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Distinctive Features of the Differentiated Phenotype and Infiltration of Tumor-reactive Lymphocytes in Clear Cell Renal Cell Carcinoma

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Abstract

Clear cell renal cell carcinoma (RCC) is considered an immunogenic tumor, but it has been difficult to identify tumor infiltrating lymphocytes (TIL) that show *in vitro* tumor recognition. We compared the characteristics of fresh RCC TIL to peripheral blood lymphocytes (PBL) or melanoma TIL. Our results demonstrated that RCC TIL contained fewer $CD27^+$ T-cells, and fewer naïve and central memory (CM) T-cells, but more effector memory (EM) T-cells than melanoma TIL or renal PBL. We hypothesized that factors in the RCC microenvironment were skewing TIL phenotype towards EM. One possibility was the expression of CD70 on nearly all human RCCs, but not melanomas. Differentiation of naïve T cells to EM cells only occurred from CD70 costimulation in concert with TCR stimulation ("signal one"), suggesting that EM TIL responding to CD70 would be enriched for T-cells reactive with local antigens, including those associated with RCC. Clonotypic analysis of T cell receptors (TCRs) in fresh RCCs showed that EM T-cells were more clonally-expanded than CM or naïve T cells, and the clonal expansion occurred at the tumor site as oligoclonal TCRs were distinct from PBL TCRs from the same patient. In addition, we found that two TCRs from the highly represented EM TIL clones, when re-expressed in fresh PBL, recognized an MHC-class II or MHC-class I- restricted antigens shared by multiple RCC lines. Our results suggest that RCC-reactive TIL do exist *in situ*, but may be difficult to recover and study due to proliferative exhaustion, driven by tumor-expressed CD70.

Introduction

Adoptively transferring tumor-infitrating lymphocytes (TIL) expanded *in vitro* back into patients with metastatic melanoma can mediate durable and complete tumor regressions (1–2). These melanoma TIL will demonstrate immunological recognition of their autologous melanoma cells in 67% of patients (3). However, this unexplained ability to generate tumor-reactive TIL from melanoma does not extend to clear cell renal cell carcinoma (RCC) as only a few have been generated from RCC (4–6), despite the fact that both cancers can respond to a variety of immunotherapies. There are many potential reasons why tumor reactive TIL might not expand and destroy the tumor they reside in, including immunosuppression by inhibitory receptors, cytokines, or by T-regulatory cells (7). Factors such as PD-1, CTLA-4, IL-10 and TGF-β have been thought to play a role in blunting anti-tumor reactivity (8–12). Yet none of these factors explains the dramatic functional differences seen between TIL expanded *in vitro* from melanomas versus RCC. With both cancers responding clinically to IL-2, ipilimumab and anti-PD-1 antibody (13–16), it would

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be hard to suggest that RCC-reactive TIL or T-cells do not exist in patients with RCC. We considered whether the failure to demonstrate tumor reactive TIL in RCC might be an artifact of the need to expand them *in vitro* for study and began looking at fresh RCC TIL. In support of this, Dietrich et al found that *in vitro* lymphocyte culture of RCC TIL had a negative impact on selecting T cell repertoire, as some highly-represented T cell populations present *in vivo* disappeared after *in vitro* stimulation by TCR analysis (17). Therefore, investigating phenotypes of T cells residing in renal tumors comparing to melanoma would help us to understand the differences between these two malignancies. Recently, CD70 (normally expressed by activated immune cells) has been identified as a diagnostic marker on RCC (18–19). CD70, a member of the TNF super-family, has been implicated in T cell survival and activation through interaction with its co-stimulatory receptor, CD27 (20). The role of CD70 in tumor immunology is controversial, conferring benefit when expressed by $CD8^+$ TIL administered therapeutically to melanoma patients (21), but perhaps mediating immune cell apoptosis and immune escape in glioblastoma and RCC (22–23). Most interestingly, in a CD70-transgenic murine model, Tesselaar et al. have demonstrated that constitutive expression of CD70 by Bcells resulted in exhaustion of the naïve T cell pool, depletion of T cells from lymph nodes, and death from opportunistic infection (24). Therefore, we investigated activation and differentiation states of T cells residing in renal tumors comparing to melanoma, and the possible role of RCC-expressed CD70 in RCC TIL differentiation. We also investigated whether recognition of RCC-associated cognate antigens could be playing a role in driving differentiation of the most dominant RCC TIL clones. This possibility could lead to identifying RCC-reactive T-cell clones and new RCC-associated antigens.

Materials and methods

Fresh tumor digests and tumor lines

Primary or stage IV metastatic tumors surgically resected from patients with clear cell renal cell carcinoma (RCC) were enzymatically digested with 0.1% collagenase type IV, 0.01% hyaluronidase type V and 30U/ml deoxyribonuclease type IV (Sigma Chemical) in RPMI 1640 (Life Technologies) at room temperature for 3 h. In total, tumors from 16 patients were processed, including 8 primary tumors and 8 metastatic tumors. The cells were filtered through 100-μm-nylon mesh, and separated by density gradient centrifugation using Lymphocyte Separation Medium (Organon Teknica). After digestion, cells were suspended in 90% of human serum (HS; Valley biomedical) with 10% DMSO and frozen at −70°C. Tumor lines from RCC patients were established and maintained in DMEM (Life Technologies) including 10% fetal bovine serum (FBS; Life Technologies), 10% tryptose phosphate (Sigma), 1x insulin-transferrin-selenium (Life Technologies) and 1 x serum pyruvate (Life Technologies). For T cell phenotypic analyses, fresh tumor digests were thawed, resuspended in RPMI 1640 with 10% HS, and cultured overnight at 37°C, 5% CO_2. Non-adherent cells were then harvested for staining with corresponding antibodies. For non-T cell analyses, the fresh tumor digests were thawed, blocked with purified IgG, and labeled with corresponding antibodies immediately.

Similarly procured fresh tumor digests of melanoma and melanoma lines were obtained from Surgery Branch Laboratories (National Cancer Institute), and the tumor lines were maintained in RPMI 1640 with 10% FBS. All melanomas used in this study are stage IV metastatic melanomas, including lung, liver or kidney metastases.

