activities suggest their direct involvement in immune control of cancers and infections.

γδ T cells can be selectively activated by naturally occurring or synthetic phosphoantigens, and drugs that enhance their accumulation into *stressed* cells, offering new avenues for the development of γδ T cell-based immunotherapies.

The recent development of small drugs selectively activating Vγ9Vδ2 T lymphocytes, which upregulate endogenous phosphoantigens, has enabled investigators to design experimental approaches of cancer immunotherapies; several ongoing phase I and II clinical trials are focused on the role of direct bioactivity of drugs and of adoptive cell therapies involving phosphoantigen-activated Vγ9Vδ2 T lymphocytes in humans. In this review, we focus on the recent advances of the activation/expansion of γδ T cells *in vitro* and *in vivo* that may represent a promising target for the design of novel and highly innovative immunotherapy in patients with different types of cancer.

Keywords: Vγ9Vδ2 T cells, aminobisphosphonates, phosphoantigens, cancer, immunotherapy.

INTRODUCTION

Human γδ T lymphocytes comprise several cell subsets defined by their cell surface T cell receptor (TCR). The main subset of γδ cells in peripheral blood of healthy adults expresses the Vγ9Vδ2 TCR and has unusual antigen specificity and effector functions [1]. Most Vγ9Vδ2 T cells respond to nonpeptide antigens, named phosphoantigens, which do not require association with HLA or HLA-related presenting molecules to be recognized. Phosphoantigens arise from metabolic pathways leading to isoprenoids biosynthesis both in mammalian and microbial cells, Fig. (**1**) [2]. These pathways are frequently up-regulated in human tumor cells, providing a rich source of phosphoantigens able to stimulate Vγ9Vδ2 T lymphocytes (for phosphoantigen biosynthesis see [3]). In addition, Vγ9Vδ2 T cells also express the NKG2D co-receptor which facilitates activation, upon interaction with its cell ligands MICA/B and/or ULBP1-3, expressed on *stressed* (infected or transformed) cells [4]. When activated, Vγ9Vδ2 T cells mediate a strong cytolytic activity directed against the phopsphoantigen-producing tumor cells, they also release pro-inflammatory chemokines (e.g. MIP-1, RANTES), T-helper cell type 1 (Th1) cytokines (e.g. IFN-γ, TNF-α), and finally proliferate in the presence of IL-2. This immunological response efficiently reduces tumor cell growth *in vitro* and in animal models, justifying the assessment of γδ T cell-based cancer immunotherapies [5]. Despite the virtues of their antitumor bioactivity, e.g. HLA-unrestricted killing of most human tumor cell types, human Vγ9Vδ2 T cells had not been specifically mobilized in any cancer vaccine or cancer immunotherapy so far. The recent development of small drugs selectively activating Vγ9Vδ2 T lymphocytes, such as synthetic phosphoantigens and anti-osteolytic bisphosphonates, which upregulate endogenous phosphoantigens, has enabled investigators to design experimental approaches of cancer immunotherapies based on activation of Vγ9Vδ2 T cells [6, 7]. Several ongoing phase I and II clinical trials currently aim at assessing the direct bioactivity of drugs such as phosphoantigen or bisphosphonates and of adoptive cell therapies involving phosphoantigen-activated Vγ9Vδ2 T lymphocytes in humans Fig. (**2**).

The diseases that are currently under investigation encompass hormone-resistant prostate cancer, metastatic renal cell carcinoma, chronic myeloid leukaemia and follicular lymphoma [8-10]. Newer trials targeting glioblastoma multiform, colon carcinoma, hepatocarcinoma and $HER2^+$ mammary carcinoma are likely to arise soon.

ADAPTIVE IMMUNE RESPONSES OF γδ T CELLS

Vγ9Vδ2 T cells display a central and effector memory phenotype due to the ubiquitous presence of the source of prenyl pyrophosphate antigens, suggesting that the development of immunological memory could be reasonably different from that of conventional CD4 and CD8 αβ T cells that recognize foreign peptide antigens. Vγ9Vδ2 T cells in healthy children expand from approximately 0.5% at birth to approximately 8% between the ages of 1 and 10 years, in the absence of a parallel thymic wave [11, 12], resulting in the skewing of the γδ T cell repertoire to a predominance of Vγ9Vδ2 T cells with a memory phenotype in most but not all subjects [12]. Although questions remain as to exactly how γδ T-cell memory operates, several studies now provide strong evidence for its existence.

The prerequisite of memory response of γδ T cells has been first demonstrated by Chen and coworkers in rhesus monkeys [13, 14], in fact Vγ9Vδ2 T cells responded to *in vivo* mycobacterial infections and were able to robustly expand in numbers upon re-infection. Following primary *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) infection, normal monkeys exhibited up to a 200-fold increase in Vγ9Vδ2 T cells with a peak at day 28 and upon re-infection,

