

Immunotherapy of Cancer Employing $\gamma\delta$ -T Cells: New Challenges, New Opportunities

Richard D. Lopez*

Bone Marrow Transplantation Program, THT-541, University of Alabama at Birmingham, 1900 University Boulevard, Birmingham, AL 35294, USA

Abstract: Particularly within the last half decade, the field of $\gamma\delta$ -T cell cancer immunotherapy has enjoyed a major expansion reflected in the growing number of publications in this area. The increased efforts of numerous investigators — with their accordingly varied strategies and approaches — is occurring largely on account of key biological, technological and pharmaceutical advances, all of which have converged in such a manner as to now give clinicians and scientists a variety of highly rational, yet practical options as they design and execute human clinical trials intended to exploit the innate antitumor properties of endogenous (i.e., patient-derived) $\gamma\delta$ -T cells for the immunotherapy of a wide variety of human malignancies. This review is not intended to serve as a comprehensive survey of the growing field as this has been expertly reviewed in the recent past. Rather, this review will attempt to highlight some of the newly recognized biological issues — and by extension, practical concerns — which have become central to the field. One such critical issue relates to the findings that in only some patients is it possible to efficiently activate and/or expand endogenous $\gamma\delta$ -T cells either *in vivo* or *ex vivo*, irrespective of the method used to stimulate these cells. This is in contrast to what is observed in normal healthy donors where robust *ex vivo* expansion or activation of $\gamma\delta$ -T cells is readily achievable. In light of such observations, the emerging general consensus is that there may exist a poorly-defined “cancer-associated $\gamma\delta$ -T cell impairment” occurring in patients — an impairment which might preclude the widespread use of strategies which rely upon activating or expanding potentially tumor-reactive endogenous $\gamma\delta$ -T cells. With this in mind, we discuss new strategies being developed to address this emerging challenge. This includes the development of models allowing for the adoptive transfer of tumor-reactive *allogeneic* (donor-derived) $\gamma\delta$ -T cells obtained from otherwise healthy individuals. This, as well as other challenges — both biological and practical — will be discussed in the context of developing the next generation of human clinical trials intended to exploit the innate antitumor properties of $\gamma\delta$ -T cells.

CELL-BASED IMMUNOTHERAPY OF CANCER: CURRENT UNDERSTANDING AND LIMITATIONS

The view that cellular immune responses might be exploited for the treatment of human malignancies is not new. Interestingly, to date, the majority of studies in this regard have focused primarily upon exploiting *adaptive* cellular immune responses which are directed against tumor-specific or tumor-associated antigens. This includes a number of important studies designed to generate tumor-specific cytotoxic CD8+ $\alpha\beta$ -T lymphocytes (CTL) utilizing specific peptide antigens, as well as other studies designed to develop tumor-specific immune responses employing dendritic cell-based vaccination strategies [1-4]. However, these and similar approaches which rely upon adaptive immunity (e.g., major histocompatibility complex (MHC)-restricted, antigen-specific responses) suffer from several potential shortcomings.

First, these strategies presuppose that an antigen selected as a target for cell-based immunotherapy is indeed tumor-specific — that is, the antigen is expressed only in tumor cells, but not in normal tissues. Moreover, various antigens

which might serve as therapeutic targets may not be ideal on account that they may be expressed only by a proportion of malignant cells.

Second, it is well established that various cancer cells can either downregulate the expression of MHC molecules, or suffer from defects in assembly and expression of MHC molecules [5-7]. Accordingly, it has been proposed that cancer cells expressing little or no MHC molecules can selectively escape recognition by MHC-restricted CTL, a view which is partially supported by the clinical observations that reduced expression of MHC class I on breast cancer cells or on diffuse large B-cell lymphoma cells may be associated with poorer clinical outcomes [8-11].

With this in mind, particularly in the context of developing novel cell-based approaches for the treatment of advanced or recurrent cancers, it becomes especially important to consider and explore tumor antigen-*independent* (innate) cellular immune responses mediated by such cells as natural killer (NK) cells and $\gamma\delta$ -T cells.