All cell lines included in the study were generated at Surgery Branch, NCI, and tested and authenticated by HLA genotyping. The cell lines were routinely confirmed with their HLA typing and antigen expression by flow cytometry and co-culture assays, respectively.

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Antibodies

For immunophenotypic analyses, monoclonal antibodies (MoAbs) including FITC-labeled anti-human IgG isotypes, CD8 (clone SK-1), CD16 (clone 3G8), CD19 (clone HIB19), CD69 (clone L78) or Lin 1, PE-labeled anti-human IgG isotypes, CD62L (clone Dreg56), CD70 (Ki-24), or CD25 (clone 2A3). PE-cy7-labeled anti-human IgG isotypes, or CD3 (clone SK7), APC-labeled anti-human IgG isotypes, CD45RO (clone UCHL1), CD28 (clone CD28.2), CD11c (S-HCL-3), or CD14 (clone MϕP9), and APC-cy7-labeled anti-human IgG isotypes or CD4 (clone SK3) were purchased from BD Pharmingen. APC-labeled anti-human CD27 (M-T271) Ab was purchased from eBiosciences.

The effect of plate-bound human CD70

Purified human CD70 fused with murine CD8 alpha (CD70-muCD8, Ancell Corporation; 0.03–10μg/ml) or controls were plated onto a 96-well flat-bottom plate that was pre-coated with anti-mouse CD8 alpha or PBS alone (Ancell; 10μg/ml) overnight at 4°C. The plate was blocked with PBS containing 1% BSA (Sigma) for 30 min at RT, and washed with PBS three times before adding T cells.

Peripheral blood lymphocytes (PBL) from RCC patients were thawed and cultured in RPMI 1640 with 10% HS overnight. Non-adherent cells were harvested the next day, labeled with fluorescence-conjugated Abs CD3, CD62L and CD45RO. Cells were incubated for 30min at 4°C, washed with PBS containing 1% BSA, and sorted for $CD3^+CD62L^+CD45RO^-$ (naïve) or $CD3^+CD62L^+CD45RO^+$ (central memory) subpopulations on FACS Aria (BD Biosciences). The purity of sorted populations was confirmed by analyzing a small portion of cells on FACS Canto II (BD Bioscieces). After sorting, cells were plated at 2×10^5 cells per well and cultured for 5 days. On day 5, cells were labeled with fluorescence-conjugated Abs CD3, CD62L and CD45RO, and analyzed on FACS Canto II.

Clonotypic analysis of renal fresh tumor digests and peripheral blood

Effector memory (EM; $CD3^+CD62L^-CD45RO^+$), central memory (CM) or naïve T cell subpopulations were sorted from renal fresh tumor digests or PBL as described above. Total RNA from each subpopulation was purified with an RNeasy mini kit (Qiagen). 5′ RACE reaction was performed by SMARTer RACE cDNA amplification kit (Clontech) following the manufacturer's instructions. The RACE cDNAs (~800bp) were obtained with primers complementary to the constant region of TCR alpha or beta chain and then inserted into the pCR2.1 vector by TA cloning (Life Technologies). Primers for the TCR alpha or beta chain were synthesized by Life Technologies, and their sequences were 5′-GCCACAGCACTGTTGCTCTTGAAGTCC or 5′-CAGGCAGTATCTGGAGTCATTGAG, respectively. After TA cloning, 96 colonies were picked from each 5′ RACE product of both TCR alpha and beta chains and their variable regions and complementarity determining region 3 (CDR3) were sequenced.

Retroviral production and transduction of anti-CD3 stimulated PBL

cDNAs encoding selected full-length TCR alpha chains and beta chains were cloned into the pMSGV1 plasmid, which isa derivative of the murine stem cell virus-based splice-gag vector (pMSGV), as described in previous publications with some modifications (25). Briefly, full-length TCR alpha and beta chain cDNAs were amplified by PCR using the pairs appropriate to corresponding sequences of each TCR alpha chain and beta chain with a P2A sequence used as the spacer in between.

To produce retrovirus, 293gp cells were transfected with 9 μg of pMSGV1-TCR and 3μg of plasmid RD114 using Lipofectamine 2000 (Life Technologies; 60μl). Two days later, the supernatants were harvested and used to transduce anti-CD3 stimulated PBL. PBL from

allogeneic donors were stimulated with soluble OKT-3 (50ng/ml) and IL-2 (300 IU/ml) for 2 days before transduction was performed. The stimulated cells were added to 24-well plates initially coated with RetroNectin (Takara) and subsequently pre-coated with retrovirus by spinoculation (2000×g, 32°C, 2hrs) or incubation at 37°C for 4hr at 5×10^5/ml. The plates were then centrifuged at 1000 × g for 10 min, and incubated overnight at 37°C in a 5% CO_2 incubator. This procedure was repeated the next day and cells were split as necessary to maintain cell density between0.5 and 1×10^6 cells/ml. Transduction efficiency was determining by analyzing Vβ expression of retrovirally-transduced cells if the antibody was available.

Cytokine release and blocking assay

Retrovirally-transduced cells (1×10^5) were co-cultured with 5×10^4 autologous renal tumor lines with or without transduction with the class II transcriptional activator (CIITA) at 37°C, 5% CO_2 overnight and tested for IFN-γ secretion. In some experiments, HLA-matched allogeneic renal tumor lines were included in the assay.

For the blocking assay, RCC cells (5×10^4 cells in 100 μl culture medium) were incubated with each blocking mAb at a concentration of 10μg/ml for 30 min. at 37°C in a flat-bottom 96-well plate. T cells ($1 \sim 5 \times 10^4$ cells/well) were then added and incubated with target cells overnight at 37°C. The supernatants were harvested and assayed for IFN- concentration by ELISA.

Statistical analysis

The two-tailed unpaired t-test with Welch's correction was used for comparing T cell population phenotypes, and lineage 1 negative cell populations in renal and melanoma tumors.