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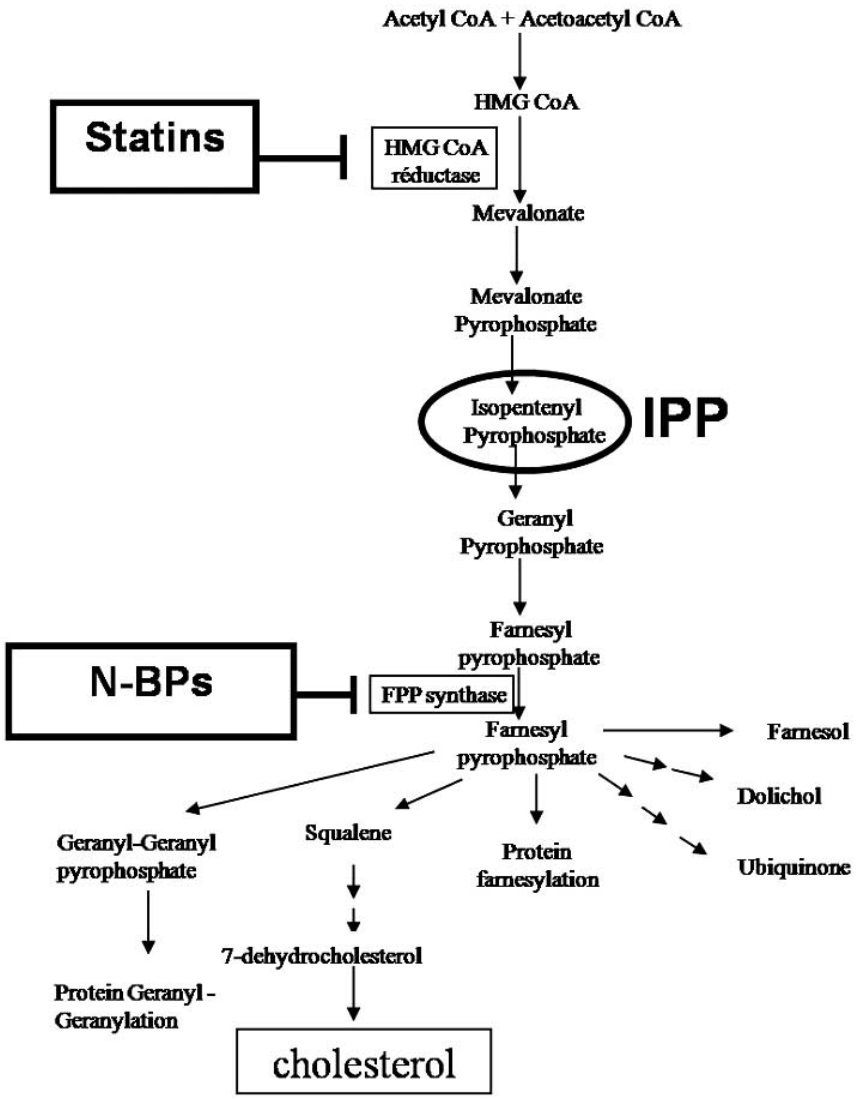

Fig. (1). The mevalonate pathway for isoprenoid biosynthesis.

In mammalian cells, isopentenyl pyrophosphate (IPP) is synthesized by the mevalonate (MVA) pathway, in which MVA is an intermediate product of a reaction catalyzed by the enzyme β-hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA) reductase. HMG-CoA reductase is the key enzyme of the MEV pathway and is subject to feedback regulation by downstream metabolites. Moreover, HMG-CoA reductase is the target of statins, which thus inhibit production of downstream metabolites. The downstream enzyme farnesyl pyrophosphate (FPP) synthase converts IPP to geranyl pyrophosphate (GPP) and FPP intermediates, and is inhibited by aminobisphosphonates (N-BPs). Inhibition of FPP synthase by N-BPs results in accumulation of upstream IPP at concentrations able to efficiently activate γδ T lymphocytes.

(Adapted from Gober HJ, Kistowska M, Angman L, Jenö P, Mori L, De Libero G: Human T-cell receptor γδ cells recognize endogenous mevalonate metabolites in tumor cells. J. Exp. Med. (2003) 197(2):163-168 2003 Rockefeller University Press).

these cells expanded 9 times more than the primary response showing earlier response (as early as 4–6 days after infection). The expansion of Vγ9Vδ2 T cells correlated with the decrease of BCG septicaemia during both primary infections and re-infections. This observation was also confirmed after aerosol immunization of rhesus monkeys with BCG and subsequent aerosol infection with virulent *Mycobacterium tuberculosis* (Mtb), in lung expanded numbers of Vγ9Vδ2 T cells was found.

We studied γδ cell responses in children infected with Mtb, on the grounds that tuberculosis in children is usually due to primary infection and represent an important condition for studying γδ T cell responses upon primary or subsequent exposures to microbial antigens. We found that Vγ9Vδ2 T cells require *in vivo* priming to be able to expand *in vitro* upon subsequent antigen stimulation. Similar to the experiments in monkeys, Vγ9Vδ2 T cell proliferation strongly decreases after chemoprophylaxis or chemotherapy, which is attributable to the reduction in bacterial load [15].