INNATE ANTITUMOR ACTIVITY OF $\gamma\delta$ -T CELLS AND TUMOR IMMUNOSURVEILLANCE: BIOLOGICAL RATIONALE FOR $\gamma\delta$ -T CELL-BASED IMMUNOTHERAPIES

Unlike $\alpha\beta$ -T cells which recognize specific peptide antigens presented by MHC molecules, $\gamma\delta$ -T cells in contrast can recognize generic antigens which can be expressed by

*Address correspondence to this author at the Bone Marrow Transplantation Program, THT-541, University of Alabama at Birmingham, 1900 University Boulevard, Birmingham, AL 35294, USA; Tel: (205) 975-0323; Fax: (205) 975-8394; E-mail: Richard.Lopez@ccc.UAB.edu

stressed cells, including cells which have undergone malignant transformation. Indeed, cancerous cells are now known to display a number of stress-induced antigens which while neither tumor-specific nor tumor-derived *per se*, can nonetheless serve as recognition determinants for human and mouse $\gamma\delta$ -T cells [12-17]. Although a functional homology between mouse and human $\gamma\delta$ -T cells has yet to be firmly established in this specific regard, the complementary study of both mouse and human $\gamma\delta$ -T cells has yielded important insight into how $\gamma\delta$ -T cells recognize and kill malignantly transformed cells *in vitro* and *in vivo*. Thus, it is now evident that both mouse and human $\gamma\delta$ -T cells utilize various pairings of specific $\gamma\delta$ -T cell receptor chains — often in combination with key co-receptors — to interact with determinants commonly expressed on tumor cells which are susceptible to $\gamma\delta$ -T cell-mediated killing [16, 17].

$\gamma\delta$ -T CELL ANTITUMOR IMMUNOSURVEILLANCE HIGHLIGHTED IN ANIMAL MODELS

The ability of $\gamma\delta$ -T cells to recognize and kill a variety of malignant cells in a tumor antigen-independent manner (innate immune response) has contributed to the emerging view that $\gamma\delta$ -T cells provide protective immunosurveillance against cancer. This view is supported by reports that mice lacking $\gamma\delta$ -T cells are more susceptible to the development of chemically-induced cutaneous tumors and are likewise, less able to resist challenges with tumorigenic melanoma or squamous-cell carcinoma cell lines [18-20]. Additional earlier studies have also established that $\gamma\delta$ -T cells in mice provide a degree of antitumor immunosurveillance against spontaneously-arising malignancies of hematolymphoid origin [21].

Very recently, utilizing the TRAMP transgenic mouse model of prostate cancer, we have extended these important findings by establishing that $\gamma\delta$ -T cells are also capable of providing protective immunosurveillance against spontaneously arising non-cutaneous solid tumors of epithelial origin [22]. In these studies, TRAMP mice — which spontaneously develop prostate adenocarcinoma — were backcrossed with $\gamma\delta$ -T cell-deficient mice (TCR δ ^{-/-}) yielding TRAMP \times TCR δ ^{-/-} mice, a proportion of which developed more extensive disease compared to TRAMP mice with normal $\gamma\delta$ -T cell development.

In addition to the studies performed in the setting of murine prostate cancer, recent data from our laboratory also establish that $\gamma\delta$ -T cells do indeed provide protective immunosurveillance against the mouse plasmacytoma/myeloma cell line MOPC-315, a cell line which has been used extensively in studies of the pre-clinical, immunological, and pharmacological aspects of myeloma. In these unpublished studies (manuscript in preparation), healthy wild-type BALB/c mice were treated with GL3, an antibody directed against the mouse $\gamma\delta$ -T cell receptor (TCR) which is known to inactivate mouse $\gamma\delta$ -T cells *in vivo* [23-25]. GL3-treated mice and control mice were then challenged with equivalent numbers of MOPC-315 cells (originally derived in BALB/c mice and thus, tumorigenic in wild-type BALB/c mice). Tumor burden was then assessed in each mouse by measuring the serum concentration of the monoclonal immunoglobulin (IgA λ ²³¹⁵) produced by MOPC-315 cells. In these studies, mice treated with GL3 developed significantly larger tumor burdens compared to control mice when challenged with equivalent numbers of MOPC-315 cells.