Results

Phenotypic differences between fresh tumor digests from renal cell cancers (RCC) and melanomas

To understand the differences between renal tumors and melanomas, we compared the phenotypes of immune cells residing in fresh tumor digests from these two malignancies. No differences were observed in the percentages of T cells (CD8 or CD4 subpopulations), NK cells or B cells when comparing renal tumors with melanomas. Interestingly, renal fresh tumors contained a significantly lower percentage of CD27-expressing T cells than melanomas and peripheral blood lymphocytes (PBL) from patients with RCC (Figure 1A). Moreover, when analyzing the differentiation and activation states of T cells residing in tumors by expression of CD62L and CD45RO (Figure 1B), melanoma fresh tumor digests contained significantly more naïve and central memory (CM) subpopulations than renal fresh tumor digests, as demonstrated by higher percentages of $CD62L^+CD45RO^-$ T cells and $CD62L^+CD45RO^+$ T cells, respectively. In contrast, renal tumors contained significantly more effector memory (EM; $CD62L^-CD45RO^+$) cells than melanomas. The differences were observed in both CD8 and CD4 T cell subpopulations. No differences were observed between primary and metastatic renal cancers. In addition, PBL from patients with RCC exhibited a less differentiated T cell phenotype than renal tumors, as demonstrated by a higher percentage of naïve and CM T cells and a lower percentage of EM T cells. Furthermore, a higher percentage of T cells from renal tumors expressed a late-differentiation marker, CD57, than those from melanomas (Data not shown). Our results demonstrate that T cells which reside in renal tumors are more terminally differentiated.

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CD70 expression on renal tumors versus melanomas and its effect on naïve T cells

Because renal fresh tumor digests contain fewer CD27-expressing T cells, we further analyzed CD70 expression in renal tumors compared to melanoma. RCC resident B cell and NK populations all contained a higher percentage of $CD70^+$ cells than melanomas (data not shown). However, the most striking difference was the consistent presence of CD70-expressing cells in Lineage 1 negative cell populations (i.e. non-immune cells) in renal fresh tumor digests compared to melanoma (Figure 2A). Further analysis of CD70 expression on our renal cancer and melanoma cell lines confirmed previous studies in which renal tumors expressed CD70 on their surface (Figure 2B; (18)) while melanomas did not. The correlation between CD70 expression in Lin1-populations and T cell phenotypes was also analyzed and the data suggest that the higher percentage of EM and lower percentage of CM T cells were associated with higher level of CD70 expression (Supplemental Figure 1). We further tested the effect of CD70 using anti-CD3 antibody and purified human recombinant CD70 protein coated onto plastic plates. As shown in Figure 2C and 2D, naïve human T cells differentiated when they received both a TCR signal and costimulation by CD70 as demonstrated by increased EM and CM T-cells and diminished naïve T cells. This effect was CD70 dose-dependent. These data suggested that the tonic costimulation by RCC-expressed CD70 would have maximal effect on T-cell clones only when T cells were engaging their cognate antigen in the RCC tumor microenvironment. Therefore, T-cell clonotypes reacting with RCC-associated antigens might be those with the most differentiated phenotype.

Clonal diversity of T cell subpopulations in renal tumors

To avoid the effects of differential clonal expansion *in vitro* and best reflect the in situ situation, we examined the clonotypic diversity in RCC TIL subpopulations by randomly selecting and sequencing T-cell receptor alpha and beta chains from fresh TIL. The most over-represented TCRs were then re-expressed in PBL and tested for reactivity with the RCC from which the TCR were obtained. EM, CM and naïve T-cell populations were FACS sorted from freshly dispersed RCCs, RNA extracted and cDNAs were synthesized and sequenced using primers complementary to the constant regions of either the alpha or beta chains. Approximately 70 alpha chain and 70 beta chain sequences were analyzed from each T-cell subpopulation. Figure 3 shows representative results from one RCC in which freshly sorted EM TIL contained oligoclonal TCRs as demonstrated by 3 highly represented TCR alpha chains (TRAV14*03TRAJ3*01, TRAV13-1*01TRAJ40*01 and TRAV38-1*01TRAJ23*01) and 3 dominant TCR beta chains (TRBV13*01TRBD2*01TRBJ2-1*01, TRBV9*01TRBJ2-7*01 and TRBV27*01TRBD1*01TRBJ2-5*01). CM T cells showed less pronounced oligoclonality (2 TCR alpha chains and 1 TCR beta chain) and naïve T cells had a diversified repertoire. Interestingly, the specific oligoclonal TCRs found in EM TIL were never found in either the CM or naïve T cell populations. In total, renal fresh tumor digests from 4 RCC patients were analyzed and 3 showed distinct oligoclonality in their sorted EM cells. We used the same methods to examine the diversity of EM T-cells in the peripheral blood and renal tumors of the same patient. As shown in Figure 4, the diversity and clonotypes of TCR alpha (Figure 4A) and beta chains (Figure 4B) were totally different between the RCC TIL and the PBL. Clonal expansion not only occurred more extensively and more frequently in EM T cells from fresh tumor digests than from PBL, but the overrepresented TCR alpha or beta chains present in the renal fresh tumor digests were not found in the patient's PBL. Conversely, the only oligoclonal TCR alpha chain in renal PBL was not present in the renal fresh tumor digest. Therefore, oligoclonal EM T cells from renal fresh tumor digests were distinct from PBL. This supports the hypothesis that the oligoclonality of EM T cells may be the product of the highly stimulatory renal tumor microenvironment.

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Functional analysis of oligoclonal EM TCRs

The presence of over-represented oligoclonal EM TCRs allowed us to isolate full-length TCR alpha and beta chains, pair them in all possible combinations in bicistronic retroviral vectors, and test their reactivity against renal tumors by transducing allogeneic PBLs. All three RCC patients with oligoclonal TCRs in their EM populations were examined and their tumor reactivity was tested. Testing only a total of 8 TCR alpha chains and 8 TCR beta chains from these three patients, tumor-reactive TCRs were identified in two of them. Tumor reactivity of TCRs from patient RV was shown in Figure 5. Nine retroviruses representing all the possible pairing of 3 TCR alpha chains and 3 beta chains, and used to transduce to anti-CD3 stimulated allogeneic PBL. One TCR, consisting of TRAV24 and TRBV24, could confer reactivity against class II transactivator (CIITA)-transduced, HLA-class II expressing autologous tumor cells (RCC/CIITA) but not parental RCC line (Figure 5A). This reactivity could be blocked by anti-HLA class II and anti-HLA DR antibodies, but not anti-HLA class I antibody (Figure 5B). When it was tested against multiple renal tumor lines, TRAV24/TRBV24-transduced PBL recognized 2 of 4 HLA-DRB1*01-matched tumor lines when induced to express HLA class II by CIITA transduction (RCC #2 and RCC #12; Figure 5C). No tumor recognition was detected when tumor lines did not express HLA class II. In addition, an HLA-DRB1*01^{-} tumor line (RCC #13) was not recognized by TRAV24/TRBV24-transduced PBL. Furthermore, control PBL transduced with GFP did not recognize any of these tumors. These results suggested that the reactivity of TRAV24/TRBV24-transduced PBL was restricted by HLA-DRB1*01 and recognized a shared antigen expressed by 3 of 5 HLA-DRB1*01^{+} renal tumors.