In humans, oral vaccination with tea, that provide a source of the alkylamine antigen ethylamine [16] or intradermal vaccination with BCG [17] increased Vγ9Vδ2 T-cell responsiveness *in vitro*, suggestive of the presence of memory features of Vγ9Vδ2 T cells. Similarly to αβ T cells, Vγ9Vδ2 T lymphocytes comprise distinct populations distinguishable on the basis of surface markers, effector functions

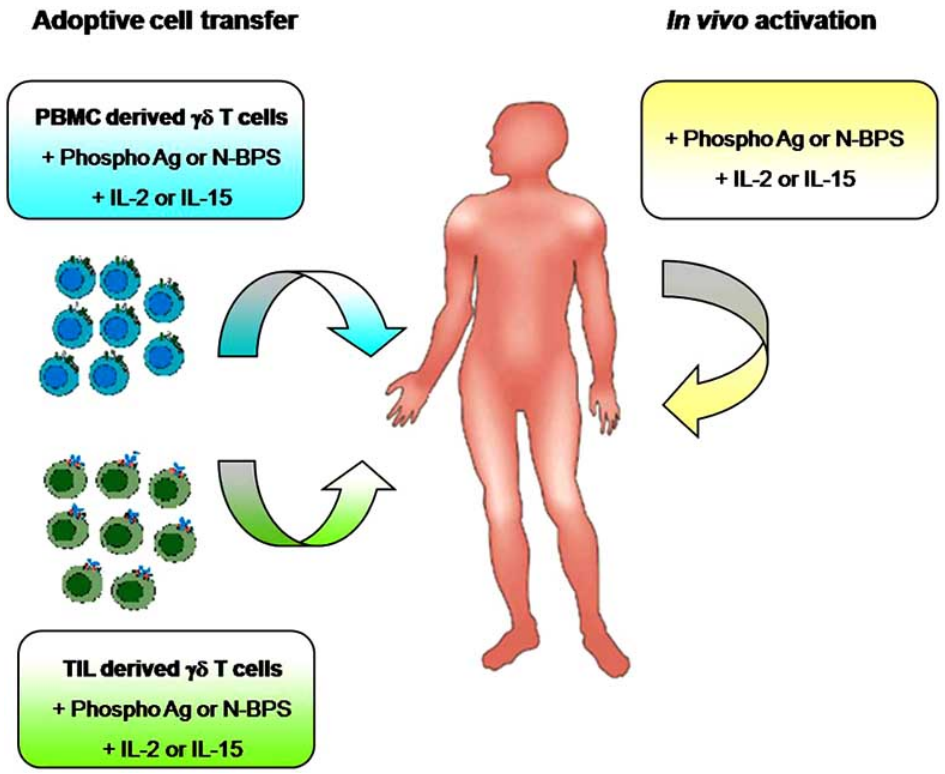

Fig. (2). Strategies for Vγ9Vδ2 T cell–based immunotherapy of cancer.

Strategies for Vγ9Vδ2 T cell-based immunotherapy include the adoptive cell transfer of *ex vivo* expanded Vγ9Vδ2 T cells and the *in vivo* activation of Vγ9Vδ2 T cells by phosphoantigens (e.g., bromohydrin pyrophosphate/Phosphostim) or aminobisphosphonates (N-BPs) and low-dose IL-2 and/or IL-15. Vγ9Vδ2 T cells obtained from the peripheral blood lymphocytes (PBL) or from tumor-infiltrating lymphocytes (TIL) can be readily activated and expanded *in vitro* by phosphoantigens (bromohydrin pyrophosphate) or aminobisphosphonates in the presence of IL-2 and/or IL-15.

and trafficking properties. Naive (T_{naive}, $CD45RA^+CD27^+$) and central memory (T_{CM}, $CD45RA^-CD27^+$) cells home to secondary lymphoid organs and lack immediate effector functions, whereas the so-called effector-memory (T_{EM}, $CD45RA^-CD27^-$) and terminally differentiated (T_{EMRA}, $CD45RA^+CD27^-$) cells home to sites of inflammation where they display immediate effector functions such as cytokine production and cytotoxicity [18, 19]. Interestingly, a recent study by Sun and coworkers [20] showed that also NK cells conceivably possess attributes of adaptive immunity, which may have major implications for the generation of immunological memory. The mechanisms involved in the generation of γδ memory phenotype and the mechanisms for their maintenance *in vivo* and *in vitro* open a new perspective of their use in immunotherapy of cancer.

BISPHOSPHONATES, Vγ9Vδ2 T CELLS AND CLINICAL APPLICATIONS

Bisphosphonates (BPs) are synthetic drugs that prevent bone resorption and are used for the treatment of Paget's disease, tumour-associated bone diseases and osteoporosis. Due to their composite mechanism of action, BPs are emerging as compounds with an extensive use in an increasing number of clinical conditions. Based on their chemical structure, BPs are traditionally divided into two pharmacological classes with distinct molecular mechanisms of action: nitrogen-containing (N-) and non-nitrogen containing (non-N) drugs. The more potent N-BPs have bulkier side chains characterized by a nitrogen moiety either in an alkyl chain (e.g., alendronate and ibandronate) or within a heterocyclic structure (e.g., risedronate and zoledronic acid) [reviewed in 21].

