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Pre-selection of PD-1+ Tumor-Infiltrating CD8+ T cells improves the efficacy of adoptive T-cell Therapy

Diego Salas-Benito[1,2], Noelia Casares[2,3], Pablo Sarobe[2,3], Juan José Lasarte[2,3] and Sandra Hervas-Stubbs[2,3]*

[1]Oncology Department, University Clinic, University of Navarra, Spain.
[2]Instituto de Investigación Sanitaria de Navarra (IdISNA), Spain.
[3]Program of Immunology and Immunotherapy, Center for Applied Medical Research (CIMA), University of Navarra, Spain.

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***Correspondence:**

Dr. Sandra Hervas-Stubbs, Center for Applied Medical Research (CIMA), University of Navarra, 31008-Pamplona, Spain; Telephone: 00-34-9481947004;
E-mail: mshervas@unav.es

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Immunotherapies with autologous T cells have become a powerful treatment option for cancer patients. These include the adoptive transfer of naturally-occurring tumor-specific T cells isolated from tumor infiltrates, the so-called tumor-infiltrating lymphocytes (TIL), and the transfer of T lymphocytes that have been genetically modified to express a transgenic T-cell receptor (tg-TCR) specific for a tumor antigen, or a chimeric antigen receptor (CAR) composed of a single-chain variable fragment, derived from a monoclonal antibody (mAbs) against a surface tumor antigen, fused to endo-domains of T-cell signaling molecules[1].

TIL therapy has had a long history of development with multiple clinical trials in centers around the world that consistently have demonstrated long-lasting clinical response rates (~50%) in advanced melanoma[2–10] and, more recently, in cervical cancer[11]. Critical requirements for the efficacy of TIL therapy are the lymphodepletion prior to TIL administration and the systemic administration of IL-2. Lymphodepleting preconditioning depletes Tregs and removes cellular "sinks" whereas IL-2 systemic administration supports the persistence of the infused TILs in vivo. TIL therapy, in a tripartite regimen that include lymphodepleting chemotherapy, cell transfer and high-dose of IL-2, has been shown to be capable of mediating durable tumor regression at rates greater than that of IL-2 alone (Overall response rate ≈ 50% and Complete response ≈ 20%)[4,7–10]. The toxicities of treatment are transient and manageable and result of the known adverse effects of nonmyeloablative chemotherapy and administration of high-dose IL-2[8].

A clear advantage of TIL treatment is the broad nature of the T-cell recognition against both defined and un-defined tumors antigens, and in the context of all possible MHC molecules, rather than the single specificity of tg-TCR- or CAR-transduced T cells, and the limited MHC coverage of tg-TCR T cells. On-target/off-tumor toxicity is relatively infrequent in TIL therapy while it is a major problem encountered with genetically modified T-cell therapies. Candidate patients for TIL therapy are needed to have an accessible and operable tumor, so TILs will be isolated from the tumor, while for generation of re-directed T cells circulating patient's lymphocytes can be used.

Recently it has been shown that TILs recognize the neoantigens

that arise as a consequence of tumor-specific mutations[12–15]. Importantly, some studies suggest that neoantigen-reactive T cells are likely the dominant player inducing tumor regressions after TIL therapy[12,14], which may also explain the low on-target/off-tumor toxicity of this therapy. TIL therapy should work better in tumors with high mutation rate, including skin and small cell lung cancers (strongly related to UV-radiation exposure and smoking, respectively), and tumors with microsatellite instability or mismatch repair-deficiency, as well as in tumors of viral origin (i.e., cervical cancer and head and neck tumors, associated with the human papilloma virus).

One of the major constraints of TIL therapy is that the TIL-manufacturing process is laborious and time-consuming. Ex vivo expansion of TILs is done in two stages[16]. The first stage involves the expansion (in multi-well cultures) of TILs from tumor fragments, or digested tumor cell suspensions, in the presence of high dose of IL-2. A total tumor lesion size of about 3 cm in diameter is required to successfully expand TILs. In the original protocol this initial culture lasts 4-5 weeks. Then, TILs are tested for recognition of autologous tumor cells (freshly frozen tumor digest or the tumor cell line). This assay is mostly based on measuring specific IFNγ secretion by ELISA on supernatant from TIL/tumor cell co-culture. The reactive wells are then selected and pooled yielding a cellular product ("pre-REP" TIL) that is then used to generate the final TIL infusion product following a "rapid expansion protocol" (REP) for two additional weeks. The REP involves activating TILs using soluble anti-CD3 mAbs in the presence of 200:1 ratio of irradiated PBMC feeder cells (either autologous or allogeneic feeders). Two days later, IL-2 is added to expand activated TILs for another 12 days. A typical REP results in 1,000-fold to 2,000 fold expansion of TILs during the 2-week culture period. Approximately, 50 x 10^6 pre-REP TILs are needed to obtain the required number of cells for therapy. This method is known as the "selected TIL" approach and has been the basis of most of the TIL clinical trials at the National Cancer Institute (NCI)[4]. However, this procedure is lengthy and limited by the need to isolate autologous tumor cells or tumor cell lines that are not available in many cases. In addition, the REP drives further T-cell differentiation that can affect the survival and proliferative capacity of TILs in vivo shortly after infusion[17].