Using the same α/β TCR pairing approach, we also identified a tumor-reactive EM TCR in Patient DS. As shown in Figure 6A, allogeneic PBL were retrovirally transduced with 9 TCRs, and one TCR (TRAV14/TRBV13) could confer low-level reactivity against the autologous tumor line. This reactivity could be blocked by HLA-class I Ab, but not HLA-class II or HLA-DR (Figure 6B), and it recognized two HLA-A3 matched renal tumor lines (Figure 6C), suggesting the reactivity could be HLA-A3 restricted. Because of the availability of the antibody against TRBV13 (Vβ23), we were able to fluorescence-sort Vβ23^{+} cells from RCC TIL and analyze the sequences of alpha chains (Table 1). Among 68 sequences analyzed, 44 had the same variable region, TRAV14 and 43 of them shared the identical CDR3 region with our reconstructed candidate TCR∀. These results demonstrated that the tumor-reactive EM TCR (TRAV14/TRBV13) was a naturally occurring TCR.

Discussion

The current study investigated the differences between RCC and melanoma TIL. By phenotypic analyses of the TIL from both maligancies, we found that they were significantly different in phenotype, with a much more differentiated profile in RCC TIL (i.e. more EM T cells, and less CM and naïve T cells) than in melanoma TIL or PBL from patients with RCC. T cells residing in renal tumors also contained significantly higher percentage of CD57-expressing T cells than melanoma (data not shown), suggesting that RCC TIL were mostly late in the differentiation sequence. This advanced state of differentiation could account for the failure of tumor-reactive RCC TIL to expand i*n vitro*. Our studies of fresh RCC also confirmed the published finding that the tumor cells themselves express CD70 (18–19), the member of tumor necrosis factor family. In a normal host, expression of CD70 is mainly restricted to lymphoid tissues such as active T cells, B cells or dendritic cells, and the interaction between CD70 and its receptor CD27 can lead to different immune response outcomes depending on the timing, context and intensity (26). Several studies have suggested that CD27–CD70 co-stimulation positively modulates T cell activation, differentiation and survival, and therefore maintains antigen-specific T cell responses (27–29). Using CD27-deficient mice, Hendriks J, *et al* showed that these mice infected with

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influenza virus had decreased numbers of effector T cells in the lung. They also demonstrated that co-stimulation of CD27–CD70 contributed to maturation of T cells and promoted $CD8^+$ virus-specific T cell responses in acute viral infection (27). Furthermore, CD27 promotes proliferation and survival of activated T cells (29). On the contrary, some groups have advocated that CD70 induces apoptosis in T cells which serves as one of the immune escape mechanisms of CD70-expresssing tumors such as RCC and glioblastoma (22–23). However, in our study, we did not observe an increase in steady-state apoptosis in RCC TIL (data not shown). Another situation where there is a negative impact of CD70 has been in chronic viral infection, with poor T cell responses and prognosis being associated with CD70 expression in human immunodeficiency virus-positive patients (30). This closely reflects the CD70-transgenic mouse model, where immunosuppression and opportunistic infections are seen, associated with a terminally differentiated T-cell repertoire (24). In addition, when the CD70/CD27 pathway is blocked, viral control was improved in a mouse chronic lymphocytic choriomeningtitis virus (LCMV) infection model (31–32). These data led us to the hypothesis that RCC-expressed CD70 was driving RCC TIL (in concert with tumor associated cognate antigen) to terminal differentiation. The presence of populations of such tumor-reactive TIL could be compatible with clinical responses to T-cell directed immunotherapies for RCC, but their advanced differentiation state might prevent them from being successfully expanded for study, characterization or adoptive therapy. This hypothesis was also attractive in light of the clinical observation that RCC patients responding to high dose IL-2 and then relapsing virtually never respond to additional IL-2 therapy (33). This might be due to clonal exhaustion of the most RCC-reactive T-cells during their first IL-2 therapy. We have been unable to identify RCC that completely lack CD70 expression where sufficient fresh tumor is available to see if this TIL differentiation profile is absent. Therefore we looked at melanomas which uniformly lack CD70 and where there is also good evidence of the presence of “signal one” from tumor associated antigens. This latter requirement prevented us from looking at other tumor histologies which express CD70. Because nearly all RCC expressed CD70 and the evolution of the T-cell differentiation profile was apparently already completed in patients with clinically evident disease, it was necessary to pursue circumstantial evidence to support this hypothesis. Constitutive CD70 expression in mice transgenic for a single TCR reactive with viral antigen did not result in T-cell exhaustion unless the mice were exposed to the viral antigen (24). For T-cells to show a state of advanced differentiation in the tumors of RCC patients but not in their blood, it was likely that they were encountering cognate antigen in the tumor microenvironment. Furthermore, it was possible that the most expanded and over-represented clonotypes in this environment would be those seeing antigens which were dominant or abundant on tumor. Isolating them and characterizing their reactivity could lead to new antigen discovery but their advanced proliferative history might be an impediment to their isolation and characterization. This led to the strategy of rescuing their TCRs and re-expressing them in fresh T-cells to allow their characterization.

