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CD137 accurately identifies and enriches for naturally-occurring tumor-reactive T cells in tumor

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Abstract

Purpose—Up-regulation of CD137 (4-1BB) on recently activated CD8+ T-cells has been used to identify rare viral or tumor antigen-specific T-cells from peripheral blood. Here, we evaluated the immunobiology of CD137 in human cancer and the utility of a CD137-positive separation methodology for the identification and enrichment of fresh tumor-reactive tumor-infiltrating lymphocytes (TILs) or tumor-associated lymphocytes (TAL) from ascites for use in adoptive immunotherapy.

Experimental Design—TILs from resected ovarian cancer or melanoma were measured for surface CD137 expression directly or after overnight incubation in the presence of tumor cells and homeostatic cytokines. CD137^{pos} TILs were sorted and evaluated for anti-tumor activity *in vitro* and *in vivo*.

Result—Fresh ovarian TILs and TALs naturally expressed higher levels of CD137 than circulating T-cells. An HLA-dependent increase in CD137 expression was observed following incubation of fresh enzyme-digested tumor or ascites in IL-7 and IL-15 cytokines, but not IL-2. Enriched CD137^{pos} TILs, but not PD-1^{pos} or PD-1^{neg} CD137^{neg} cells, possessed autologous tumor-reactivity *in vitro* and *in vivo*. In melanoma studies, all MART-1-specific CD8+ TILs up-regulated CD137 expression after incubation with HLA-matched, MART-expressing cancer cells and antigen-specific effector function was restricted to the CD137^{pos} subset *in vitro*. CD137^{pos} TILs also mediated superior anti-tumor effects *in vivo*, compared to CD137^{neg} TILs.

Conclusion—Our findings reveal a role for the TNFR-family member CD137 in the immunobiology of human cancer where it is preferentially expressed on tumor-reactive subset of TILs, thus rationalizing its agonistic engagement *in vivo* and its use in TIL selection for adoptive immunotherapy trials.

Keywords

Tumor infiltrating lymphocyte (TIL); CD137 (4-1BB); Adoptive Immunotherapy; Tumor antigen; Enrichment

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Introduction

The principle that naturally-occurring T-cells with anti-tumor potential exist in human cancer has rationalized the application of immunotherapy in oncology. The clinical efficacy of non-specific immunomodulatory agents, including immunostimulatory cytokines and antibodies that block negative immunoregulatory molecules or engage agonistic receptors on T-cells, supports the notion that pre-existent cancer immunity can be unleashed and amplified to mediate tumor regression. Additionally, the association between intratumoral T-cell accumulation and improved survival in cancer(1–3) predicts a role for tumor-specific T-cell activity in tumor control. While the identification, validation and study of spontaneous tumor-reactive tumor-infiltrating lymphocytes (TILs) in oncology remains challenging, it may provide a better understanding of the immunobiology of cancer and aid in designing improved immunotherapies.

In melanoma, heterogenous populations of natural tumor-reactive TILs can be enriched from resected lesions through optimized cell culture(4). TILs represent sound cellular candidates for adoptive immunotherapy since they are often enriched for tumor-reactive T-cells, heterogeneous in antigen specificity, potent in antitumor activity after *ex vivo* manipulation, and can be administered in large cell numbers(5). Adoptive TIL therapy can mediate dramatic, durable tumor regression in melanoma when administered after recipient lymphodepletion with ~50%–72% objective response rates reported(6–9). Ovarian cancers can also yield tumor-reactive TILs(10, 11), with encouraging clinical results reported(12–14). These results support the further pursuit and optimization of TIL-based immunotherapy for these and other cancers, although identification and expansion of natural tumor-reactive TILs for therapy still represents a challenge.

Following antigen-induced stimulation, human T-cells undergo dynamic functional and phenotypic changes, including upregulated surface expression of multiple activation-associated molecules, including CD25, CD69, CD38 and others. This provides the opportunity to identify and isolate antigen-specific T-cells through antibody binding of the upregulated determinant and subsequent enrichment by magnetic separation or fluorescence-activated cell sorting (FACS). CD137 (4-1BB, TNFSFR9) is a TNFR-family member with costimulatory function that was originally identified as an inducible molecule expressed on activated mouse and human CD8+ and CD4+ T-cells(15–17). CD137 signaling regulates T-cell proliferation and survival, particularly within the T-cell memory pool(18–20), can upregulate Bcl-X_L anti-apoptotic protein expression(21) and supports CD8+ T-cell expansion(19, 22). Because CD137 expression by T-cells is activation-dependent, the capture of CD137^{pos} T-cells from healthy donor blood can be utilized as a sensitive approach for rapid identification and isolation of rare circulating antigen-specific CD8+ T-cells, though this requires *ex vivo* stimulation with defined viral or tumor antigen(23). CD137^{pos} cytomegalovirus, Epstein-Barr virus, influenza or human adenovirus-specific T-cells can be isolated from donor blood by a similar strategy(24). This strategy can facilitate identification of new immunogenic epitopes from pre-defined antigens(23), however its great potential has not been fully explored.

To isolate and study naturally-occurring tumor-reactive T-cells, tumors represent a more promising reservoir than blood. An increased relative frequency of defined tumor antigen-specific T-cells reside in tumor(25), and although tumor cells present defined tumor antigens, whole-exome sequencing in various solid tumors has revealed that tumor cells harbor a vast, heterogeneous array of patient-specific mutated antigens that can be recognized by TILs with tumor-rejecting capability(26). Unlike in blood, naturally-occurring tumor-reactive TILs may express activation-associated molecules as a product of direct interaction with tumor cells(27), thus eliminating the need for *ex vivo* stimulation with

defined antigens to reveal this subset. Based upon these findings, we hypothesized that CD137, an activation-induced T-cell agonist, may play a role in the biology of tumor-reactive T-cells in solid human cancers.