More recent protocols reduce the initial expansion period down to a few weeks before the cells are subjected to the REP and circumvent measuring tumor reactivity using bulk "unselected TILs" for further amplification[18]. This approach, known as the "young TIL methods", increases the intention-to-treat-patients and has an Overall response similar to that reported by the "selected" TIL approach in refractory melanoma patients (40-50%)[6,19,9]. However, there is a significant risk of obtaining final TIL products with lower anti-tumor efficacy and a clinical trial with large number of patients comparing selected TILs with unselected TILs is needed.

The current culture technologies have rendered TILs with high tumor reactivity and demonstrated efficacy in patients with melanoma[2–8] and, more recently, in patients with cervical cancer[11]. Tumor-reactive TILs have also been efficiently expanded from head and neck cancer metastases[20] . However, this method has not been able to consistently yield TIL products with proven antitumor functions in patients with kidney, breast, ovarian or gastrointestinal cancer[21–26], probably due to the lower frequency of tumor-reactive T lymphocytes in these types of tumors (in contrast to melanoma) and the outgrowth of non-tumor specific T cells present in the infiltrate.

A critical parameter in TIL therapy is ensuring that the starting TIL culture has significant tumor reactivity and maintained this reactivity after ex vivo expansion. During the expansion process, there is an interclonal competition with different T-cell clones increasing or decreasing in frequency. Given this interclonal competition, the more tumor-specific clones in the starting culture, the greater the chance of maintaining tumor-specific clones at an appreciable frequency in the final product. Therefore, enrichment for tumor-specific T-cell subsets immediately before the expansion phase might eventually enhance the tumor reactivity of the final cellular product and simplify the TIL production process.

Recently, biomarkers, such as PD-1, LAG-3, TIM-3 and CD137 (4-1BB), have been proven to be able to distinguish tumor-specific T cells from other unrelated T cells present in the tumor infiltrate without knowing the specific antigen targeted[27–29]. The original observation came from the group of Dr. Yang at the NCI [27]. They observed that the tumor digests from melanoma patients that generate highly tumor reactive TILs exhibited a higher percentage of PD-1^+ CD8 T cells. Interestingly, $CD8^+$PD-1^+ T cells directly sorted from tumor digests showed much higher tumor-reactivity after expansion than their counterparts $CD8^+$PD-1^-, suggesting that the tumor reactive CD8 T cells resided within the $CD8^+$PD-1^+ TIL population. In another study by Ye et al. CD137 was preferentially detected on the tumor-reactive subset within TILs from ovarian cancer and melanoma patients[28]. In this study, an HLA-dependent increase in CD137 expression was observed in TILs following incubation of fresh enzyme digested tumors. The $CD137^+$ and $CD137^-$ fraction (including CD4 and CD8) were sorted and expanded for 8-10 days. Only enriched $CD137^+$ TILs, but not $CD137^-$ cells, exhibited autologous tumor reactivity. These observations were subsequently confirmed by Dr. Rosenberg's group, who demonstrated that, in addition to PD-1 and CD137, LAG-3 and TIM-3

also identify tumor-reactive CD8 T cells directly from the tumor digests, including mutated neoantigen-specific CD8 T cells and, importantly, without the previous knowledge of their antigen specificities[29]. Since CD137, LAG3 and TIM-3 were largely confined to the PD-1$^+$ CD8 TIL subset[29], PD-1 expression in the fresh tumor more comprehensively identify the full repertoire of naturally-occurring tumor reactive CD8 TILs.

Together, these results strongly suggest that the selection of TIL by PD-1 expression prior to expansion can lead to highly tumor reactive TIL products and increase the efficacy of TIL Therapy. However, the clinical benefit of pre-enriched tumor-specific TILs has not yet been evaluated. Recently, in two mouse models of solid tumors, we have shown that the enrichment and separate amplification of PD-1$^+$ CD8 TILs improve the antitumor efficacy of TIL therapy[30]. Surprisingly, despite the high proportion of tumor-reactive T cells in bulk CD8 TILs before expansion, only T cell products derived from PD-1-selected CD8 TILs, but not those from PD-1$^-$ or bulk CD8 TILs, were able to specifically recognize tumor cells and control tumor progression in vivo. The fold-expansion of PD-1$^+$ CD8 TIL was 10 times lower than that of PD-1$^-$ cells, suggesting that outgrowth of PD-1$^-$ cells was the limiting factor in the tumor specificity of cells derived from bulk CD8 TIL. This, together with the bystander nature of PD-1$^-$ TILs, highlights the importance of separately expanding PD-1$^+$ CD8 TILs to obtain T-cell products with high anti-tumor activity.