Several RCC TIL did indeed show individual T-cell clonotypes in their effector memory populations that constituted up to 19% of all the TCR randomly selected and cloned with 5′RACE. Such clonotypic dominance was not seen in naïve or central memory TIL from the same patients. The most over-represented TCR-α and TCR-β chains in a patient’s TIL were then transduced in random pairs to produce putative α/β TCRs for testing against the autologous tumor line. Two of three patient TIL subjected to this extensive process produced α/β TCR that recognized the autologous tumor. In one case where an antibody to the TCR-β chain was available, we were further able to show that our TCR pairing was likely the native pairing when sequencing demonstrated that nearly all TIL sorted for the selected TCR-β contained the exact TCR-α we had selected with tumor recognition. Still, not all patients and over-represented TCR species could be shown to react with autologous tumor. This could be due to technical limitations, or the TCR of some TIL may be

recognizing antigens presented by antigen presenting cells rather than tumor itself. Yet in extensive studies of bulk RCC TIL, there are only rare instances of finding tumor reactive T-cells, so this experience in a small number of cases seems unusual.

In addition to possibly elucidating one reason why RCC TIL do not show tumor recognition after expansion for study, this approach may lead to a general technique for finding T-cell clones with tumor recognition from RCC and perhaps other tumors. Only melanoma routinely yields tumor reactive TIL, while all other cancers studied do so very infrequently. This does not illuminate the reasons for the singular behavior of melanoma, nor provide answers about other cancers with do not display CD70. Future studies will examine the phenotype of fresh TIL from other cancers, looking for evidence of advanced T-cell differentiation or alternate explanations. The ability to find individual T-cell clones with tumor reactivity can be exploited by cloning their TCR and retrovirally re-engineering it into the T-cells of other patients using widely available and efficient techniques. We do not yet know the antigens recognized by the T-cell clones we found. Future antigen identification efforts will be applied to receptors with the highest avidity, and broad and shared reactivity, preferably restricted by MHC Class I. Additional studies attempting to correlate CD70 levels or TIL differentiation states in RCC with their response to immunotherapy would also be of use in corroborating the hypothesis proposed by these studies. In summary, these studies suggest a novel mechanism to explain how tumor-reactive TIL can reside in RCC, potentially participate in responses to immunotherapy, yet not be recovered when expanded *in vitro* for study. The use of TCR cloning and re-expression in peripheral blood lymphocytes allows for the further study of RCC TIL and points to a means of utilizing these findings in new adoptive cell therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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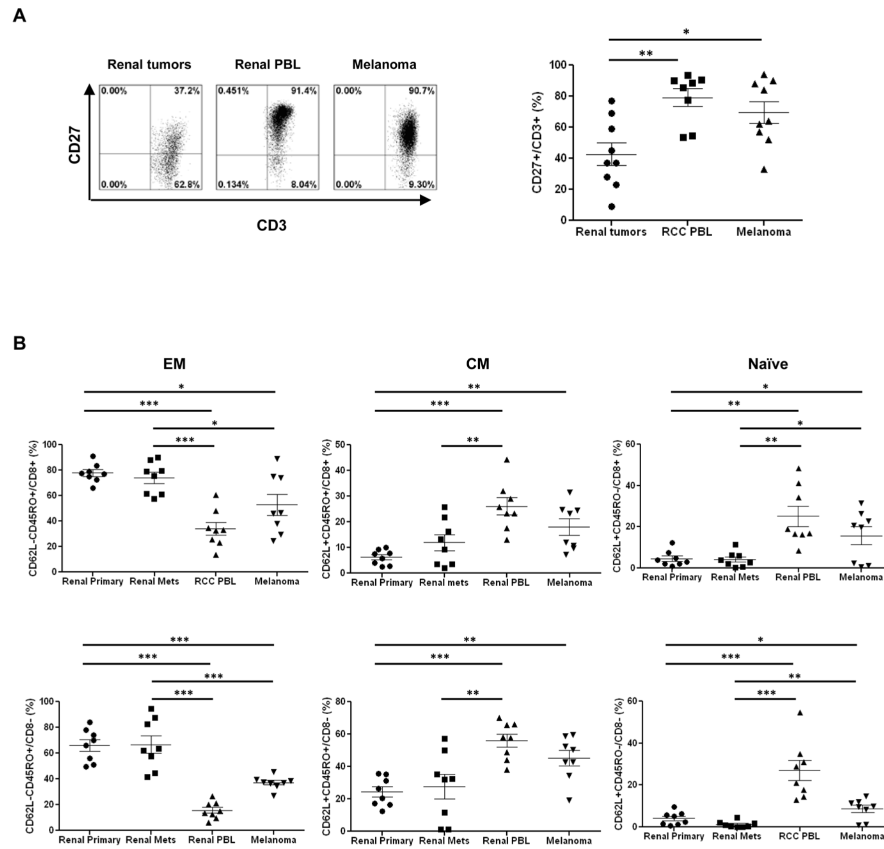

Figure 1.
Differentiation and activation states of T cells in renal fresh tumor digests comparing to those in renal peripheral blood lymphocytes (PBL) or melanoma tumor digests. Fresh tumor digests from 9 (A) or 8 (B) renal cell carcinoma (RCC) patients, PBL from 9 (A) or 8 (B) different RCC patients, and fresh tumor digests from 9 (A) or 8 (B) melanoma patients were thawed and cultured in RPMI with 10% HS overnight. Non-adherent cells were harvested, labeled with fluorescence-conjugated antibodies anti-CD3 and CD27 (**A**), or CD3, CD8, CD62L, and CD45RO (**B**), and then analyzed on FACS Canto II. Data shown in A was gated on live $CD3^+$ populations while data shown in B was gated on live $CD3^+CD8^+$ or $CD3^+CD8^-$ populations. FACS plots in A represented CD27 expression in RCC tumors, RCC PBL or melanomas. EM, CM and naïve were effector memory, central memory, and naïve T cells, respectively. Unpaired, two-tailed student t-tests were performed for statistical analyses. *, p 0.05; **, p 0.01; ***, p 0.001.

