



# Potential use of $\gamma\delta$ T cell-based vaccines in cancer immunotherapy

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Immunotherapy is a fast advancing methodology involving one of two approaches: (1) compounds targeting immune checkpoints and (2) cellular immunomodulators. The latter approach is still largely experimental and features *in vitro* generated, live immune effector cells, or antigen-presenting cells.  $\gamma\delta$  T cells are known for their efficient *in vitro* tumor killing activities. Consequently, many laboratories worldwide are currently testing the tumor killing function of  $\gamma\delta$  T cells in clinical trials. Reported benefits are modest; however, these studies have demonstrated that large  $\gamma\delta$  T-cell infusions were well tolerated. Here, we discuss the potential of using human  $\gamma\delta$  T cells not as effector cells but as a novel cellular vaccine for treatment of cancer patients. Antigen-presenting  $\gamma\delta$  T cells do not require to home to tumor tissues but, instead, need to interact with endogenous, tumor-specific  $\alpha\beta$  T cells in secondary lymphoid tissues. Newly mobilized effector  $\alpha\beta$  T cells are then thought to overcome the immune blockade by creating proinflammatory conditions fit for effector T-cell homing to and killing of tumor cells. Immunotherapy may include tumor antigen-loaded  $\gamma\delta$  T cells alone or in combination with immune checkpoint inhibitors.

**Keywords:** immunotherapy,  $\gamma\delta$  T cells, cancer, antigen-presenting cells, vaccine

## HUMAN BLOOD $\gamma\delta$ T CELLS

Human blood  $\gamma\delta$  T cells constitute a minor subset (1–5%) of total T cells that is characterized by their T-cell antigen receptors (TCR) encoded by rearranged V-gamma and V-delta genes. Among blood  $\gamma\delta$  T cells, those expressing V $\gamma$ 9V $\delta$ 2–TCR greatly outnumber other  $\gamma\delta$  T-cell subsets whereas in peripheral tissues V $\gamma$ 9V $\delta$ 2+ T cells are relatively scarce. Their numbers are substantially increased in blood of patients suffering from various infectious diseases, indicating an involvement of V $\gamma$ 9V $\delta$ 2+ T cells in antimicrobial immunity. Indeed, early work revealed that V $\gamma$ 9V $\delta$ 2+ T cells are selective for a microbial non-peptide metabolite, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), found in numerous commensal and pathogenic microbes, including bacteria, *Plasmodium* and *Toxoplasma* species but not in man (1, 2). The same type of  $\gamma\delta$  T cells also responds (albeit at  $>10^4$ -fold higher concentrations) to the structurally related compound isopentenyl pyrophosphate (IPP), a metabolite of the mevalonate pathway in eukaryotes including man. Of interest, IPP was suggested to be increased in stressed cells, such as tumor cells, and its interaction with a specific IPP- (and HMBPP-) binding protein (BTN3A/CD277) leads to V $\gamma$ 9V $\delta$ 2+ T-cell activation (reviewed by the groups of E. Adams, E. Scotet, and T. Hermann in this Research Topic). IPP levels can be elevated artificially by addition of amino-bisphosphonates (such as zoledronate; see below) that inhibit an intracellular IPP-metabolizing enzyme. Consequently, treatment of PBMC with zoledronate results in the selective activation and outgrowth of V $\gamma$ 9V $\delta$ 2+ T cells. The exquisite selectivity for HMBPP/IPP distinguishes V $\gamma$ 9V $\delta$ 2+ T cells from  $\alpha\beta$  T cells that recognize a myriad of short peptides under MHC restriction. In fact, the V $\gamma$ 9V $\delta$ 2–TCR endows a sizable army of blood  $\gamma\delta$  T cells to become immediately mobilized in response to a single

class (“phosphoantigens”) of danger signals produced by microbes and possibly tumor cells (3). Relevant to the discussion below, this “mono-selectivity” allowed us to study the TCR-mediated functionality and migration properties of the entire V $\gamma$ 9V $\delta$ 2+ T-cell population.