Here, we explored the immunobiology of CD137 in spontaneous immune responses against human cancer. Further, we investigated the application of a rapid, tumor-based CD137 isolation approach for selective enrichment of activated tumor-reactive TILs with potent anti-tumor function *in vitro* and *in vivo* using optimized conditions of short-term *ex vivo* culture in the presence of homeostatic cytokines and cancer cells.

Results

Naturally-occurring CD137 expression by TILs and TALs of ovarian cancer

To evaluate CD137 expression T-cells from human cancer, baseline CD137 surface expression was evaluated by flow cytometry on T-cells derived directly from either enzyme-digested solid tumor (TILs), ascites (TALs) or peripheral blood from patients with ovarian cancer. CD137 expression level was significantly higher on TILs than on CD3⁺ T-cells from blood ($0.2\% \pm 0.054$, $n=6$, $p<0.0057$; Fig. 1A, B). TILs expressed CD137 at variable levels among samples (range of 0.9%–20%, mean = $7.8\% \pm 5.7$; $n=12$; Fig. 1B). The frequency of CD137^{pos} TALs in ascites ($1.82\% \pm 1.04$, $n=13$) was also significantly higher than in blood ($p<0.0016$), but lower than in solid tumor ($p<0.0012$). Thus, a hierarchy exists in CD137 expression, with highest frequencies detected in intratumoral T-cells, followed by T-cells in loose association with tumor and, lastly, by blood T-cells not directly interacting with tumor cells. CD137 was expressed by both CD4⁺ and CD8⁺ T-cell subsets from solid or ascites tumor, with greater frequencies detected in TILs (Fig. 1C). There was no difference in the frequency of CD137^{pos} cells between the CD4⁺ or CD8⁺ T-cell subsets in either TIL or TAL ($p>0.05$; Fig. 1C). Thus, patients with ovarian cancer naturally harbor T-cells with an activated CD137^{pos} phenotype, preferentially in tumor sites.

Increased CD137 expression by fresh TILs and TALs after overnight incubation

T-cell clones stimulated *in vitro* with cognate peptide in the presence of IL-7 and IL-15 cytokines upregulate CD137 expression within 5 hours, with peak expression after 24 hours which returns to baseline levels after 72 hours(23). To stimulate the broad and undefined repertoire of tumor-reactive cells in TIL or TAL, autologous tumor cell targets are required. Single cell suspensions achieved by enzyme-digestion of fresh solid human ovarian cancer, or cells directly from ascites, are comprised of both CD3⁺ TILs or TALs, respectively, and EpCAM⁺ cancer cells (Fig. 2A). The latter represent a rich autologous cell source for tumor antigen presentation and stimulation of T-cells in culture. To determine whether incubation of TILs and autologous tumor cells in the presence of T-cell growth factors results in increased CD137 expression by TILs, fresh enzyme-digested tumors were incubated in the presence of recombinant human IL-7 and IL-15 (50 ng/ml each) or IL-2 cytokine (50 IU/ml) and CD3⁺ TILs monitored for surface CD137 expression. The 24 hour time point was selected for analysis based upon our results showing peak CD137 expression achieved within 24–48 hours of culture, with a rapid decline after 72 hours in both cytokine conditions (not shown). In the IL-7/IL-15 condition, a significant increase in CD137^{pos} CD3⁺ TIL frequency was observed after overnight incubation ($p=0.048$; Fig. 2B), with increased levels in six of eight samples tested. Notably, the CD137^{pos} CD8⁺ TIL fraction of was significantly increased ($p=0.016$) in all samples. CD137^{pos} CD4⁺ TIL frequency did not significantly increase ($p=0.207$), and culture of enzyme-digested tumor in media without cytokines had no significant impact on CD137 expression by CD3⁺ ($p=0.56$), CD4⁺ ($p=0.50$) or CD8⁺ TILs ($p=0.09$, $n=7$). Further, incubation of enzyme-digested tumors in IL-2, a T-cell growth factor commonly utilized in TIL expansion, also had no significant

impact on CD137 expression by either CD3+ ($p=0.09$), CD4+ ($p=0.25$) or CD8+ TILs ($p=0.17$, $n=6$; Fig. 2C).

Similar to TILs, CD3+ TALs from fresh ascites displayed increased CD137 when incubated under IL-7/IL-15 conditions ($p=0.019$); 9/12 samples showed increases of ~1.5 to 10-fold (Fig. 2D). Both CD8+ and CD4+ TALs trended towards having enhanced CD137 fractions, but only the CD4+ TAL subset reached a level of significance ($p=0.028$). Incubation in IL-2 significantly increased the CD137^{pos} CD3+ TAL fraction, but was not directly attributable to either CD4+ or CD8+ subset (Fig. 2E). CD137^{pos} frequency was not increased when peripheral blood T-cells were cultured under identical conditions (Fig. 2F). CD137 upregulation was HLA-dependent since CD137 expression was diminished when enzyme-digested tumor was cultured overnight in IL-7/IL-15 cytokines in the presence of anti-HLA-ABC blocking antibodies (Fig. 2G). Thus, CD137 expression on T-cells from enzyme-digested tumors is up-regulated in an activation-induced, MHC-dependent manner *ex vivo* in the presence of homeostatic cytokines, IL-7 and IL-15.