$\gamma\delta$ -T CELL ANTITUMOR IMMUNOTHERAPY HIGHLIGHTED IN ANIMAL MODELS

While the studies noted above have contributed to the emerging view that $\gamma\delta$ -T cells provide protective innate immunosurveillance against certain malignancies, they have also provided a strong rationale for developing immunotherapy models to assess how the innate antitumor properties of $\gamma\delta$ -T cells might be exploited clinically. Using xenograft models whereby human tumor cell lines were first introduced into immunodeficient mice, a number of investigators have clearly shown that adoptively-transferred human $\gamma\delta$ -T cells were effective in controlling disease in such models [26-29]. In addition, we have recently been able to show using a fully syngeneic mouse prostate cancer model that adoptively-transferred $\gamma\delta$ -T cells are not only effective at controlling experimentally established disease, but that adoptively-transferred $\gamma\delta$ -T cells clearly home to and localize within established tumors — a key biological correlate in cell-based immunotherapy models [22]. Importantly, in this model, disease-bearing mice treated intravenously with syngeneic $\gamma\delta$ -T cells displayed superior survival compared to untreated mice.

CLINICAL APPLICATIONS OF HUMAN $\gamma\delta$ -T CELLS: ALTERNATE STRATEGIES, SIMILAR INTENT

Given the recognized capacity of $\gamma\delta$ -T cells to innately kill malignant cells both *in vitro* and *in vivo*, efforts are now actively underway to develop and refine the means to exploit the antitumor properties of $\gamma\delta$ -T cells for clinical purposes. Several factors suggest that $\gamma\delta$ -T cell-based immunotherapies could be applicable to a wide variety of human cancers. First, there is the growing number of reports which have established that human $\gamma\delta$ -T cells can indeed recognize and kill a wide variety of malignant human cell lines ranging from those of epithelial origin (e.g., breast, prostate, colorectal, pancreatic, lung, glioblastoma and other cell lines), to include those of hematolymphoid origin as well (lymphoma and myeloma cell lines) [30-39]. Second, it has also been shown that $\gamma\delta$ -T cells isolated from tumors removed from patients (i.e., tumor-infiltrating lymphocytes) retain *in vitro* lytic activity against human cancer cells — yet almost uniformly fail to kill non-malignant human cell lines [36, 37]. This is a key point to be made in the particular context of studies in which $\gamma\delta$ -T cells are to be administered therapeutically.

Although it remains to be determined specifically how $\gamma\delta$ -T cells might best be employed clinically, two general approaches are currently being taken in this regard. One approach includes strategies primarily designed to activate or expand *in vivo* within patients, their own endogenous $\gamma\delta$ -T cells. This approach is based upon the recognition that either bisphosphonates (which are commonly used to prevent skeletal fractures in cancer patients) or synthetic phosphoantigens can stimulate human as well as simian (but not murine) $\gamma\delta$ -T cells leading to their expansion and activation *in vitro* and *in vivo* [40-47]. As of now, studies have been conducted or are ongoing which have focused upon employing pharmacological agents such as the aminobisphosphonates pamidronate (Aredia[®]) or zoledronate (Zometa[®]) or synthetic phosphoantigens such as bromohydrin pyrophosphate (BrHPP, Phosphostim[™]) administered in conjunction with interleukin (IL)-2 [43, 47-52]. Importantly, recently published results from a phase I clinical trial strongly support the view that activated $\gamma\delta$ -T cells found in zoledronate-

treated patients contribute either directly or indirectly to the clinical responses observed in patients with hormone-refractory prostate cancer [53]. These findings are in agreement with earlier studies in which objective responses were also seen in patients with hematolymphoid malignancies who were treated with pamidronate and low-dose IL-2 [43].

Alternatively, the innate antitumor properties of human $\gamma\delta$ -T cells might also be exploited through the adoptive transfer of $\gamma\delta$ -T cells first expanded *ex vivo*, then subsequently reinfused into tumor-bearing patients. Indeed, recent advances by ourselves and others have now made possible the large-scale *ex vivo* expansion of human $\gamma\delta$ -T cells which importantly retain potent innate antitumor activity against a variety of human cancer cell lines *in vitro* [38, 39, 50, 54, 55]. As importantly, human $\gamma\delta$ -T cells expanded *ex vivo* also have been shown to retain potentially clinically useful antitumor activity *in vivo* [29]. This was demonstrated using a SCID mouse model in which animals harboring human cancer cells were found to have reduced tumor burdens and prolonged survival when treated with human $\gamma\delta$ -T cells first expanded *ex vivo* using the aminobisphosphonate alendronate (Fosamax®).