BPs may slow the progression of bone lesions or prevent bone metastasis *in vivo* preclinical models [22, 23]. Moreover, N-BPs have been tested for a wide range of solid and haematopoietic cancers. Pamidronate has been used and demonstrated efficacy versus placebo upon all major end points but only for breast cancer and multiple myeloma [24]. Zoledronic acid (ZA) is a newer N-BP with a tertiary amino group included within a ring structure. It can be considered the most potent and widely used intravenous BP that prevent the delayed onset of skeletal related event (SRE) in patients with bone metastases from any type of tumour and also for the treatment of hypercalcemia of malignancy [25-28]. In particular, ZA is approved for the treatment of patients with bone metastasis from breast cancer, hormone refractory prostate cancer, as well as other solid tumors and multiple myeloma [29]. Preclinical data also suggest that ZA has antitumor properties and a very recent study on 1803 premenopausal women with endocrine-responsive early breast cancer showed that addition of ZA to adjuvant endocrine therapy improves disease-free survival and results in a relative reduction of 36% in the risk of disease progression [30]. Finally, a very interesting property of N-BPs has been discovered, which might be important in antitumor immune responses:

this class of compounds is able to activate Vγ9Vδ2 T cells. In fact, it has been shown that N-BPs act at molecular level and are potent inhibitors of the mevalonate pathway enzyme farnesyl pyrophosphate synthase (FPPS) [31-33], that leads to the biosynthesis of isopentenyl pyrophosphate (IPP), thereby causing the intracellular accumulation of IPP and in consequence exerting strong activation of Vγ9Vδ2 T cells. Statins, such as mevastatin, which inhibit the upstream enzyme hydroxymethyl-glutaryl coenzyme-A (HMG-CoA) reductase, Fig. (**1**) [34, 35], blocks the N-BPs-induced accumulation of IPP and limits Vγ9Vδ2 T cell activation [32]. N-BPs have gained additional importance as they have been shown to inhibit tumor cell adhesion, invasion, and proliferation and to induce apoptosis of a variety of human tumor cell lines *in vitro*. More importantly, N-BPs also have been shown to exhibit direct antitumor activity *in vivo* by inhibiting cancer growth through antiangiogenic, anti-invasive, and immunomodulatory actions. Although several mechanisms have been proposed to explain the N-BPs' antitumor effect, the inhibition of FPPS is of crucial importance in the expansion and activation of γδ. N-BPs do not act bind to the γδ T cell receptor but may indirectly activate γδ T cells *via* IPP production in monocytes and macrophages. The classical explanation for this antigen presenting cells (APCs) requirement is that N-BPs act preferentially on cells showing high pinocytic activity such as monocytes, dendritic cells (DCs) and macrophages. In fact, it has been demonstrated that although monocytes, macrophages and DCs are not required for Vγ9Vδ2 T-cell expansion driven by soluble non-peptide ligands, the response is nevertheless substantially enhanced by cell to cell contact from such accessory cells [36].

However, more and more studies suggest instead a significant contribution of APCs to this process. For instance activation of primary Vγ9Vδ2 T cells by N-BPs strictly requires monocytic adherent cells, and species specific interactions are needed for optimal engagement of γδ T cells in such a setting [37, 38].

ANTI-TUMOR RESPONSE OF γδ T CELLS: *IN VITRO* STUDIES

Conventional strategies for treatment of solid tumors or haematological malignancies are based on chemotherapy, radiotherapy or a combination of both. Vγ9Vδ2 T cells have become prominent players with a broad anti-tumor activity for their ability to expand *in vitro* upon phosphoantigens or N-BPs treatment in an appreciable and desiderable numbers suitable for *in vivo* transfer in human cancer patients and for their ability to interact with and efficiently kill tumor cells of different origin. Tumor target cell recognition in general involves interaction of the reactive Vγ9Vδ2 TCR with mevalonate pathway-derived metabolites [2, 39], or an F1-ATPase-related structure complexed with one of its ligands, a delipidated form of apolipoprotein A1 [40], that unlike in normal cells is ectopically expressed on the surface of hemopoietic tumors and renal cancer cell lines. Moreover, Vγ9Vδ2 T cell cytotoxicity is tightly regulated by NK-like receptors for MHC class I ligands and some previous studies have indicated the importance of NKG2D-MICA/B interactions for tumour cell recognition and cytotoxic activity by Vγ9Vδ2 T cells [4, 39, 41, 42]. Finally, cytotoxic Vγ9Vδ2 T cells express the CD16 and therefore mediate antibody-dependent cell-mediated cytotoxicity (ADCC) activity toward antibody-coated tumor targets [43,44]. Upon recognition, Vγ9Vδ2 T cells kill tumor targets *via* a number of mechanisms including death receptor/ligands interactions with TRAIL and FasL, and by release of perforin/granzymes or cytokines such as IFN-γ and TNF-α [45-47].

In this review, we report on few representative studies, selected from the vast body of data in the literature and that highlight the role played by Vγ9Vδ2 T cells toward tumoral cells.

Wrobel *et al.* [39] investigated on the ability of γδ T cells to kill various tumour cells including autologous tumors. Most epithelial tumors were susceptible to allogeneic γδ T cell lysis and the lysis of resistant cells was enhanced by treatment of tumor cells with aminobisphosphonates or by preactivation of γδ T cells with phosphoantigens. Similarly, Mattarollo and coworkers [48] demonstrated that pretreatment with low concentrations of chemotherapeutic agents or ZA sensitized solid tumor-derived cell lines to rapid killing by Vγ9Vδ2 T cells with levels of cytotoxicity approaching 90%. In addition, ZA enhanced the chemotherapy-induced sensitization of tumor cells to Vγ9Vδ2 T cell cytotoxicity, resulting in almost 100% lysis of tumour targets in some cases.

Corvasier and coworkers [49] found the presence of Vγ9Vδ2 T cells in the majority of colon cancer tumor-infiltrating lymphocytes (TIL) populations and Vγ9Vδ2 T cell clones from colon cancer patients or allogeneic healthy donors were able to efficiently kill colon cancer cell lines, thus contributing to the immunosurveillance of these tumors and suggesting the development of novel strategies for treatment of colon cancer patients.