CD137-expressing T-cells from primary tumor possess autologous tumor reactivity

To assess whether CD137^{pos} T-cells from fresh tumor are tumor-reactive, enzyme-digested tumor or ascites were cultured overnight in IL-7/IL-15 cytokines, and CD137^{pos} T-cells enriched by positive magnetic bead separation. After column purification, >70% of T-cells were CD137^{pos}. CD137-enrichment was achievable from enzyme-digested tumors and ascites samples (representative data; Fig. 3A). CD137^{pos} and CD137^{neg} fractions were cultured for 8–10 days in media containing 600 IU/ml IL-2 and then tested for reactivity against autologous tumor cells in standard overnight co-culture assays. CD137^{pos} TILs or TALs secreted IFN- γ cytokine in response to autologous tumor cell stimulation; CD137^{neg} counterparts did not respond (Fig. 3B, C). CD137 upregulation was antigen-driven since CD137^{pos} and CD137^{neg} TILs and TALs did not spontaneously secrete IFN- γ , and non-specific responses to HLA-mismatched tumors were restricted to CD137^{neg} fractions.

To confirm that reactivity in CD137^{pos} T-cells from ovarian cancer is peptide/HLA-dependent, we identified an ovarian TAL line (1555) from an HLA-A2+ donor which recognized autologous tumor, as well as a shared antigen expressed by the HLA-A2+ tumor cell line, OVCAR5. In overnight co-culture, 1555TALs secreted IFN- γ in response to autologous tumor or OVCAR5 stimulation, but not to non-HLA matched lines (Supplementary Fig. 1A). Reactivity was inhibited by addition of anti-HLA-ABC blocking antibodies to the OVCAR5 co-culture, implicating a class-I restricted CD8+ TAL response. Prior to co-culture, ~1% of CD8+ 1555TALs expressed CD137. Following culture with OVCAR5, ~8% of CD8+ TALs were CD137^{pos} (Supplementary Fig. 1B). Consistent with inhibited cytokine responses, antibody blockade of MHC class I decreased the fraction of CD137^{pos} TALs by ~75%. Thus, short-term culture of TILs or TALs from primary tumor in the presence of autologous or HLA-matched tumor cells results in activation-induced CD137 upregulation, which can serve as a biomarker for the identification and enrichment of T-cells with HLA-restricted tumor reactivity.

Tumor antigen-specific TILs upregulate CD137 after stimulation with tumor

Due to the paucity of tumor antigen-specific TILs of known specificity in ovarian cancer, we used four established HLA-A2+ melanoma TIL lines, three with defined MART-1:26–35 peptide specificity, as an *in vitro* model system to test whether CD137 identifies the tumor antigen-specific T-cell population in TIL. Prior to stimulation, MART-1 specific T-cells were detectable by tetramer staining at various frequencies in TILs #1, #2 and #3 (Fig. 4A) and CD137 expression was low (average of $0.9 \pm 0.6\%$) on tetramer-positive and negative TIL populations. After overnight co-culture with the HLA-A2+ MART-1-expressing

melanoma line 624mel, but not HLA-A2-negative 938mel, ~90% of MART-1 tetramer-positive TILs expressed surface CD137. TIL#2 and TIL#3 also upregulated CD137 expression in the tetramer-negative population suggesting shared tumor antigen recognition. TIL#2 also possessed a subset of tetramer-negative TILs with cross-reactivity against 938mel in repeated assays. TIL#4, which lacks detectable MART-1-specific T-cells but possesses shared HLA-A2-restricted tumor antigen reactivity, upregulated CD137 in the tetramer-negative population upon stimulation with 624mel, but not 938mel.

To assess whether CD137^{pos} TILs in melanoma possess antigen-specific anti-tumor reactivity, TIL#2 was stimulated with 624mel cells overnight and subsequently enriched for CD137^{pos} and negative fractions (Fig. 4B). Nearly all MART-1 tetramer-positive cells were contained within the CD137^{pos} T-cell fraction (Fig. 4A). One week later, TIL fractions were stimulated with peptide-pulsed T2 APCs or HLA-matched melanoma lines, 526mel and 624mel, or mismatched line, 938mel. Consistent with CD137 expression and tetramer staining results, CD137^{pos} TILs secreted IFN- γ when stimulated with MART-1 peptide-pulsed T2 cells, or 526mel and 624mel lines, but not when stimulated with irrelevant peptide-pulsed T2 cells or with HLA-A2-negative 938mel cells (Fig. 4B). CD137^{neg} TILs did not respond to MART-1 peptide or tumor stimulation. Thus, the tumor-stimulated CD137^{pos} T-cell fraction from melanoma TIL lines identifies a subset of T-cells with known and unknown tumor antigen-specificity.

CD137^{pos}, but not CD137^{neg}, TILs inhibit tumor growth in vivo

We next evaluated the capacity of CD137-enriched TILs to inhibit tumor outgrowth *in vivo*. After overnight co-culture of TIL#2 and 624mel cells, CD137^{pos} and CD137^{neg} fractions were isolated and rested in media for 10 days. CD137^{pos}, CD137^{neg} or non-manipulated TILs were subcutaneously co-injected with an equal number of firefly luciferase-transfected 624mel cells in the hindquarters of immunodeficient NOD/SCID/IL-2R γ ^{null} (NSG) mice in a modified Winn assay. Tumor outgrowth was measured by photon emission imaging and caliper measurement. Three weeks post-inoculation, a strong delay in tumor outgrowth was observed in mice treated with CD137^{pos} TIL compared to CD137^{neg} and non-manipulated TIL groups as measured by bioluminescence signal detection (Fig. 4C, $p=0.00037$, CD137^{pos} versus CD137^{neg}; $p=0.038$, CD137^{pos} versus non-manipulated TILs). Four weeks after inoculation, measurable tumor emerged in mice that received CD137^{neg} or non-manipulated TILs; no palpable tumors were detected in mice receiving CD137^{pos} TILs until 42 days after inoculation. At the end of study, non-manipulated TILs mediated a modest inhibition in tumor outgrowth compared to CD137^{neg} TILs, though not statistically significant ($p=0.375$, Fig. 4D). In contrast, a significant reduction in measurable tumor outgrowth was observed in CD137^{pos} TIL-treated mice relative to CD137^{neg} ($p=0.016$) and non-manipulated TIL groups ($p=0.0354$). These results were confirmed by direct morphological sizing of tumors resected from euthanized mice (Fig. 4E).