These and related advances have made possible the design and execution of early phase clinical trials including two recently reported studies in which patients with renal cell carcinoma were treated with *ex vivo* expanded autologous $\gamma\delta$ -T cells, demonstrating the feasibility and tolerability of such an approach [52, 56]. Currently at our institution, we are conducting a phase I clinical trial in which patients with advanced breast cancer are to be treated with autologous tumor-reactive $\gamma\delta$ -T cells which are *ex vivo* expanded utilizing an alternative method of $\gamma\delta$ -T cell expansion [54, 57].

EMERGING EVIDENCE OF “DAMAGED” OR EXHAUSTED ENDOGENOUS $\gamma\delta$ -T CELLS IN PATIENTS

Despite early advances leading to the first generation of clinical trials, evidence is now accumulating suggesting that there exists a potential major obstacle to autologous $\gamma\delta$ -T cell-based strategies — irrespective of whether $\gamma\delta$ -T cells are to be activated pharmacologically *in vivo*, or first *ex vivo* expanded, then reinfused. This relates to $\gamma\delta$ -T cells themselves found within patients. It was first reported that when compared to healthy donors, endogenous $\gamma\delta$ -T cells can be substantially decreased in numbers in the peripheral blood of patients newly diagnosed with certain cancers [58]. We have since confirmed and extended these findings having now shown that a numerical deficit — sometimes quite striking — can exist in the $\gamma\delta$ -T cell compartment of some patients newly diagnosed with certain cancers. This includes patients with glioblastoma [31], prostate cancer [59], as well as patients with breast cancer or lung cancer (manuscripts in preparation).

In addition — and possibly more importantly — it appears that in only a proportion of patients is it possible to efficiently activate and/or expand endogenous $\gamma\delta$ -T cells either *in vivo* or *ex vivo*. This is in contrast to what is observed in normal healthy donors where robust expansion or activation of $\gamma\delta$ -T cells is readily achievable. Indeed, in earlier clinical trials and in ongoing studies, it is sometimes necessary to “pre-screen” study subjects using small-scale *in vitro* $\gamma\delta$ -T cell proliferation assays thus allowing for the identification and selection of those patients in whom $\gamma\delta$ -T cells can be activated [43]. As presented

at the 2008 $\gamma\delta$ -T cell Conference (Marseille, France, May 21-23), a number of investigators — including ourselves — now report that irrespective of the specific activation or expansion methods employed, $\gamma\delta$ -T cells obtained from a substantial proportion of tumor-bearing individuals appear to respond poorly to activation stimuli. Thus, the emerging general consensus is that there may exist a poorly-defined “cancer-associated $\gamma\delta$ -T cell impairment” in patients — an impairment which might possibly render unfeasible strategies which rely exclusively upon the innate antitumor properties of autologous $\gamma\delta$ -T cells.

We have since been able to corroborate these findings using a mouse model designed to directly assess this issue (manuscript in preparation). In these studies, when compared to healthy control animals, mice bearing syngeneic tumors were found to have fewer circulating peripheral blood $\gamma\delta$ -T cells, while manifesting no significant changes in peripheral blood $\alpha\beta$ -T cell counts, or other relevant hematological parameters. Moreover, we observed that a substantially greater proportion of $\gamma\delta$ -T cells isolated directly from the spleens and peripheral blood of tumor-bearing mice were actively undergoing apoptosis and importantly, expressed surface markers consistent with activation. We have since been able to demonstrate a strong correlation between tumor burden and the proportion of $\gamma\delta$ -T cells actively undergoing apoptosis, observing that animals with higher tumor burdens had in turn, a greater proportion of $\gamma\delta$ -T cells actively undergoing apoptosis. Entirely consistent with the observations made in humans, $\gamma\delta$ -T cells isolated from tumor-bearing mice expanded very poorly, if at all, in contrast to $\gamma\delta$ -T cells isolated from healthy control mice.