Viey and coworkers [50] demonstrated that Vγ9Vδ2 T lymphocytes expanded from most renal cell carcinoma (RCC) patients display a selective lysis against autologous primary renal tumoral cells. Moreover, the authors found that the Vγ9Vδ2 T cells *in situ* are an important component of TIL in RCC. In another study Viey *et al.* [51] have investigated the differentiation status and the migration potential of tumor-infiltrating Vγ9Vδ2 T lymphocytes from RCC patients. The repertoire of tumor-infiltrating Vγ9Vδ2 T cells from RCC patients was characterized by a dominant $CD27^-$ $CD45RA^-$ subset. Tumor-infiltrating Vγ9Vδ2 T cells migrated with higher efficiency toward primary renal tumor cells, requiring the CXCL12/CXCR4 interaction.

Pages *et al.* [52] have previously reported that the presence of T_{EM} cells within colorectal cancer correlates with the absence of pathologic evidence of early metastases and with prolonged survival. However, Inman *et al.* [53] have investigated on the levels of γδ T cells in 248 clear cell RCC (ccRCC) tumor specimens, but did not find any correlation between γδ levels and clinico-pathologic prognostic features of ccRCC aggressiveness as well as cancer-specific outcomes and survival. Vγ9Vδ2 cells exhibit differential migration capacities according to their localization, their differentiation status, and the tumor microenvironment parameters that may influence their use in immunotherapy.

Burjanazde *et al*. [54] investigated on the ability of phosphoantigens and ZA to expand γδ T cells from normal donors or patients with multiple myeloma (MM) and evaluated the *in vitro* anti-MM cytotoxicity of phosphoantigen-expanded γδ T cells from patients with MM. They found that, in patients with MM, γδ T cells counts did not differ from those in healthy donors, and 14 day culture of γδ T cells in the presence of phosphoantigen plus IL-2 determines a 100-fold expansion of these cells. These expanded T cells are able to kill 13/14 myeloma cell lines as well as primary myeloma cells, but not normal CD34 hematopoietic stem cells.

Finally, Alexander *et al*. [55] investigated on the cytotoxic potential of γδ T lymphocytes against squamous cancer cell head and neck (SCCHN) and characterized the phenotype of γδ T lymphocytes responsible for tumor killing. They found that expanded $CD56^+$ γδ T cells, obtained from PBMC stimulated with IPP and IL-2, mediated antitumor cytotoxic effects and the killing of tumor cells by $CD56^+$ γδ T cells was associated with increased expression of CD107a, a degranulation marker, suggesting that these cells are functionally capable of SCCHN killing and that they may play an important role in immunotherapy. Moreover, they observed that γδ T lymphocytes are not only important for tumor lysis but also play a role in enhancing the cytotoxic potential of NK cells.

IN VIVO ANIMAL STUDIES: MOUSE AND NON-HUMAN PRIMATES

Although in a transgenic adenocarcinoma of the mouse prostate (TRAMP) model, it was established that adoptively transferred γδ T cells provided protective immunity and their rational use for clinical trials in prostate cancer [56], mouse do not represent a suitable model to study γδ T cells, due to their different antigen recognition repertoire. Therefore, adoptive transfer of human γδ T cells into severe combined immunodeficient (SCID) mice has been widely used as a suitable system and several studies have demonstrated the ability of human γδ T cells, upon adoptive transfer, to improve the survival of SCID mice inoculated with tumoral cells, such as Daudi lymphoma, nasopharyngeal carcinomas and melanoma.

The two major subsets of γδ T cells, Vδ2 and Vδ1, differ in their *in vivo* anti-tumor efficacy, depending on their different migratory properties and on the different type of tumor [57, 58]. Studies in SCID/human (hu) models indicate that although both Vδ2 and Vδ1 cells can clear human melanoma cells in grafted mice with SCID when injected within the tumor, only Vδ1 cells can clear tumor cells when injected systemically [58]. However, such a functional dichotomy between Vδ1 and Vδ2 cells may not apply for all solid tumors, as suggested by enhanced elimination of melanoma [59] or RCC by Vδ2 cells in very similar SCID/hu models. In mice with SCID/hu PBL, Vγ9Vδ2 PBLs activated *in vivo* can migrate to and clear human kidney tumors grafted subcutaneously. These seemingly discrepant results could be explained by the up-regulation of skin-homing receptors on activated Vγ9Vδ2 T cells and/or by the different levels of tumor-induced inflammation in the above models, leading in some cases to *in situ* migration of Vγ9Vδ2 cells, which have a strong tropism for inflammatory sites. In an infectious context, Wang *et al*. [60] have reported efficient clearance of intraperitoneal bacterial infections in SCID mice following adoptive transfer of Vγ9Vδ2 PBLs, in accordance with their *in vitro* anti-bacterial effector properties.