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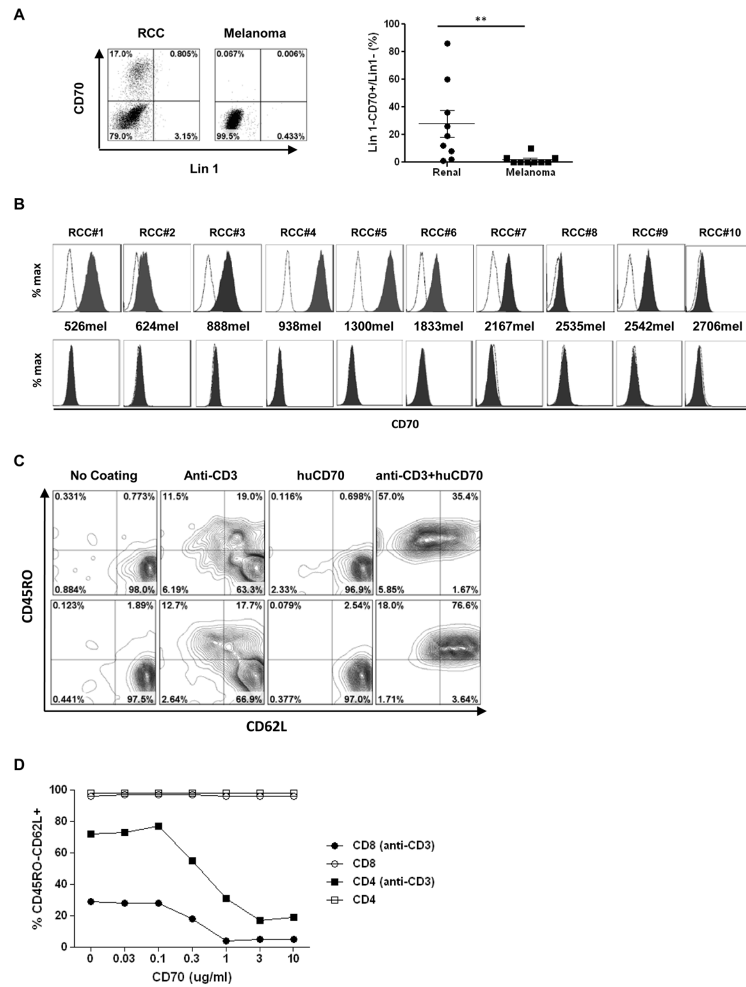

Figure 2.

CD70 expression on tumors and the effect of purified CD70 on naïve CD8$^+$ or CD4$^+$ T cells. **A.** CD70 expression on non-immune cells in renal fresh tumor digests comparing to melanoma. Fresh tumor digests were labeled with fluorescence-conjugated Lineage 1 cocktail antibodies (Lin 1) and CD70, and then analyzed on FACS Canto II. Lin 1$^-$ cells represent non-immune cells in the fresh tumor digests. FACS plots represented CD70 expression in RCC or melanoma. **B.** CD70 expression on renal tumor lines and melanoma lines. Tumor lines established from 10 RCC patients and 10 melanoma patients were thawed and grown till their confluency. The tumor cells were then harvested and labeled either with PE-conjugated anti-CD70 Abs (filled histogram) or isotype control (open histogram), and analyzed on FACS Canto II. **C.** The effect of purified CD70 on naïve CD8$^+$ or CD4$^+$ T cells. Peripheral blood mononuclear cells (PBMC) were thawed and cultured in RPMI with 10% HS overnight, and sorted for CD8$^+$CD62L$^+$CD45RO$^-$ or CD4$^+$CD62L$^+$CD45RO$^-$ naïve T cell populations on FACS Aria. Sorted naïve CD8 or CD4 T cells (2×10^5/well) were added to the 96-well plate that were pre-coated with anti-human CD3 (10μg/ml) and/or purified human CD70 protein (10μg/ml) and cultured in RPMI + 10%HS + IL-2 (50cu/ml) for 5 days. 5 days later, T cells were labeled with fluorescence-conjugated Abs, CD3, CD8, CD4, CD62L and CD45RO, and analyzed on FACS Canto II. **D.** The dose-dependent effect of purified CD70 on naïve CD8$^+$ or CD4$^+$ T cells. Sorted naïve CD8$^+$ or CD4$^+$ T cells were cultured with different doses of purified human CD70 protein (0–10μg/ml) in the presence

or absence of anti-CD3 Ab (10μg/ml). The experiments in C and D were performed multiple times and gave similar results.

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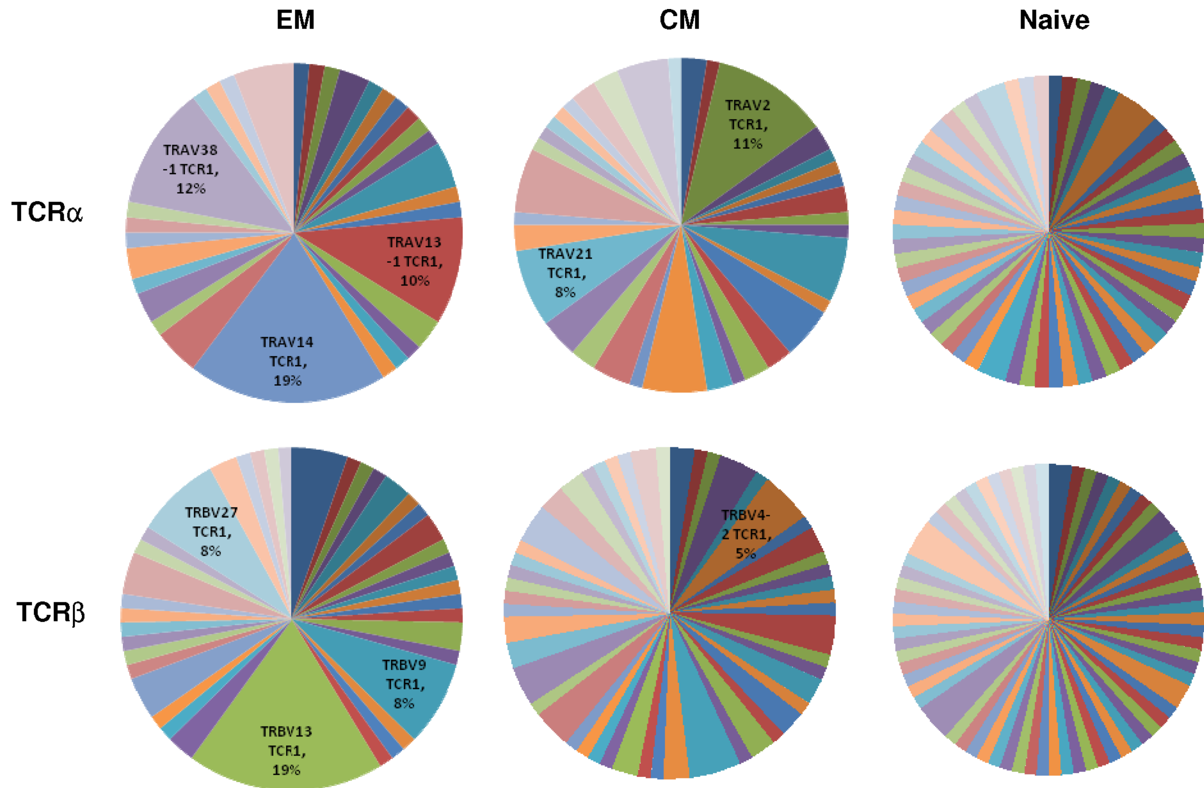