## DISCOVERY OF $\gamma\delta$ T-APC: $\gamma\delta$ T CELLS WITH ANTIGEN-PRESENTATION FUNCTION

The myriad of effector and memory T cells present in our body can be distinguished based on their functional and migratory profiles. In fact, the characteristic functions of individual T cells are intimately related to their migratory properties, as exemplified by the distinct chemokine receptor profiles decorating individual T helper subsets (4, 5). For example, the chemokine receptor CXCR5 identifies follicular B helper T ( $T_{FH}$ ) cells that are specialized in orchestrating T cell-dependent antibody responses within the follicular compartments of secondary lymphoid tissues (6, 7). Our research on human blood  $\gamma\delta$  T cells (V $\gamma$ 9V $\delta$ 2–TCR+  $\gamma\delta$  T cells) began with the realization that treatment of  $\gamma\delta$  T cells with phosphoantigens induced the rapid and transient expression of CCR7 (8), the chemokine receptor enabling the rendezvous between naïve/central memory T cells and mature DC within lymph nodes (9). Immunological analyses revealed their presence in the T-cell zone but also B-cell follicles, the latter location suggesting that  $\gamma\delta$  T cells affect humoral responses. Indeed, and similar to  $T_{FH}$  cells, co-culture of  $\gamma\delta$  T cells with tonsillar B cells resulted in massive production of antibodies (8). In support, another lab identified CXCR5 on activated  $\gamma\delta$  T cells (10) and, recently, we and others reported the expression of the IL-21 receptor, linking follicular  $\gamma\delta$  T cells with  $T_{FH}$  cells and their B-cell targets (11, 12).

CCR7 expression is also in line with the view that activated  $\gamma\delta$  T cells team up with  $\alpha\beta$  T cells and/or DC in the T-cell compartment of secondary lymphoid tissues. In fact, short-term (1–3 days) activation of  $\gamma\delta$  T cells with the phosphoantigens IPP or HMBPP resulted in *de novo* expression (or up-regulation) of cell surface proteins normally associated with DC, including antigen presentation (MHC class I and II), co-stimulatory (CD40, CD80, CD86), and adhesion (CD11a, CD11b, CD11c, CD18, CD54) receptors. This observation led to detailed investigations into the possibility that activated (CCR7<sup>+</sup>)  $\gamma\delta$  T cells behaved like *bona fide* antigen-presenting cells (APC). Indeed, short-term activated  $\gamma\delta$  T cells were capable of processing simple (tetanus toxoid) and complex (*M. tuberculosis* PPD) protein antigens and inducing antigen-specific immune responses in primary, autologous  $\alpha\beta$  T cells (13). Activated  $\gamma\delta$  T cells did this equally well as donor matched monocyte-derived DC (moDC). Of note, short-term activated  $\alpha\beta$  T cells failed to express DC markers and were unable to present peptide antigens to  $\alpha\beta$  T cells, although under special conditions cloned T-cell lines were reported to induce responses in antigen-specific responder T-cell lines (14). Subsequent studies revealed that short-term activated  $\gamma\delta$  T cells were also capable of antigen cross-presentation, a process describing the induction of CD8<sup>+</sup>  $\alpha\beta$  T-cell responses to extracellular antigens. The precise mechanisms underlying antigen cross-presentation require further clarification, although it is generally assumed that extracellular antigens need to overcome intracellular membrane barriers in order to access the proteasome in the cytoplasm, which produces the peptide substrates for loading onto nascent MHC class I molecules (15). This process is in clear contrast to the classical MHC I pathway involving the processing of endogenous self- or microbe-derived antigens. Obviously, CD8<sup>+</sup> T cells-mediated killing of tumor and infected cells depends on cross-presentation capabilities of DC involved in processing corresponding cell debris. Activated  $\gamma\delta$  T cells are excellent cross-presenting APC, as shown by us (16, 17) and another laboratory (18). In fact, they seem to be able to do so more consistently than donor matched moDC, which may be explained by the reduced lysosomal activity in  $\gamma\delta$  T cells (17). After all, being a subtype of phagocytes, DC are very efficient in antigen uptake and intracellular degradation. Of note,  $\gamma\delta$  T cells were shown to phagocytose opsonized particles (*E. coli*, cell debris, microbeads), suggesting that phagocytosis, in addition to macropinocytosis, can contribute to antigen processing in this novel type of APC (18, 19). Induction of CD8<sup>+</sup> T-cell responses by peptide-pulsed  $\gamma\delta$  T cells was also demonstrated by another group (20, 21). Finally, activated  $\gamma\delta$  T cells resemble professional APC in their ability to trigger antigen-specific responses in naïve (antigen inexperienced) T cells. In summary, all these findings document unexpected properties that are normally associated DC and prompted us to designate activated (but not freshly isolated, resting) V $\gamma$ 9V $\delta$ 2<sup>+</sup> T cells as  $\gamma\delta$ T-APC (1).