In 1999, we were the first to demonstrate that in non-human primates the infusion of phosphoantigen triggers a transient but large expansion of circulating Vγ9Vδ2 T cells, inducing the production of high levels of Th1 cytokines [61]. Later, Sicard *et al*. [62] reported the *in vivo* expansion and activation of Vγ9Vδ2 T cells following injection of a synthetic phosphoantigen Phosphostim™ and low doses of IL-2 in a preclinical non-human primate model, suggesting that IL-2 administration is a strict requirement for Vγ9Vδ2 T cell expansion *in vivo*. Moreover, Casetti *et al*. [63] found that *in vivo* combined treatment with Vγ9Vδ2 T cell-stimulatory drugs, including N-BPs, and low doses of IL-2 was also able to induce both $CD27^-$ effector and $CD27^+$ naive and central memory Vγ9Vδ2 T cell subsets in the peripheral blood of treated macaques and hence, to contribute to the development of both innate and adaptive immune responses. Thus, N-BPs therapy in combination with low doses of IL-2 could be useful to promote and enhance the potent antiviral and antitumor responses of Vγ9Vδ2 T lymphocytes.

Using the protocol described by Kabelitz *et al*. [59], we have recently investigated on the ability of newly synthesized small series of N-BPs structurally related to ZA, to activate γδ T cells in SCID mice [64]. We identified a compound, called 6a, which shows a higher capability than ZA to activate Vγ9Vδ2 T cells either *in vitro* or in the SCID/hu model *in vivo* and to improve survival of these reconstituted immunodeficient mice to injection of a breast cancer tumor cell line. Thus, taking into account the unique pharmacological profile of 6a, characterized by a potent activation of Vγ9Vδ2 T cells and by a remarkable pro apoptotic effect both *in vitro* and *in vivo*, 6a may represent a promising anticancer drug which can be added to the traditional chemotherapy of a wide range of tumor types.

IN VIVO HUMAN STUDIES AND CLINICAL TRIALS

Despite the relative complexity of immunotherapy, such treatment may have a role, in conjunction with other therapies in the treatment of cancers refractory to conventional treatments alone. Clinical studies have recently shown that adding immunotherapy to chemotherapy has survival benefits in comparison to chemotherapy alone [65], an example being in the setting of monoclonal antibody/chemotherapy combination therapy [66-68]. Moreover, chemotherapeutic agents can sensitise tumors to immune cell mediated killing, for instance increasing sensitivity of tumor cells to subsequent cytotoxicity by T cells *via* up-regulation of death receptors DR5 and Fas, ligands to TRAIL and FasL respectively. Based on the above reported *in vitro* and in preclinical animal models *in vivo*, it can be speculated that the intentional activation of Vγ9Vδ2 T cells may be of significant clinical benefit in the treatment of different forms of cancer. This might be achieved for instance by stimulating Vγ9Vδ2 T cells in cancer patients through injection of phosphoantigens or N-BPs and IL-2, as recently performed in hematological malignancies [69] and in solid tumors [70,

71]. Alternatively, it could be achieved by adoptive transfer of *ex vivo* expanded autologous Vγ9Vδ2 T cells derived from cancer patients Fig. (**2**) [72, 73]. These T cells could be obtained from patient blood, because PBL-derived Vγ9Vδ2 T cells exhibited a similar reactivity than TIL toward colon cancer cells [49], even if *ex vivo* expanded, tumor infiltrating Vγ9Vδ2 T cells migrate toward primary renal tumor cells with higher efficiency than their peripheral blood expanded counterparts [51]. Both these approaches have been proven to be well tolerated [69-73]. In theory, an effective T cell-based immunotherapy might involve a double strategy for the potential usage of γδ T cells: the *in vivo* therapeutic application of γδ T cell-stimulating N-BPs together with low-dose IL-2, possibly followed by an adoptive cell transfer of *in vitro* expanded γδ T cells.

γδ T cells may have APC functions, as demonstrated by Moser *et al.*, IPP-stimulated Vγ9Vδ2 T cells express and maintained the features of APC during prolonged culture periods, except for the expression of CD40, process endogenous self or microbial proteins or exogenous antigens, in the form of soluble proteins, cell debris or whole microorganisms and activate naive CD4 or CD8 T cells. This further aspect of APC features of γδ T cells suggest their use as live vaccines in immunotherapy for patients suffering from tumors or persistent infections [74, 75].

Finally, recent advances in designing protocols for adoptive transfer of Vγ9Vδ2 T cells in some malignancies have been considered in conjunction with monoclonal antibody therapy. For instance, Tokuyama *et al.* [44] have demonstrated that $CD16^+$ Vγ9Vδ2 T cells can be obtained from peripheral blood of healthy subjects or from patient with malignancy and after *ex vivo* expansion with ZA plus IL-2 their tumor cell killing effects could be enhanced by rituximab (active against CD20 positive tumors) and trastuzumab (active against HER2 expressing cancers), suggesting their potential therapeutic application in cancer patients.

Below, we briefly report on the results of clinical trials with γδ T cell-based immunotherapy.