Adoptive TIL therapy has been explored extensively for melanoma, but less so for ovarian cancer where isolation of tumor-reactive T-cells from tumors is more challenging(10–13). To determine whether CD137 selection of T-cells derived from ovarian cancer yields a cell population with anti-tumor potency *in vivo*, tumor-reactive HLA-A2-restricted TALs were stimulated with OVCAR5 cells under IL-7/15 conditions and enriched for CD137 by positive magnetic selection for use in a modified Winn assay. After tumor stimulation, 34% of TALs expressed CD137 (Supplementary Fig. 2A) and were subsequently enriched to 90% CD137^{pos} (Supplementary Fig. 2B). After 10 days, non-manipulated, CD137^{pos} or CD137^{neg} TALs were subcutaneously co-inoculated with OVCAR5 tumor cells into the hind quarters of NSG mice and monitored for tumor outgrowth by caliper-based measurement. Measurable tumors were detectable in mice receiving CD137^{neg} TALs 32

days after inoculation, while detectable tumor growth was delayed until day 52 in mice receiving either non-manipulated or CD137^{pos} TALs (Supplementary Fig. 2C). Two months after inoculation, tumor outgrowth was significantly inhibited in the CD137^{pos} TAL group compared to the CD137^{neg} group ($p=0.035$), with a similar but non-significant trend for non-manipulated TILs ($p=0.076$). Increased inhibition of tumor outgrowth was observed in mice receiving CD137^{pos} TALs compared to non-manipulated TALs, but not to the level of statistical significance ($p=0.498$). Caliper-based sizing results were confirmed by direct morphological sizing of lesions harvested from euthanized mice (Supplementary Fig. 2D).

CD137, not PD-1, accurately identifies tumor-reactive TILs

Programmed cell death-1 (PD-1, PDCD1, CD279) receptor is a negative immunoregulatory molecule expressed by activated T-cells that plays an important role in the immunobiology of cancer(28, 29). PD-1 can be a useful biomarker for enriching tumor-specific T-cells from fresh melanomas where there is an observed higher tumor-specific IFN- γ production by PD-1+CD8+ TILs compared with PD-1^{neg} T-cells(30). We measured TILs from fresh enzyme-digested ovarian tumors for their relative expression of CD137 and PD-1 either fresh or after overnight incubation in IL-7/15. Prior to incubation, up to 60% of TILs expressed surface PD-1 (mean of $46\% \pm 13$), while only $3.6\% \pm 1.7$ expressed CD137 ($n=3$; representative data shown in Fig. 5A). After overnight culture in IL-7/15, the percentage of CD137^{pos} TILs increased to $4.6\% \pm 1.2$ (Supplementary Table 1), while $50\% \pm 13$ expressed PD-1. Under both conditions, only a small portion of PD-1+ TILs co-expressed CD137; the majority was CD137^{neg}. Small frequencies of CD137^{pos} cells were also detected in the PD-1^{int/neg} fraction. Similar phenotypic results were observed using HLA-A2-restricted, tumor-reactive melanoma TIL lines that were stimulated with 624mel cells. Functional assessment of FACS-sorted CD137^{pos}PD-1+, CD137^{pos}PD-1^{neg}, and CD137^{neg}PD-1+ TIL subsets revealed a dichotomy in tumor antigen-specific reactivity. CD137^{pos}PD-1+ and CD137^{pos}PD-1^{neg} TILs responded specifically to stimulation with either the HLA-matched, MART-1+ 624mel or MART-1 peptide pulsed T2 cells with modestly greater activity observed from CD137^{pos}PD-1+ TILs; CD137^{neg}PD-1+ TILs did not respond to either stimulus (Figs. 5B,C). This data, coupled with the reproducible observation that CD137^{pos} TIL (but not CD137^{neg} +/- PD-1) enrichment leads to comprehensive isolation of tumor-reactive TILs in melanoma and ovarian cancer (Figs. 3B, 4B), indicates that CD137 more accurately identifies the subset of TILs and TALs with antitumor activity than PD-1.

Discussion

Here, we investigated the role of CD137 (4-1BB, TNFSFR9), an activation-induced costimulatory molecule that promotes T-cell proliferation and survival(18–20), in tumor-associated T-cell biology. Using primary leukocytes from patients with ovarian cancer, we observed a preferential increase in the frequency of naturally-arising T-cells with a CD137^{pos} activation phenotype at sites of tumor. CD137^{pos} T-cells were found in both solid tumors and ascites, with higher frequencies seen in solid tumors where TILs are in direct contact with tumor cells. Few CD137^{pos} T-cells were observed in the resting peripheral blood, as noted elsewhere(23, 31, 32). Our results indicate that ovarian cancers may act as natural sinks for enrichment of spontaneously-arising tumor antigen-specific T-cells, analogous to melanoma(25), and support the notion that ovarian cancer cells are immunogenic. This is consistent with the observed improved survival in patients with ovarian cancers with evidence of intraepithelial T-cell accumulation(1, 33), as well as the positive association between patient survival and the presence of intraepithelial cells expressing activated effector T-cell phenotypes (CD45RO, TIA-1, granzyme B) in high-grade serous ovarian cancer(27). Our results imply that CD137 expression might predict improved prognosis in ovarian cancer, a hypothesis that is under active investigation.