Various mechanisms likely contribute to the observed numeric or functional defects occurring in $\gamma\delta$ -T cells isolated from either tumor-bearing mice or humans [60]. However, we favor a model in which activation-induced cell death (AICD) plays a major role accounting for either the numerical deficits or the poor activation observed in $\gamma\delta$ -T cells found in tumor-bearing hosts. Indeed, under certain conditions $\gamma\delta$ -T cells can be particularly sensitive to AICD and can quite readily be induced to undergo apoptosis upon activation [54, 61-65]. We accordingly propose that in the setting of cancer, $\gamma\delta$ -T cells are lost as a consequence of AICD, this resulting from repeated encounter with tumor cells which express a variety of stress-induced self antigens which can be recognized by (and thus stimulate) reactive $\gamma\delta$ -T cells. Consequently, it is this repeated mitogenic stimulation (i.e., AICD) which eventually drives tumor-reactive $\gamma\delta$ -T cells to undergo apoptosis. This model is particularly appealing if one considers that tumor cells — which are not effectively eradicated by adaptive immune responses — persist and thus remain as a source of chronic mitogenic stimulation for tumor-reactive $\gamma\delta$ -T cells. Studies in both humans and mice are actively ongoing in our laboratory to directly test this hypothesis and to more clearly elucidate the cellular and molecular mechanisms by which this may be occurring.

RATIONALE FOR EMPLOYING ALLOGENEIC (DONOR-DERIVED) $\gamma\delta$ -T CELLS RATHER THAN AUTOLOGOUS $\gamma\delta$ -T CELLS

With the above concerns in mind, we have now developed an alternative approach to autologous $\gamma\delta$ -T cell-based immunotherapies. In essence, rather than relying upon

potentially “damaged” autologous (i.e., patient-derived) $\gamma\delta$ -T cells for immunotherapy, we are taking an approach which allows us to adoptively transfer *allogeneic* (donor-derived) $\gamma\delta$ -T cells obtained from healthy donors. We reason that tumor-reactive $\gamma\delta$ -T cells obtained from healthy donors will be “undamaged” and in theory more effective. Moreover, donor-derived tumor-reactive $\gamma\delta$ -T cells will be available in essentially limitless numbers from a healthy donor (after *ex vivo* expansion) and thus in theory can be delivered repeatedly to the tumor-bearing hosts. However, as noted below, the adoptive transfer of allogeneic $\gamma\delta$ -T cells (or any T cell subset) into a tumor-bearing host is unlikely to be successful in the absence of specific immunological maneuvers first undertaken explicitly to permit this.

CONVENTIONAL VIEW: ALLOGENEIC HEMATOPOIETIC STEM CELL (HSC) TRANSPLANTATION AND DONOR LYMPHOCYTE INFUSIONS (DLI)

Traditionally, allogeneic hematopoietic stem cell (HSC) transplantation (also referred to as bone marrow transplantation, or BMT) has been reserved primarily for the treatment of malignancies of hematology origin such as the acute leukemias or certain subtypes of lymphoma [66]. In conventional HSC transplantation, antitumor effects are provided by high-dose chemotherapy and/or radiation delivered as part of the transplant conditioning. However, it is also evident that secondary nonspecific immune-mediated “graft-versus-tumor” effects also contribute to disease control. Though the mechanisms by which this occurs are not well understood, it is evident that competent donor-derived (allogeneic) immune effector cells play a key role in the graft-versus-tumor effects seen in the setting of allogeneic HSC transplantation in selected diseases.

Indeed, given the powerful antitumor effects of donor-derived immunity, in certain diseases it is not uncommon following allogeneic HSC transplantation to deliver a so-called “donor lymphocyte infusion” (DLI) in order to either induce (promote) or sustain remission after transplantation [66-68]. This, however, is commonly performed by introducing crude, unfractionated preparations of donor-derived peripheral blood lymphocytes containing primarily $\alpha\beta$ -T cells. Accordingly, and not unexpectedly, such maneuvers commonly result in the development of sometimes life-threatening uncontrollable graft-versus-host disease (GVHD) in the recipient [67].

“ $\gamma\delta$ -T CELL DLI” AS A NEW PARADIGM: ALLOGENEIC HSC TRANSPLANTATION AS A PLATFORM PERMITTING THE SUBSEQUENT CELLULAR THERAPY OF CANCER EMPLOYING DONOR-DERIVED, TUMOR-REACTIVE $\gamma\delta$ -T CELLS