Multiple Myeloma and Non-Hodgkin Lymphoma

A pilot study by Wilhelm and coworkers [69] firstly reported the potential for Vγ9Vδ2 T cell activation for the immunotherapy of patients with hematologic B-cell malignancies (8 patients with MM and 11 patients with non-Hodgkin lymphoma) that failed conventional therapies. Initially, they treated 10 patients with 90 mg pamidronate intravenously on day 1 followed by increasing dose levels of continuous 24-hour intravenous infusions of IL-2 (0.25 to 3 million IU/m^2) from day 3 to day 8. Even at the highest IL-2 dose level *in vivo*, Vγ9Vδ2 T-cell response was found and only 1 patient achieving stable disease. However, when further patients were enrolled, who were selected by positive *in vitro* proliferation of Vγ9Vδ2 T cells in response to pamidronate/IL-2 and received a modified treatment schedule (90 mg pamidronate intravenously on day 1 followed by 6-hour bolus intravenous IL-2 infusions from day 1-6), significant *in vivo* activation/proliferation of Vγ9Vδ2 T cells was observed in 5 patients (55%), and objective responses were achieved in 3 patients (33%). Only patients with significant *in vivo* proliferation of Vγ9Vδ2 T cells responded to treatment, indicating that Vγ9Vδ2 T cells might contribute to this anti-lymphoma effect. Overall, administration of pamidronate and low-dose IL-2 was well tolerated.

Hormone-Resistant Prostate Cancer

Our group recently initiated a phase I clinical trial to determine the anti-tumor effects of the single or combined administration of ZA plus IL-2 in patients with metastatic hormone-refractory prostate cancer (HRPC) [71], based on previous studies showing that ZA combined treatment is able to shift Vγ9Vδ2 T cells toward an activated effector-memory-like state [70]. Nine patients were enlisted to each arm and they received 4 mg ZA intravenously followed or not by subcutaneous infusions of IL-2 (0.6 million IU) on the same day, at 21-day intervals over a 12 months period. Neither treatment showed appreciable toxicity. Most patients were treated with ZA and IL-2, but conversely only two treated with ZA displayed a significant long-term shift of peripheral Vγ9Vδ2 T cells toward an activated effector-memory-like state producing IFN-γ and perforin. These patients also maintained serum levels of Vγ9Vδ2-derived TRAIL. Moreover, the numbers of effector-memory Vγ9Vδ2 T cells showed a statistically significant correlation with declining prostate-specific antigen (PSA) levels and objective clinical outcomes that comprised three instances of partial remission and five of stable disease. By contrast, most patients treated only with ZA failed to sustain either Vγ9Vδ2 T cell numbers or serum TRAIL, and showed progressive clinical deterioration. Thus, ZA and IL-2 represents a novel, safe, and feasible approach to induce immunologic and clinical responses in patients with HRPC, potentially providing a substantially increased window for specific approaches to be administered.

Renal Cell Carcinoma

There have been two different γδ T cell-based clinical trials in patients with metastatic RCC, and both have used adoptive transfer of *ex-vivo* expanded Vγ9Vδ2 T cells. Kobayashi *et al.* [72] have conducted a pilot study of adoptive immunotherapy using *in vitro* activated Vγ9Vδ2 T cells against advanced RCC to evaluate the safety profile and possibly anti-tumor effects. Seven patients were treated weekly with *ex-vivo* activated, autologous γδ T cells and IL-2. Five of the 7 patients enrolled in the study showed an increased population of peripheral γδ T cells and 5 patients showed an increase of absolute number of peripheral blood γδ T cells after a series of the therapy. After 6 to 12 cycles of treatment, no severe adverse reaction was observed and prolongation of tumor doubling time was seen in 3 out of 5 patients, paralleled by an increase of γδ T cells *in vivo* and a high response to antigen *in vitro*. These results indicate that the adoptive immunotherapy using *in vitro* activated autologous Vγ9Vδ2 T cells was well tolerated and induced anti-tumor effects.

Bennouna *et al.* [73] conducted a phase I study to determine the maximum-tolerated dose and safety of Innacell γδ™, an autologous cell-therapy product based on Vγ9Vδ2 T lymphocytes. Innacell γδ™ is manufactured *in vitro* from an autologous PBMC preparation, by a single stimulation with the synthetic phosphoantigen Phosphostim™ followed by a

2-week period of culture and expansion with IL-2. Innacell γδ™ contains 95% of T lymphocytes, of which a high proportion (mean of 76% of total cell number) is of the Vγ9Vδ2 phenotype. Tolerability and effectiveness of Innacell γδ™ were evaluated after administration of the cell-therapy product either by itself or in combination with repeated subcutaneous injections of a low dose of IL-2 sufficient to induce activation of Vγ9Vδ2 cells, for 27 cycles at 3-weeks interval. The data collected in this study indicate that repeated infusions of Innacell γδ™ either alone or with co-administration of IL-2 is well tolerated up to the dose of 4×10^9 Vγ9Vδ2 T cells. Moreover, 6 out of the 10 treated patients showed stabilized disease. Thus Innacell γδ™ retains valuable potential for further evaluation in metastatic renal cell carcinoma and the treatment of other types of cancer refractory to conventional treatments.

CONCLUSIONS: PROBLEMS AND PITFALLS

The discovery that γδ T cells can be selectively activated by naturally occurring or synthetic phosphoantigens, or drugs that enhance their accumulation into *stressed* cells, offers new avenues for the development of γδ T cell-based immunotherapies. Currently, several protocols based on the *in vivo* activation of Vγ9Vδ2 T cells with phosphoantigens or N-BPs or the adoptive transfer of *in vitro* expanded Vγ9Vδ2 T cells are in development for the treatment of some tumors Fig. (**2**). However, there are several potential problems which could hamper the possibility to use Vγ9Vδ2 T cells.