Figure 3.

TCR clonotypes in effector memory (EM), central memory (CM) or naïve T cells from renal fresh tumor digests. Fresh tumor digests from RCC patient DS were thawed and cultured in RPMI+10%HS overnight. The non-adherent cells were stained with fluorescence-labeled CD3, CD62L and CD45RO, and FACS-sorted to EM ($CD62L^{-}CD45RO^{+}$), CM ($CD62L^{+}CD45RO^{+}$) and naïve cell ($CD62L^{+}CD45RO^{-}$) subpopulations. Approximately, 70 sequences of TCR alpha chains and beta chains in each population were evaluable after 5′RACE using gene-specific primers. Each pile chart shows the composition of TCR alpha or beta chains. Each sector represents a single alpha or beta chain with a unique CDR3.

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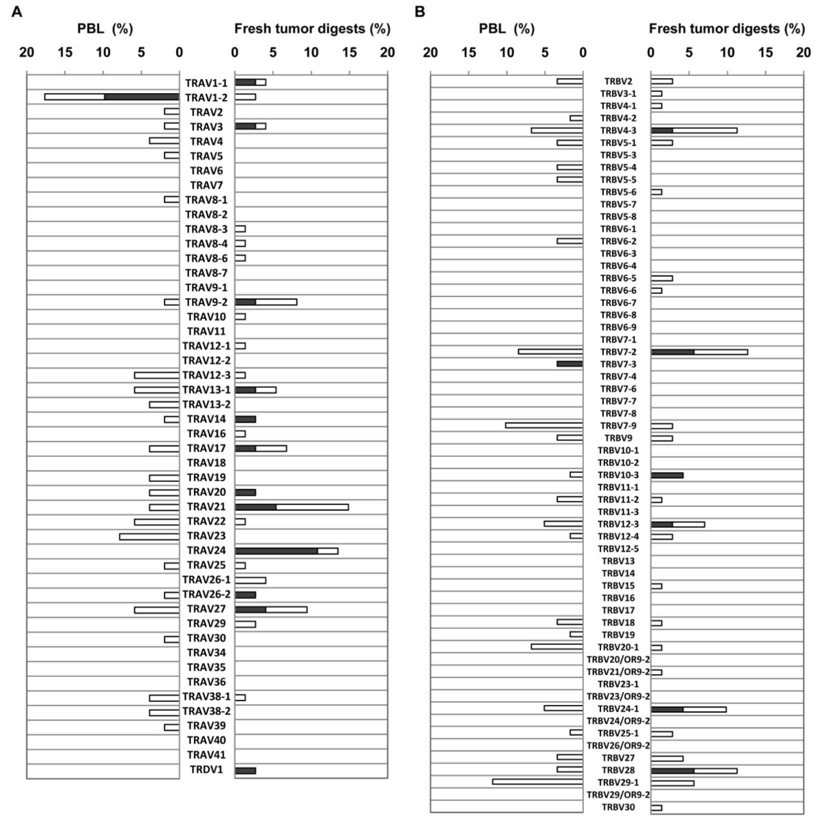

Figure 4.

Frequencies of EM TCR alpha chains (**A**) and beta chains (**B**) from fresh tumor digests compared to PBL from RCC patient RV. Each single bar represents the frequency of a single variable TCR alpha or beta chain. The grey bar within each bar represents the frequency of the dominant single TCR alpha or beta chain with a unique CDR3.

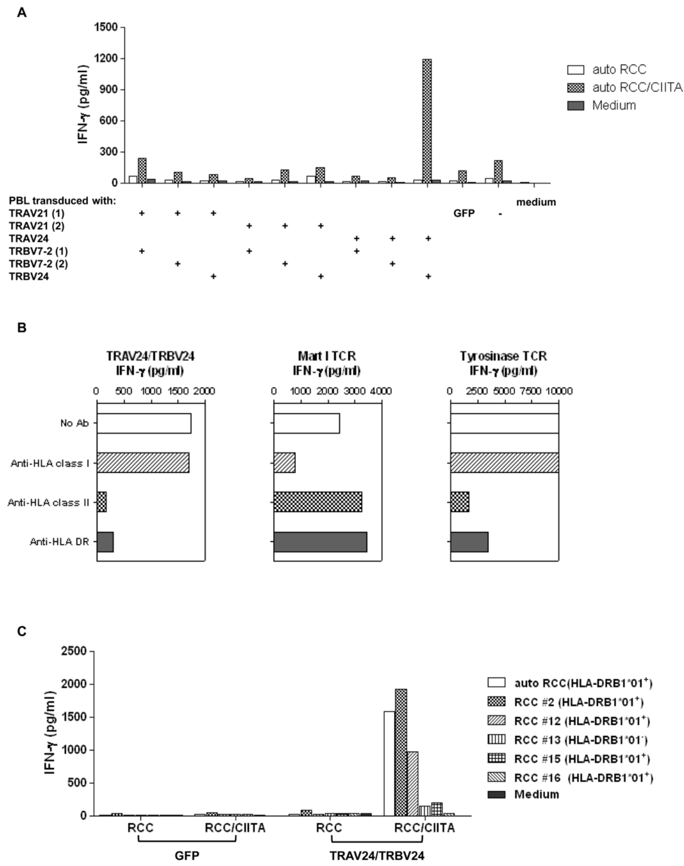

Figure 5.