In the past,  $\gamma\delta$  T cells have been portrayed as uniquely situated at the cross-road between innate and adaptive immunity, bridging both worlds by means of expressing recombined TCR involved in  $\gamma\delta$  T-cell activation and target cell lysis (hallmarks of adaptive immunity) while at the same time recognizing non-peptide antigens (phosphoantigens) in an MHC-unrestricted fashion similar to pattern recognition receptors (hallmarks of innate cells) (22,

23). Our findings with  $\gamma\delta$ T-APC add to this functional dichotomy, although proof of APC functions under *in vivo* conditions is still missing. Because  $\gamma\delta$  T cells respond to stress, e.g., elevated levels of IPP in tumor cells and microbes harboring HMBPP, one could envisage that the phosphoantigen-induced killing of target cells by mobilized  $\gamma\delta$  T cells is accompanied by the processing of tumor/microbial antigens and the subsequent induction of  $\alpha\beta$  T-cell responses. Activated  $\gamma\delta$  T cells are known for their abundant secretion of proinflammatory cytokines (TNF $\alpha$ , IFN $\gamma$ ) and, accordingly,  $\gamma\delta$ T-APC-mediated differentiation of CD4<sup>+</sup>  $\alpha\beta$  T cells resulted in effector cells dominated by a Th1-type cytokine profile (13). It will be important to see if under appropriate co-stimulatory conditions,  $\gamma\delta$ T-APC are also capable of generating alternative Th subsets. It is worth mentioning, however, that  $\gamma\delta$ T-APC are not equipped with the plethora of receptors sensing environmental cues as DC and, thus, are unlikely to compete with the numerous highly specialized DC whose inherent function it is to control T-cell functions in all aspects of immunity (health and disease). Instead, it appears that the APC function of  $\gamma\delta$  T cells is limited to situations where they become activated by their TCR ligands (phosphoantigens).

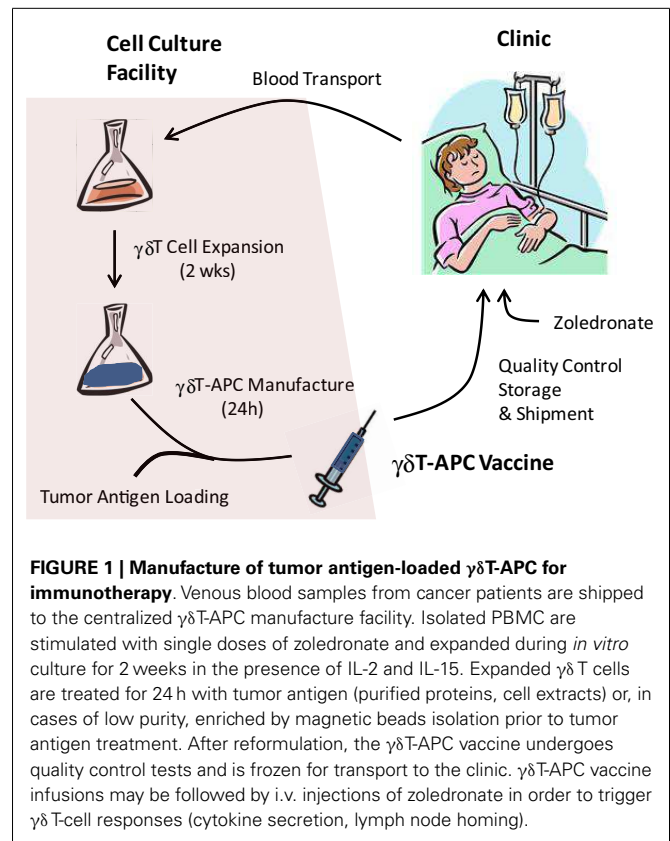
#### METHODS FOR THE GENERATION OF ANTIGEN-PRESENTING $\gamma\delta$ T-APC