We have now constructed various mouse models in which the allogeneic HSC transplant procedure itself has been relegated to a supporting role — essentially serving now as the therapeutic platform for the subsequent delivery of therapeutic donor-derived $\gamma\delta$ -T cells. For example, in one model, tumor-bearing BALB/c mice (harboring syngeneic tumors) first undergo allogeneic HSC transplant performed using bone marrow stem cells obtained from C57BL/6 mice. This is performed not as a therapy, but rather to first establish immunological chimerism in tumor-bearing BALB/c mice, thus allowing for the subsequent transfer of donor-derived (C57BL/6) $\gamma\delta$ -T cells. Indeed, after tumor-

bearing mice undergo HSC transplantation, we are able to adoptively transfer large numbers of donor-derived $\gamma\delta$ -T cells into these tumor-bearing recipient mice. Importantly, allogeneic donor-derived $\gamma\delta$ -T cells are not rejected by the host (recipient), and moreover, donor-derived $\gamma\delta$ -T cells do not cause GVHD in the recipient animals — all despite the full MHC-mismatch of the host-donor strain combinations. Ongoing studies have now clearly established that tumor-bearing BALB/c mice which subsequently undergo an allogeneic HSC transplant using bone marrow derived from C57BL/6 mice manifest excellent disease control and survival, particularly if they receive multiple infusions of C57BL/6 donor-derived $\gamma\delta$ -T cells (i.e., $\gamma\delta$ -T cell DLI). In contrast, mice undergoing the same HSC transplant but which receive no $\gamma\delta$ -T cell DLI develop more disease and display far poorer survival (unpublished data). Thus, we interpret these findings to support our model that it is not the allogeneic HSC transplant procedure itself, but rather the subsequent delivery of donor-derived $\gamma\delta$ -T cells which accounts for the improved disease control and survival.

In the context of the above discussion, the clinical transfer of allogeneic tumor-reactive $\gamma\delta$ -T cells must now be considered seriously for a number of compelling reasons. First, tumor-reactive $\gamma\delta$ -T cells expand robustly from virtually all healthy individuals. Accordingly, the availability of $\gamma\delta$ -T cells for use as a DLI will not be a limiting factor in the performance of such studies. Second, $\gamma\delta$ -T cells appear incapable of mediating GVHD as they appear incapable of recognizing MHC-defined allogeneic donor-host disparities [69, 70]. Most importantly, advances in the field of clinical HSC transplantation have made it possible now to perform allogeneic HSC transplants with ever-decreasing morbidity and mortality. Thus, in theory it would be possible to carry out the adoptive transfer of allogeneic $\gamma\delta$ -T cells in the setting of a state-of-the-art nonmyeloablative HSC transplant (commonly, but imprecisely referred to as a “mini-transplant”) — a strategy explicitly developed to achieve donor-host T-lymphocyte chimerism with minimal regimen-related toxicity [71-76]. Indeed, data clearly show that such allogeneic HSC transplants can be performed now even in high-risk populations, such as elderly patients or patients with serious medical co-morbidities.

In theory then, the establishment of donor-host immunological chimerism followed by the repeated infusion of tumor-reactive donor $\gamma\delta$ -T cells may form the basis of the next generation of experimental allogeneic HSC transplantation strategies. Importantly, these and other innovative approaches can now be tested directly — especially now that the technological means exist to expand *ex vivo* the large numbers of highly pure donor-derived $\gamma\delta$ -T cells which will likely be needed for such studies [52, 54, 57].

Finally, indirect evidence provides additional support for the view that allogeneic $\gamma\delta$ -T cells may indeed be potent mediators of antitumor effects. Though an observational study, it was nevertheless noted that long-term leukemia-free survival as well as overall survival was significantly greater in patients, who, after transplant, developed increased $\gamma\delta$ -T cell blood counts compared to patients who developed low or normal $\gamma\delta$ -T cell blood counts. These findings can be interpreted to indicate that $\gamma\delta$ -T cells may be able to mediate clinically relevant graft-versus-leukemia effects [77].

FUTURE DIRECTIONS

As discussed above, although $\gamma\delta$ -T cell-based immunotherapies hold great promise, certain challenges present themselves and remain to be negotiated in order that clinical trials can be designed and performed in a rational and practical manner.