Our findings implicate a novel role for CD137, an immunologic agonistic, in the biology of tumor-reactive TILs. The observation that the spontaneously-arising tumor-reactive TIL subset selectively expresses CD137 in tumors helps explain in part the demonstrated anti-tumor effects of agonistic anti-CD137 antibodies in preclinical studies(34), and the observation that CD137-signaling protects human TILs from activation-induced cell death, thereby enhancing net antitumor activity *in vitro*(35, 36). Mouse studies show that the hypoxic tumor microenvironment upregulates CD137 expression by TILs in a hypoxia-inducible factor (HIF)-1 α dependent manner(32), in line with our findings, and revealing an opportunity for CD137-targeted immunotherapy in solid tumor. In an open-label, ascending, multi-dose phase I-II study, treatment of subjects with melanoma, renal cell carcinoma (RCC), or ovarian carcinoma with single-agent anti-CD137 antibody BMS663513 showed clinical activity including partial remissions and sustained stable diseases with a manageable toxicity profile(37); a phase II randomized study in previously treated melanoma patients with stage IV disease was terminated due to high incidence of grade 4 hepatitis(38). Still, the pro-T-cell survival function and antitumor activity of anti-CD137 agonists appear to occur by mechanisms independent from that of blocking antibodies directed against negative immunoregulatory molecules such as PD-1 and CTLA-4 which mediate antitumor activity on their own(28, 29). Thus, CD137 agonists appears to be a strong candidates for use in combination with anti-PD-1 and/or anti-CTLA-4 therapy, or other immune enhancing immunotherapies such as vaccines, cytokines, immunologic chemotherapy, other agonistic antibodies and adoptive T-cell therapy(39).

Identification and enrichment of naturally-occurring, tumor-reactive TILs for use in clinical trials has commonly relied upon prolonged microculture of a small number of independent tumor fragments in IL-2 cytokine and subsequent screening of emerging TILs for reactivity against autologous or HLA-matched allogeneic cancer cell lines(4). The limited number of fragments grown and low detection sensitivity of this method poses the risk of excluding tumor-reactive TILs and simultaneously carrying non-reactive TILs through to the final cell product. Using cell separation to enrich for CD137^{pos} cells from either enzyme-digested tumor specimen or TILs co-cultured with HLA-matched tumor cell lines, we have defined a new methodology for rapid, comprehensive isolation of tumor-reactive TILs from various tumor types that is not dependent upon *ex vivo* stimulation with defined antigens. Here, CD137 expression reflects specific TCR-triggered activation resulting from tumor antigen encounter. Only CD137-enriched TILs and TALs produce IFN- γ after exposure to autologous tumor cells; CD137^{neg} TILs or TALs do not, showing that the tumor-reactive TIL fraction is enriched while the non-reactive fraction is eliminated. The lack of non-specific IFN- γ production by CD137^{pos} TILs against HLA-mismatched tumor lines, and the inhibition of IFN- γ secretion with anti-HLA class I antibody during specific tumor stimulation, excludes the possibility that immune recognition is mediated by an allogeneic response, nor the impact of agonistic anti-CD137 antibody binding. Indeed, we are unaware of any report to date that has demonstrated that the 4B4-1 anti-CD137 clone utilized in our studies possesses T-cell agonistic properties, though others antibodies do(34). The observed inhibition of both IFN- γ responses and CD137 upregulation by HLA class-I antibodies in our study implies a more prominent role for CD8⁺ TILs in the anti-tumor response, although both CD4⁺ and CD8⁺ TILs in fresh enzyme-digested ovarian tumor express CD137. In other studies, the anti-tumor effects of CD137 agonists also appear primarily dependent upon CD8⁺ T-cells although other lymphocytes, such as CD4 and NK cells, have been implicated elsewhere but this may be tumor model-dependent. A role for CD8⁺ T-cells in CD137^{pos} TIL anti-tumor activity is also ascribed in our study of established CD8⁺ melanoma TIL lines with MART-1 antigen-specificity which recognized HLA-matched, MART-1-expressing melanoma cells, where CD137 is upregulated by all MART-1 peptide-specific TILs following tumor encounter. This finding also indicates that TILs upregulate CD137 upon encounter with defined tumor antigen-derived epitopes, however the

observation that melanoma TILs lacking MART-specificity, but possessing known reactivity against 624mel cells, similarly upregulate CD137 after stimulation with tumor, indicates that TILs with heterogeneous defined and unknown tumor antigen-specificity can collectively be rapidly identified and comprehensively enriched via CD137 cell separation.