Major problems with *in vivo* immunization are anergy induction, immune exhaustion and/or activation induced cell death (AICD). Prenyl pyrophosphate and N-BPs antigens can be presented by any cell including non-professional APCs because of the broad expression of the proposed antigen-presenting molecule and the lack of requirement for antigen processing. However, presentation by non-professional APCs that lack the proper costimulatory signals and/or cytokines is likely to lead to incomplete activation and AICD. This perspective appears to happen in monkeys because intravenous immunization can only be performed 2–4 times before there is no further Vγ9Vδ2 T-cell response [62]. Even one intravenous immunization in the absence of exogenous IL-2 probably leads to loss of Vγ9Vδ2 T-cell function, given that the side effects of N-BPs are primarily seen only with the first injection. Recovery of proliferative responses appears to be very slow, as minor Vγ9Vδ2 T-cell expansions were seen 6 months after cessation of therapy in one individual. This induction of anergy and/or AICD probably also reflects the absence of Toll-like receptor ligands with current immunization approaches. Other problems may affect adoptive immunotherapy with *ex vivo*-expanded Vγ9Vδ2 T cells in fact γδ T cells, like αβ T cells, appear to have a homeostatic set point [76, 77].

Adoptive cell transfer (ACT) after host preconditioning by lymphodepletion represents an important advance in cancer immunotherapy. The efficacy of ACT-based tumor immunotherapy can be improved by the removal of the host immune system, several mechanisms might underlie the augmented efficacy of tumor-reactive T cells in the lymphopaenic environment. These factors include the elimination of immunosuppressive cells such as $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells which have the capability to inhibit phosphoantigen-activation of Vγ9Vδ2 [78], the depletion of endogenous cells that compete for activating cytokines and the increased function and availability of APCs [79]. Preclinical and clinical studies have identified multiple mechanisms contributing to successful adoptive immunotherapies, including host-related factors, as well as the phenotypic and functional characteristics of the tumor-reactive T cells used for transfer. Mouse models have now shown that early effector T cells mediate better *in vivo* anti-tumor responses than intermediate and late effector T cells on the basis of their increased proliferative and survival potential, receptiveness to homeostatic and co-stimulatory signals, homing to secondary lymphoid tissues and ability to secrete IL-2 [80]. In humans, mounting evidence seems to support the preclinical finding that less-differentiated T cells are the ideal cells for ACT [81-83]. Because the prenyl pyrophosphate antigens are so ubiquitous, Vγ9Vδ2 T cells begin to terminally differentiate at a relatively young age, such that one can find clonally expanded Vγ9Vδ2 T cells in middle age [84] and many older individuals are anergic or weakly responsive to stimulation with phosphoantigens [85]. Taken together, these findings indicate that the current criteria for selection of T cells for ACT, including release of IFN-γ or cytolysis alone, are suboptimal. Prenyl pyrophosphate antigens being produced by the tumor itself could also be a problem, as continued antigenic stimulation can lead to anergy of Vγ9Vδ2 T cells, as has been observed in patients with chronic tuberculosis or toxoplasmosis [86, 87].

Despite some mitigated results, cancer immunotherapies are still promising. The impressive increase of fundamental knowledge about tumor escape mechanisms now draws a clearer- yet incomplete- map of the many pitfalls on the way to therapeutic success. Innovative immunotherapeutic strategies harnessing γδ T cells have already been launched thanks to the rapid development of highly selective, small molecular weight drugs. In this endeavour, integrating the many different problems that have already been identified and those most likely to appear, may help to optimize the design of better cancer immunotherapies targeting γδ T cells.

DISCLOSURES

Francesco Dieli is founding member of a University of Palermo spin-out company (TetraPharm S.r.l.), in which he has a share of equity and to which he acts as scientific advisor in a nonexecutive capacity. Francesco Dieli is named inventor for several patents filed by TetraPharm S.r.l. on products who are related to those studied in this work. The other authors do not have a commercial or other association that might pose a conflict of interest.

ABBREVIATIONS

ACT	=	Adoptive cell transfer
ADCC	=	Antibody-dependent cell-mediated cytotoxicity
AICD	=	Activation induced cell death
APCs	=	Antigen-presenting cells

BCG	=	*Mycobacterium bovis Bacillus Calmette–Guerin*
BPs	=	Bisphosphonates
ccRCC	=	Clear cell RCC
DCs	=	Dendritic cells
FPPS	=	Farnesyl pyrophosphate synthase
GPP	=	Geranyl pyrophosphate
HMG-CoA	=	Hydroxymethyl-glutaryl coenzyme-A
HRPC	=	Hormone-refractory prostate cancer
IPP	=	Isopentenyl pyrophosphate
MM	=	Multiple myeloma
Mtb	=	*Mycobacterium tuberculosis*
MVA	=	Mevalonate
N-	=	Nitrogen-containing
non-N	=	Non-nitrogen containing
PSA	=	Prostate-specific antigen
RCC	=	Renal cell carcinoma
SCCHN	=	Squamous cancer cell head and neck
SCID	=	Severe combined immunodeficiency
SCID/hu	=	Severe combined immunodeficiency/ human
SRE	=	Skeletal related event
TCR	=	T cell receptor
Th1	=	T-helper cell type 1
TIL	=	Tumor-infiltrating lymphocytes
TRAMP	=	Transgenic adenocarcinoma of the mouse prostate
Treg	=	Regulatory T
ZA	=	Zoledronic acid

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Received: August 25, 2009 **Revised: January 04, 2010** **Accepted: January 04, 2010**