Oligoclonal EM TCRs from RCC patient RV could confer HLA-class II restricted tumor reactivity. **A.** IFN-γ production by PBL transduced with oligoclonal EM TCRs. Allogeneic PBL were stimulated with anti-CD3 (50ng/ml) for 2 days and transduced twice with retrovirus encoding oligoclonal EM TCRs at 0.5×10^6 cells/well in a 24-well plate. PBL retrovirally transduced with green fluorescence protein (GFP) was used as the control. 3 days after transduction, transduced cells (1×10^5) were co-cultured with 5×10^4 autologous tumors (RCC) or tumors transduced with CIITA (RCC/CIITA), which is a known transcriptional factor to upregulate HLA-class II on the cell surface as shown in Supplemental Figure 2. After overnight incubation, the supernatant was harvested and IFN-γ production was measured. GFP-transduced and untransduced PBL were used as controls. **B.** The reactivity of PBL transduced with TRAV24/TRBV24 was blocked with HLA-class II and HLA-DR Abs. PBL transduced with TRAV24/TRBV24 were co-cultured overnight with autologous RCC/CIITA in the presence of anti-HLA class I, anti-HLA class II or anti-HLA DR antibodies (10 μg/ml), and IFN-γ from the co-culture supernatant was measured the next day. PBL transduced with a HLA-class I restricted TCR recognizing MART-1 and a HLA-class II restricted TCR recognizing tyrosinase were used as controls. **C.** IFN-γ production of PBL transduced with TRAV24/TRBV24 against HLA-DRB1*01 matched or mismatched tumors.

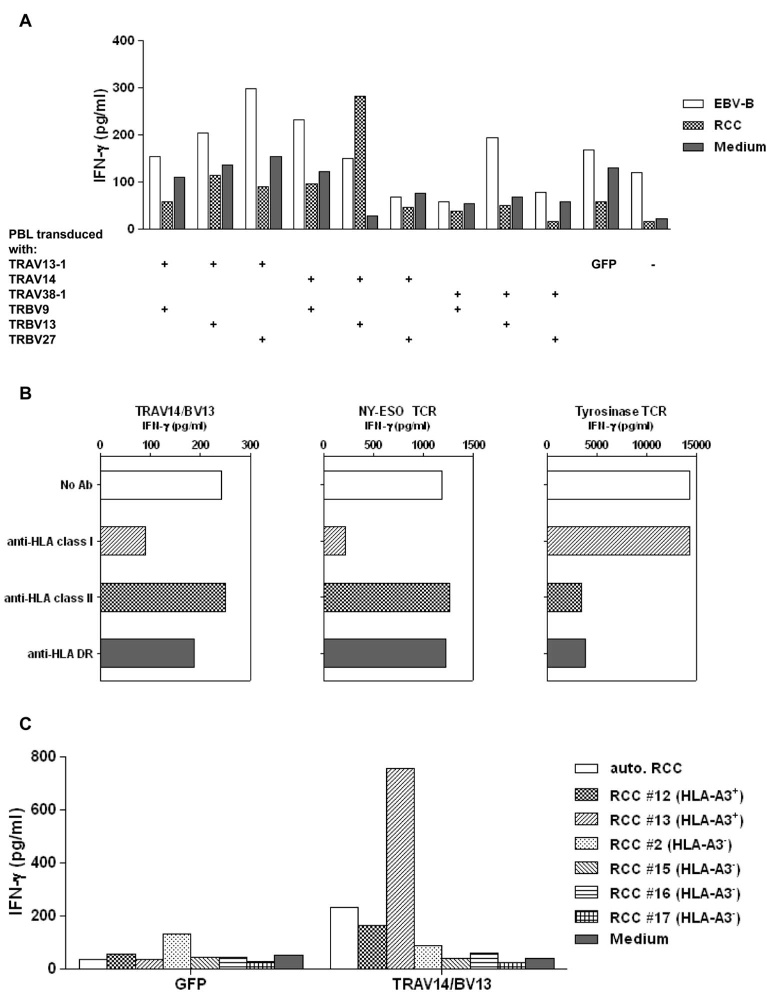

Figure 6.

Oligoclonal EM TCRs from RCC patient DS could confer HLA-class I restricted tumor reactivity. **A.** IFN-γ production by PBL transduced with oligoclonal EM TCRs. Anti-CD3 stimulated allogeneic PBL were transduced with EM TCRs, and co-cultured with autologous tumors (RCC) and Epstein-Barr virus-transformed B (EBV-B) line. IFN-γ production was measured after overnight co-culture. GFP-transduced and untransduced PBL were used as controls. **B.** The reactivity of PBL transduced with TRAV14/TRBV13 was blocked by HLA-class I Ab. PBL transduced with TRAV14/TRBV13 were co-cultured overnight with autologous RCC in the presence of anti-HLA class I, anti-HLA class II or anti-HLA DR antibodies (10 μg/ml), and IFN-γ from the co-culture supernatant was measured the next day. PBL transduced with a HLA-class I restricted TCR recognizing NY-ESO and a HLA-class II restricted TCR recognizing tyrosinase were used as controls. **C.** IFN-γ production of PBL transduced with TRAV14/TRBV13 against HLA-A3 matched or mismatched tumors.

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Table 1

5′ RACE of TCR alpha chain in Vβ23$^+$ EM populations from RCC patient DS[1]:

	Identical Variable region[2]	Identical CDR3 region
TRAV14	44	43
TRAV1-1*01	6	6
TRDV1-1*01	5	5
TRAV13-2*01	4	3
TRAV13-1*01	2	2
TRAV5*01	2	1
TRAV12-1*01	1	1
TRAV12-2*01	1	1
TRAV2*01	1	1
TRAV23*01	1	1
TRAV8-3*02	1	1

[1] 82% of Vβ23$^+$ EM populations were TRBV13, and 88% of which shared the same CDR3 region;

[2] Total sequences analyzed for alpha chain were 68.