In our preclinical models of adoptive TIL therapy, both unmanipulated and CD137-enriched TILs and/or TALs were able to slow tumor growth *in vivo*, compared to CD137^{neg} TILs, however, inhibition of tumor outgrowth was enhanced by the pre-selection of TILs for tumor-reactivity based upon tumor-stimulated CD137 expression. Whether use of agonistic anti-CD137 antibody for TIL selection yields a more potent cell product for infusion remains to be tested. Importantly, pre-CD137-selection of the tumor-reactive fraction allows concomitant elimination of non-reactive TILs that do not contribute to the anti-tumor effect and potentially compete with reactive TIL clones for key homeostatic cytokines after infusion into the lymphopenic host(5, 40). Notably, isolation of CD137^{pos} TILs for therapy as described here was conducted over a one week period; immediate CD137-enrichment followed by one week of IL-2 culture. Newly devised methods that minimize cell culture duration, expansion of melanoma TILs for therapy in the absence of tumor-reactive T-cell selection has been explored with encouraging early clinical results reported (9, 41, 42). This abbreviated method may be uniquely useful for generating TILs for use in melanoma patients where ~70% of tumors contain TILs with tumor-reactivity at detectable levels (43, 44), however even here CD137 isolation may further enrich this tumor-reactive fraction and shorten culture duration, and extend effective TIL therapy to non-melanoma cancers.

Another important finding is the elucidation that augmented CD137 expression among tumor-reactive cytotoxic CD8⁺ T-cells from enzyme-digested tumor is best achieved in the presence of IL-7 and IL-15, homeostatic cytokines that support T-cell survival and memory formation(45). Increased CD137 expression among CD8⁺ TILs in the presence of tumor cells was MHC dependent, ruling out activation-independent effects mediated by cytokine alone(46). Although IL-2 supports the expansion of TILs from resected cancer tissue *ex vivo* and has been utilized extensively in generating TIL cultures(6, 9, 12, 13, 47, 48), CD137^{pos} TIL frequencies were unchanged when cultured overnight in IL-2. The known ability of IL-2 to promote apoptosis versus the pro-survival attributes of IL-7 and IL-15 may explain this difference; a hypothesis currently being tested. Our findings rationalize the use of IL-7 and IL-15 cytokines, and reconsideration of IL-2, in clinical TIL production.

It remains feasible that other activation-induced markers may permit the selection of natural tumor-reactive TILs. Inozume et al. recently demonstrated the negative immunoregulatory molecule PD-1 to be a promising biomarker for tumor-specific TIL selection in melanoma(30). The efficacy of anti-PD-1 and PD-L1 antibodies in melanoma supports this position(28, 29). Our results confirm PD-1 expression on melanoma as well as ovarian TILs (~20–60%) and show that PD-1⁺ TILs do indeed possess anti-tumor activity. We however find that the PD-1⁺ population is comprised of distinct CD137^{pos} and CD137^{neg} subsets, analogous to animal studies(32), and that antigen-specific tumor-reactivity is restricted to the PD-1⁺CD137^{pos} TIL subset. Therefore, while tumor-reactive T-cells do reside within the PD-1⁺ population, PD-1⁺CD137^{pos} and PD-1⁺CD137^{neg} subsets are functionally distinct. This is corroborated by the observation that PD-1⁺ TILs often reside within the CD137^{neg} fraction, the fraction of TILs that does not respond to stimulation with autologous or HLA-matched tumor, or peptide-pulsed APC *in vitro*, nor efficiently control tumor outgrowth *in vivo*. We therefore conclude that while a fraction of PD-1⁺ TILs may possess specific anti-tumor reactivity, tumor-induced expression of CD137 on TILs better identifies genuine, recently activated tumor-specific T-cells and allows for their efficient enrichment.

Our results define a role for CD137 in the immunobiology of human cancer, where it represents an agonistic biomarker for naturally-occurring, tumor antigen-reactive TILs or TALs, rationalizing further investigations of CD137 agonistic engagement in cancer. CD137 marking also allows for the rapid, selective enrichment of rare tumor-reactive TIL populations for the development of effective adoptive immunotherapy. Development of a reliable closed-chamber method for enrichment and outgrowth of CD137-expressing lymphocytes represents the next step towards the improvement and application of adoptive TIL therapy for patients with various forms of cancer. Finally, this tumor-reactive TIL enrichment strategy is also positioned to support downstream translational investigations, including profiling of immunoregulatory elements in tumor-reactive TILs, and T-cell receptor gene isolation for use in antigen discovery studies and cancer therapy.

Materials and Methods

Antibodies and flow cytometric immunofluorescence analysis

Antibodies against human CD3, CD4, CD8, PD-1 and CD137 were purchased from BD Bioscience (San Jose, CA). CD45 and EPCAM antibodies were purchased from Biolegend (San Diego, CA). 7-AAD viability staining solution was purchased from BD Bioscience. HER2:369–377 peptide (KIFGSLAFL) and MART-1:26–35(27L) (ELAGIGILTV) peptide containing HLA-A2010 tetramers were purchased from Beckman Coulter, Inc. (Brea, CA). Cells were resuspended in FACS buffer consisting of PBS with 2% FBS (Gemini Bioproducts) and blocked with 10% normal mouse Ig (Caltag Laboratories) for 10 min on ice. 10^6 cells in 100 μ l were stained with fluoro-chrome-conjugated mAbs at 4°C for 40 min in the dark. For viability gating, cells were briefly stained with 7-AAD solution, washed twice and analyzed for nonviable cell exclusion using a FACS Canto II (BD Biosciences).