The use of blocking mAbs against PD-1 or PDL-1 during culture did not enhance the expansion of PD-1$^+$ TILs[30], meaning that the plausible expression of PD-1 ligands on feeder cells or activated T cells does not affect the expansion of PD-1$^+$ TILs. An important feature of tumor-infiltrating PD-1$^+$ T cells was that most of them showed an effector memory (TEM) phenotype (CD44$^+$CD62L$^-$KLRG1$^-$), whereas PD-1$^-$ TILs were largely central memory T cells (CD44$^+$CD62L$^+$KLRG1$^-$)[30]. In order to know if PD-1$^+$ TILs expand less efficiently due to their TEM differentiation state, we sorted PD-1$^+$ CD8 TILs into PD1$^+$CD62L$^+$ and PD1$^+$CD62L$^-$. Interestingly, whereas PD-1$^+$CD62L$^+$ TILs grew nearly as efficiently as PD-1$^-$ cells, PD-1$^+$CD62L$^-$ cells expanded 10 time less than their counterparts[30]. These data suggest that the TEM differentiation state of PD-1$^+$ CD8 TILs, and not the expression of PD-1 or other inhibitory receptors, is the main factor that hampers the ex vivo expansion of this subset.

TIL therapy shows positive correlations of clinical response with high amount of infused cells[6]. The reduced expansion rate of PD-1$^+$CD8 TILs could prevent obtaining the required number of cells for therapy. However, it is still unclear whether this high amount of TILs needed is a way to compensate for the loss of TIL tumor-reactivity during the expansion phase. The use of pre-enriched tumor-specific T cells may generate final TIL products with higher predictable antitumor activity, which would reduce the dose of TILs required for therapy. Another critical issue that has emerged in adoptive T-cell therapy is the inverse relationship between T-cell differentiation status and the capacity of infused T cells to proliferate, persist and mediate antitumor response[31–35]. We still do not know to what extent the reduced ex vivo expansion rates of the PD-1+CD8 TILs, as compared to PD-1$^-$ subset, may be indicative of the ability of TILs to expand and persist in vivo. Further studies are needed to determine the lifespan of expanded PD-1$^+$CD8 TILs. It would be also interesting to develop improved methods for both the ex vivo expansion and the reinvigoration of pre-enriched PD-1$^+$ CD8 TILs. Enhancing costimulation through the TNF-R family members during the REP may be beneficial. Indeed, it has been shown that addition of an agonistic anti-CD137 mAb at the beginning of the REP expands tumor-specific CD8 T-cell populations while preserving CD28 expression (associated with long telomeres)[36]. Additional costimulation via CD134/OX40 may also be beneficial based on a study in mice showing that synergistic activation of CD137 and CD134 promoted the accumulation of "super-effector" CD8$^+$ T cells expressing the IL-7 receptor that were capable of long-term survival in response to IL-7[37].

We have also shown that PD-1 precisely identifies marrow-infiltrating, myeloma-specific T cells in a mouse model of multiple myeloma[30]. This observation was further confirmed by Jing et al. that also showed that adoptive transfer of ex vivo-expanded PD-1$^+$ T cells together with a PD-L1 blocking mAbs eliminated established myeloma[38]. Recently, the group of Dr. Borrello at the Johns Hopkins hospital has carried out a pilot clinical study in Multiple Myeloma patients using cells expanded from bulk marrow-infiltrating T cells (MILs)[39]. Interestingly, the authors observed a direct correlation between the tumor-specificity of the ex vivo expanded MILs with clinical outcomes[39]. Although results hold promise, the clinical benefit of the MIL therapy is still modest. Possible contamination of MIL products with T cells unrelated to tumors may likely have diminished the efficacy of the MIL therapy. Our study[30] together with the work by Jing et al.[38], reveal a way to implement MIL therapy for the treatment of hematological tumors.

Overall, our data provide a rationale for the use of PD-1-selected TIL in adoptive T-cell therapy. These have important implications for the commercialization of TIL therapy. PD-1$^+$ CD8 T cells can be easily and rapidly isolated using FACS or magnetic technologies. The use of pre-enriched tumor-specific T cells may simplify the TIL production method and, at the same time, may help to generate T-cell products with high antitumor activity. PD-1 may enable the isolation of rare tumor-specific TILs

and allow TIL therapy to be extended to tumor types other than melanoma. Finally, at a time when great efforts are being made to identify candidate tumor antigens using next generation sequencing technologies for further identification and expansion of tumor-specific T cells, the use of distinctive biomarkers may allow the identification and isolation of the full-repertoire of tumor-reactive TILs without the previous knowledge of their antigen specificities.

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Conflict of interest statement

The authors have no financial conflicts of interest.

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