Autologous moDC have been widely used in clinical trials as cellular vaccine with limited success. In fact, despite >10 years of translational research, clinically approved, moDC-based therapies still do not exist. Bottlenecks are manifold, including the difficulty of obtaining large numbers of moDC with robust APC function. Our recent findings suggest that  $\gamma\delta$ T-APC represent a promising alternative to moDC. Many laboratories have already demonstrated how easy it is to expand  $\gamma\delta$  T cells to very large numbers during *in vitro* culture (24–35). Based on these findings, we have developed a method for the generation of *in vitro* expanded  $\gamma\delta$ T-APC that may provide the basis for their large-scale manufacture under clinical-grade conditions (36). In brief, best results, both in terms of yield and quality, are achieved when PBMC samples isolated from fresh blood are stimulated once with zoledronate and then cultured in the presence of the growth factors IL-2 and IL-15. Zoledronate is a bisphosphonate drug known for its ability to increase intracellular IPP in target cells to levels sufficient for the selective activation of V $\gamma$ 9V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T cells (37). After 14 days of culture,  $\gamma\delta$ Tells have expanded >1000-fold and >800-fold with PBMC of healthy individuals and melanoma patients, respectively, yielding 10–50 million  $\gamma\delta$  T cells per milliliter of whole blood. Day 14  $\gamma\delta$  T cells retained their functionality as assessed by cytokine secretion and proliferation in response to HMBPP. Secreted cytokines included IFN $\gamma$  and TNF $\alpha$ , which are typically produced by primary  $\gamma\delta$  T cells in response to phosphoantigens, as well as several inflammatory chemokines whereas immune inhibitory cytokines (TGF $\beta$ , IL-10) were not detected. Day 14  $\gamma\delta$  T cells also killed tumor cells during *in vitro* culture. Importantly, and similar to short-term activated  $\gamma\delta$  T cells, expanded  $\gamma\delta$  T cells largely retained many cell surface receptors normally associated with APC, including antigen presentation (MHC I and II), co-stimulation (CD80, CD86), and adhesion (CD11a, CD54) molecules. Some of these were further elevated or re-expressed

transiently (CD40 and CCR7) in response to re-stimulation with HMBPP. APC marker expression was in line with their functionality. Without need for re-stimulation, day 14  $\gamma\delta$  T cells were able to process simple (influenza M1) and complex (PPD) antigens and to induce antigen-specific  $\alpha\beta$  T-cell responses in both cultured responder cell lines and primary responder cells present in unfractionated PBMC. Stimulation with HMBPP did not further enhance their APC function although, as discussed earlier, expanded  $\gamma\delta$  T cells still largely maintained their responsiveness (proliferation, cytokine secretion) to phosphoantigens. In summary, the current state of our research documents the feasibility of transforming peripheral blood  $\gamma\delta$  T cells into almost unlimited numbers of antigen-presenting  $\gamma\delta$ T-APC. We now need to investigate the ability of day 14  $\gamma\delta$  T cells to process tumor antigens, either in purified form or as tumor cell extracts. We believe that these APC are able to do so since we have already shown that activated  $\gamma\delta$  T cells efficiently processed extracts of influenza-infected epithelial cells and induced M1-specific  $\alpha\beta$  T-cell responses (17). It is further possible that interaction of  $\gamma\delta$  T cells with tumor cells not only leads to tumor cell killing but also to tumor antigen-presenting  $\gamma\delta$ T-APC able to induce  $\alpha\beta$  T-cell responses locally (tumor tissue) or at distal sites (lymph nodes).

### A PROTOCOL FOR A FIRST-IN-MAN CLINICAL TRIAL WITH TUMOR ANTIGEN-PRESENTING $\gamma\delta$ T-APC

Current state of research supports the following protocol for a first-in-man clinical trial with tumor antigen-presenting  $\gamma\delta$ T-APC (Figure 1). Preliminary data indicate anticoagulant-treated whole blood survives a 24 h shipment period in that PBMC samples retain responsiveness to zoledronate and IL-1/IL-15; similarly, we know that tumor antigen-loaded  $\gamma\delta$ T-APC can be frozen for storage and/or shipment to cancer clinics. Therefore, it appears that cancer patients will not need to visit a centralized  $\gamma\delta$  T-cell culture facility for donating blood samples and receiving  $\gamma\delta$  T cell-based vaccine infusions. A simplified procedure of  $\gamma\delta$ T-APC handling and distribution will facilitate clinical trials involving multiple centers. Following 2 weeks on *in vitro* culture as described above, tumor antigen-loaded  $\gamma\delta$ T-APC will be prepared by culturing expanded  $\gamma\delta$  T cells for 24 h in the presence of defined tumor antigen(s) or tumor cell extracts. A personalized immunotherapy protocol will involve the treatment autologous  $\gamma\delta$ T-cell preparations with extracts from the patient's own tumor cells. After washing and reformulation, tumor antigen-loaded  $\gamma\delta$ T-APC will be divided into individual bolus samples and then frozen in liquid nitrogen for shipment to corresponding cancer clinics. The frozen tumor antigen-loaded  $\gamma\delta$ T-APC samples will then be prepared locally for i.v. infusion into cancer patients according to treatment regimens that need to be defined during clinical trials. Ideally, a single round of cell culture will provide enough tumor antigen-loaded  $\gamma\delta$ T-APC for carrying out the entire round of treatment, which may follow a prime-boost protocol. Obviously, effective cell doses need to be established before being able to apply arithmetic for the generation of expanded  $\gamma\delta$  T cells. A small sample of tumor antigen-loaded  $\gamma\delta$ T-APC will be retained for quality control that will include sterility, purity, and APC phenotype assessments. Frequently, PBMC from cancer patients yield reduced numbers of expanded  $\gamma\delta$  T cells or cell preparations containing large (>50%)