Tumor specimens and TIL generation

Patients were entered into an Institutional Review Board-approved protocol and signed an informed consent prior to tissue collection. For enzymatic digestion of solid tumors, specimen was diced into RPMI-1640, washed and centrifuged at 800rpm for 5min at 15–22°C, resuspended in enzymatic digestion buffer (0.2mg/ml collagenase and 30units/ml DNase in RPMI-1640) before overnight rotation at room temperature. Cells were then washed and used fresh or cryopreserved for later use. Ascites samples were washed and enriched for the lymphocyte/tumor fraction by Ficoll-gradient separation or RBC lysis using ACK lysing buffer (Invitrogen Life Technologies). Generation of TIL cultures was performed as described(4). Briefly, 2mm³ tumor fragments were cultured in complete media (CM) comprised of AIM-V medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 2mM glutamine (Mediatech, Inc. Manassas, VA), 100U/ml penicillin (Invitrogen Life Technologies), 100 μ g/ml streptomycin (Invitrogen Life Technologies), 5% heat-inactivated human AB serum (Valley Biomedical, Inc. Winchester, VA) and 600IU/ml rhIL-2 (Chiron, Emeryville, CA). To generate TAL lines, total ascites cells were cultured at 10^6 cells/well of 24-well plate in CM for 3–4 weeks and T-cells harvested.

Enrichment of CD137-positive cells

Fresh enzyme-digested tumors or ascites were cultured overnight in CM containing IL-7 and IL-15 (PeproTech, Rocky Hill, NJ) at 50ng/ml each or rhIL-2 (50IU/ml) overnight. For allogeneic tumor cell stimulation, heterogeneous T-cells were cultured with indicated tumor cell lines at 1:1 ratio overnight in IL-7 and IL-15. For magnetic separation, CD137^{POS} cells were then isolated using a CD137 MicroBead kit according to manufacturer's instruction (Miltenyi, Auburn, CA). For CD137/PD-1subset analyses, tumor-exposed TILs were stained with anti-PD-1 and anti-CD137 (BD Bioscience) and sorted by FACS using the BD FACS Aria II SORP high-speed cell sorter. The purity of enriched T-cells was >90% as determined

by flow cytometry. Sorted CD137^{pos}/PD-1^{pos}, CD137^{pos}/PD-1^{neg} and CD137^{neg}/PD-1^{pos} T-cells were rested in IL-2 (100 IU/ml) containing CM for ~9 days before functional assessment.

ELISA assay for T-cell function

Established tumor lines or CD45-depleted tumor cells isolated from fresh autologous ascites or enzyme-digested solid tumor cells (10⁵ cells/well) were co-cultured overnight with equal numbers of unmanipulated or enriched TILs in triplicate in 96-well U-bottom plates in 200μL CM. For specific antigen stimulation of isolated CD137^{pos} and CD137^{neg} T-cells, 10⁶ T2 cells were loaded with 1μM MART-1:26–35(27L) or p53:264–272 peptide at 37°C for 1 hour, then washed and co-cultured overnight with T-cells. For HLA-blocking, anti-HLA-ABC antibodies (BD Pharmingen) were added at 10μg/well at start of culture. Supernatants were harvested and analyzed for IFN-γ by ELISA, according to manufacturer's instruction (Biolegend). Mean cytokine concentration (pg/mL) ± SD of triplicate wells is shown.

In vivo assays

Animals were obtained from the Stem Cell and Xenograft Core of the Abramson Cancer Center, University of Pennsylvania. Six to 12-week-old NOD/SCID/γ-chain^{-/-} (NSG) mice were bred, treated and maintained under pathogen-free conditions in-house under IACUC-approved protocols. For melanoma and ovarian studies, 6 to 12-week-old female NSG mice were inoculated s.c. with 10⁶ of the indicated T-cells mixed with an equal number of either fLuc⁺ 624mel cells or OVCAR5 cells. Tumor dimensions were longitudinally measured with calipers, and tumor volumes calculated as $V = 1/2(\text{length} \times \text{width}^2)$, where length is greatest longitudinal diameter and width is greatest transverse diameter. For fLuc⁺ 624mel imaging, inoculated animals were imaged prior to and at the day of T-cell transfer and weekly thereafter. Tumors were resected immediately after euthanasia ~50–60 days after tumor cell inoculation. All in vivo assays were performed twice with similar results.

Bioluminescence imaging

Tumor growth was monitored by Bioluminescent imaging (BLI) using the Xenogen IVIS imaging system. Photons emitted from fLuc-expressing cells within animals were quantified using Living Image software (Xenogen). Mice bearing 624mel fLuc⁺ tumor cells were injected intraperitoneally with D-luciferin (150 mg/kg stock, 100 μL of D-luciferin/10 grams of body weight) in PBS and imaged under isoflurane anesthesia after 5–10 minutes. Pseudocolor images representing light intensity (blue, least intense; red, most intense) were generated using Living Image. BLI findings were confirmed at necropsy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

The existence of naturally-occurring, tumor-reactive T-cells in human cancer has rationalized the application of immunotherapy in oncology, however the immunobiology of spontaneous tumor-reactive T-cells in cancer is not well-defined, since identifying and validating these responses is difficult. In melanoma, adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs) with anti-cancer activity can mediate durable tumor regressions. Currently, lengthy methodologies with poor sensitivity are used for TIL selection, posing the risk of excluding tumor-reactive TILs and carrying non-reactive T-cells through to the final product. Here, we elucidated CD137 as a biomarker for naturally-occurring tumor-reactive T-cells in cancer and developed a rapid, accurate system to comprehensively isolate TILs with tumor-rejecting capability directly from resected tumors. Our findings implicate a role for CD137 in the immunobiology of human cancer where it is preferentially expressed on the tumor-reactive TIL subset, rationalizing its agonistic engagement *in vivo* and use in TIL selection for adoptive immunotherapy trials.