Clearly, the most pressing issue relates to whether or not autologous $\gamma\delta$ -T cell-based therapies will become an option for more than a few selected patients given the strong suggestion that the $\gamma\delta$ -T cell compartment (for whatever reason) may be numerically or functionally impaired or suppressed in a significant proportion of patients. Precisely how this impairment or suppression occurs *in vivo* is not known, though understanding this will be key to developing clinically relevant strategies to overcome this obstacle. One recent report provides important insight into this matter. In this study, it was determined that CD4+CD25+FoxP3+ regulatory T cells (Treg) play a role in suppressing the *in vitro* activation of $\gamma\delta$ -T cells. Accordingly, the authors suggest that combining Treg cell inhibition approaches with $\gamma\delta$ -T cell activation strategies may result in overcoming $\gamma\delta$ -T cell-specific suppression mediated by Treg cells [60]. Alternatively, if activation-induced cell death is indeed accounting for the impairment of $\gamma\delta$ -T cells in patients, strategies might be specifically developed to attempt to overcome this both *in vitro* and *in vivo*. Our laboratory is actively undertaking just such studies.

Related to the immunobiology of $\gamma\delta$ -T cell interactions with tumors, it will be key to gain a more clear understanding of what molecular targets are being recognized on tumor cells by $\gamma\delta$ -T cells. Presuming these targets can be identified, new strategies might be developed to selectively or non-selectively modulate or upregulate these targets, thereby potentiating $\gamma\delta$ -T cell-mediated killing of tumor cells. This might be accomplished using various combination of chemotherapy, bisphosphonates, or even biological agents as suggested by recent studies [78-81].

Another potentially fruitful area of investigation relates to the recognition that under certain conditions, $\gamma\delta$ -T cells may be capable of enhancing dendritic cell (DC) maturation, or may themselves serve as antigen presenting cells to $\alpha\beta$ -T cells [82, 83]. Provided that appropriate tumor-specific or tumor-associated antigen are selected, strategies might be developed in which adaptive immune responses directed against tumor antigens can thus be augmented through the activation of innately functioning $\gamma\delta$ -T cells.

Finally, it stands to reason that once studies progress beyond early-phase clinical trials — which typically enroll patients with end-stage or advanced cancers — the focus will shift to the study of how $\gamma\delta$ -T cells might be employed to minimize the likelihood of recurrence. In other words, one could begin to ask how adjuvant treatment strategies could be devised for patients at particularly high risk for local treatment failure such as patients who undergo only an incomplete surgical resection, or for those patients with tumors which, despite definitive therapy, are nevertheless likely to recur under any circumstance (e.g., pancreatic cancer, glioblastoma, etc.). Indeed, scenarios can be envisioned in which selected patients can be administered bisphosphonates or phosphoantigens along with IL-2 purely

in the adjuvant setting after undergoing surgery, chemotherapy or radiotherapy. One could argue that $\gamma\delta$ -T cell-based immunotherapies — or any cell-based therapies — are most likely to be effective in just such a setting of minimal residual disease. Accordingly, the development of $\gamma\delta$ -T cell-based adjuvant immunotherapies may become a particularly exciting area of exploration in the near future.

CONCLUSION

From this review, it should be evident that in dealing with the biological and clinical challenges encountered to date in the field of $\gamma\delta$ -T cell-based immunotherapy, new areas of investigation have arisen. Accordingly, clinicians and scientists now have the opportunity to extend the field in important ways allowing for the development in concert, of the next generation of clinical trials.

While the biological and technical challenges are fairly obvious, it should be noted that owing to the unique and complex nature of cell-based therapies, a number of non-biological challenges also exist and remain to be negotiated in order that clinical trials can be designed and performed in a practical and cost-effective manner — no small consideration in these times.

Particularly for clinical studies involving the adoptive transfer of *ex vivo* expanded $\gamma\delta$ -T cells, significant regulatory issues must be addressed as cellular products must be produced and administered in accordance with increasingly more stringent regulatory guidelines and standards. Related to this point is the requirement that current Good Manufacturing Practices (cGMP) facilities and cGMP-grade reagents must be used in the expansion and/or purification of $\gamma\delta$ -T cells for adoptive transfer. As a consequence, clinical trials employing these cells may become prohibitively expensive to undertake. Moreover, when clinical trials are to be performed, the question of who will pay for such clinical trials looms large — particularly in the U.S. where third party payers (i.e., insurance companies) typically will not cover the cost of experimental therapies. Clearly, this is an issue that will need to be addressed and will require a concerted effort by clinicians and scientists directly involved in this field to proactively engage with governmental funding agencies, regulatory agencies, pharmaceutical and biotechnology concerns, as well as third party payers (in the U.S.) to assure that this important work can go on.

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