numbers of non- $\gamma\delta$  T cells (mostly  $\alpha\beta$  T cells and/or NK cells) (36), suggesting that a single magnetic beads purification step could improve the vaccine function of antigen-loaded  $\gamma\delta$ T-APC. Last but not least, outcome measures for the  $\gamma\delta$ T-APC-based vaccine treatment need to be defined but will include immunological parameters determined in blood samples taken at intervals after each infusion step as well as clinical parameters (e.g., see details in EMA document)<sup>1</sup>. The above treatment protocol may be extended to include a single i.v. infusion of zoledronate 24 h after administration of each  $\gamma\delta$ T-APC vaccine infusion. We know that expanded  $\gamma\delta$  T cells retain full functionality (36). Equally important,  $\gamma\delta$  T cells in cancer patients who have received  $\gamma\delta$  T-cell infusions and even endogenous  $\gamma\delta$  T cells of cancer patients who did not receive  $\gamma\delta$  T-cell infusions were reported to respond to i.v. injections with zoledronate (32, 33, 38–43). Zoledronate is a safe medication that is frequently used to treat patients with bone disorders (44). Therefore, zoledronate may induce proinflammatory cytokine (TNF $\alpha$ , IFN $\gamma$ ) production and lymph node homing receptor (CCR7) expression and, thus, enhance the vaccine effect in infused  $\gamma\delta$ T-APC.

It is important to emphasize that  $\gamma\delta$ T-APC-based vaccines target the immune system in cancer patients and beneficial effects could include tumor control through immediate mobilization of endogenous, tumor-specific effector T cells, or establishing

<sup>1</sup>[http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2009/09/WC500003875.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003875.pdf)

tumor immunosurveillance involving the generation of long-lived, tumor-specific memory T cells (or a combination of both processes). In this regard, the here described method involving tumor antigen-loaded  $\gamma\delta$ T-APC differs fundamentally from current clinical trials exploiting the tumor killing properties of expanded  $\gamma\delta$  T cells (24–35).

## CONCLUDING REMARKS

Many questions remain, including the one related to the type of patients selected for a first-in-man clinical trial. As mentioned above,  $\gamma\delta$ T-APC-based vaccines target the patients' own immune system, indicating that patients suffering from tumors with well described immunogenicity profiles would benefit the most (45). Of course, tumor-specific immunity is a subject of intensive investigations and numerous tumor antigens have been associated with certain types of tumors. However, we are still far from understanding the complex adaptive immune processes involved in steady-state tumor control (immunosurveillance) and in tumor progression (disease). Perhaps, the most promising indication relates to conditions with prominent involvement of inhibitory immune cells (Treg cells, myeloid-derived suppressor cells), and current success with immune checkpoint inhibitors [anti-CTLA-4 Abs (Ipilimumab), anti-PD-1 Abs (Nivolumab)] may point us in the right direction (46). In fact, combination therapy with  $\gamma\delta$ T-APC-based vaccines and immune checkpoint inhibitors may even result in synergistic outcomes, i.e., blockade of inhibitory immune cells by immune checkpoint inhibitors may facilitate the stimulatory effect of  $\gamma\delta$ T-APC-based vaccines leading to enhanced tumor-specific effector T-cell responses and long-lived immunosurveillance T-cell formation. T-cell responses against melanoma are well described (47), suggesting that melanoma patients are promising candidates for a first-in-man clinical trial with  $\gamma\delta$ T-APC-based vaccines although other cancers with less well studied immunogenicity should not be excluded. We envisage that the proposed  $\gamma\delta$ T-APC-based immunotherapy will not be restricted to a single type of cancer since the majority of human cancers are considered to evoke T-cell responses (45).

At present, a single cellular vaccine product (sipuleucel-T) showing limited clinical benefits for prostate cancer patients has been approved by the FDA. Thus far, DC-based vaccines have not developed beyond the experimental stage. We believe that our proposed  $\gamma\delta$ T-APC-based therapy combines several features, including ease of manipulation and functional robustness that will make it a serious contender in the race to the first successful vaccine for use in immunotherapy of patients suffering from a broad range of cancers.

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