Figure 1. Naturally occurring CD137+ T cells exist in human ovarian cancer
 CD137 expression on TILs, TALs and PBMC from patient samples. All samples were stained with anti-CD3, CD8 and CD137 antibodies, gated on viable cells (7-AAD-) and examined by flow cytometry. (A) CD137 staining on the CD3+ T cell population compared to the isotype control on TILs, TALs and PBMC. Plots representative of staining performed on n=12 (TILs), n=13 (TALs) or n=6 (PBMC) samples. (B) CD137 expression for all patient TILs, TALs and PBMC assessed. (C) CD137 expression on viable, CD3+, CD8+ and CD4+ (CD3+ CD8-) T cell subpopulation of TILs and TALs. P values were determined by Student t-test.

Figure 2. CD137 expression on TILs and TALs is upregulated by common gamma chain cytokines IL-7 and IL-15 and inhibited by anti-HLA class I antibody
(A) A single-cell suspension of fresh enzymatically-digested solid tumor or ascites from a representative ovarian cancer patient was stained with antibodies for EpCAM (tumor cells), CD45 (leukocytes) and 7-AAD and viable cells analyzed by flow cytometry. Representative plots showing gating strategy for CD45+ leukocytes are shown. **(B)** CD137 expression on CD45-gated CD3+, CD8+ and CD4+ (CD3+ CD8-) TILs before and after overnight culturing in complete medium containing 50 ng/mL each of IL-7 and IL-15. **(C)** CD137 expression on CD3+, CD4+ and CD8+ TILs before and after overnight culture in complete media containing 50 IU/mL IL-2. **(D)** CD137 expression on CD3+, CD4+ and CD8+ TALs from ascites before and after overnight culturing in 50 ng/mL each of IL-7 and IL-15. **(E)** CD137 expression on CD3+, CD4+ and CD8+ TALs before and after overnight culturing in 50 IU/mL IL-2. **(F)** CD137 expression on CD3+ T cells in patient PBMC cultured in complete media alone or with IL-2 or IL-7 plus IL-15. **(G)** CD137 expression on CD3+ T cells in ovarian cancer patient TILs after overnight incubation with anti-HLA-ABC antibody in vitro. P values were determined by Student t-test.

Figure 3. CD137 enrichment from ovarian cancer TILs or TALs

(A) Purity of enriched CD137^{pos} T cells from ovarian cancer patient TILs and TALs cultured overnight in 50ng/mL each of IL-7 and IL-15 after CD137 magnetic cell separation (MACS). The CD137-enriched or eluted fraction of TILs (B) or TALs (C) were then cultured for 8–10 days in CM and 50IU/mL IL-2. Representative data from donor 1745 shows IFN-g production (pg/ml $\pm$ SEM) from CD137^{pos} or CD137^{neg} TIL or TAL cultures from an HLA-A2+ donor after stimulation with autologous tumor, HLA-matched (OVCAR5) or mis-matched (SKOV3) ovarian cancer lines and HLA-A2+ 624 or HLA-A2–938 melanoma cell lines, as measured by human IFN-g ELISA. Results are representative of six independent assays.

Figure 4. Enhanced potency of CD137-enriched melanoma-reactive TILs

(A) Melanoma TIL cell lines (TILs #1, 2, 3 and 4) were analyzed for CD137 expression and MART-1 tetramer positive staining on viable CD3⁺ cells by flow cytometry before and after overnight co-culture with HLA-matched (624mel) or mismatched (938mel) tumor cell lines in vitro. (B) TILs from overnight co-cultures were enriched for CD137^{pos} T cells by magnetic cell separation, further cultured for 7 days then stimulated overnight in triplicate with MART-1:26–35(27L) or irrelevant HER2 peptide-pulsed T2 cells, HLA-matched melanoma lines 526mel or 624mel or HLA-mismatched melanoma line 938mel, and supernatants measured for IFN- γ concentration. Histograms show CD137 expression on

representative TIL#2 following cell enrichment. Mean IFN- γ concentration from triplicate cultures is shown in pg/mL $\pm$ SEM. (C) NGS mice co-inoculated with firefly luciferase-transfected 624 melanoma cells and the indicated TIL population were monitored via bioluminescence signal detection or caliper measurement. Image of luciferase-transfected 624 tumor luminescence in mice receiving unmanipulated, CD137^{pos} or CD137^{neg} TILs in a subcutaneous melanoma model. (D) Caliper-based measurement of subcutaneous 624 tumor growth in vivo. Tumor volume is shown. (E) Macroscopic visualization and sizing of tumors from the various treatment groups resected after 51 days after inoculation.

Figure 5. CD137 expression is more restricted than PD-1 on fresh and tumor-stimulated TILs
(A) Representative TILs from ovarian cancer patient digested tumor (Ov1938) or a melanoma TIL line (MEL1931) were stained with 7-AAD for viability, anti-CD137, anti-CD3 and anti-PD-1 or isotype control antibodies either fresh or after overnight incubation with 50ng/mL of IL-7 and IL-15 each. For MEL1931 TILs, cells were co-cultured overnight in the presence of HLA-matched 624 melanoma cells. Stimulated MEL1931 TIL subsets were then sorted by FACS and rested in low IL-2 (100 IU/ml) conditions for 9 days prior to overnight co-culture with 624mel or 938mel tumor cells or peptide pulsed T2 cells. **(B)** IFN- γ production by sorted CD137⁺PD-1⁺ and CD137⁺PD-1⁻ subsets but not CD137⁻PD-1⁺ TIL

following stimulation with 624mel. (C) MART-1 peptide-specific reactivity was limited to CD137⁺PD-1⁺ and CD137⁺PD-1⁻ subsets. TILs subsets were co-cultured overnight with T2 cells loaded with 1uM MART-1 or p53 peptides. Mean IFN-g concentration from triplicate cultures is shown in pg/mL $\pm$ SEM.