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

Qiong J. Wang, Ken-ichi Hanada, Paul F. Robbins, Yong F. Li, and James C. Yang

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 showed that RCC TIL contained fewer CD27+ T cells, and fewer naive 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 toward EM. One possibility was the expression of CD70 on nearly all human RCCs, but not melanomas. Differentiation of naive T cells to EM cells only occurred from CD70 costimulation in concert with T-cell receptor (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 TCRs in fresh RCCs showed that EM T cells were more clonally expanded than CM or naive 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 2 TCRs from the highly represented EM TIL clones, when reexpressed 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 because of proliferative exhaustion, driven by tumor-expressed CD70. Cancer Res; 72(23); 1–11. ©2012 AACR.

Introduction

Adaptively transferring tumor-infiltrating lymphocytes (TIL) expanded in vitro back into patients with metastatic melanoma can mediate durable and complete tumor regressions (1, 2). These melanoma TILs will show immunologic 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 antitumor 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 be hard to suggest that RCC-reactive TIL or T-cells do not exist in patients with RCC. We considered whether the failure to show 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 and colleagues 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 situ disappeared after in vitro stimulation by T-cell receptor (TCR) analysis (17). Therefore, investigating phenotypes of T cells residing in renal tumors comparing with melanoma would help us to understand the differences between these 2 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 costimulatory receptor, CD27 (20). The role of CD70 in tumor immunology is controversial, conferring benefit when expressed by CD8+ TIL administered therapeutically to patients with melanoma (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 and colleagues have shown that constitutive expression of CD70 by B cells resulted in exhaustion of the
naive 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 with 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 RCC were enzymatically digested with 0.1% collagenase type IV, 0.01% hyaluronidase type V, and 30 U/mL deoxyribonuclease type IV (Sigma Chemical) in RPMI-1640 (Life Technologies) at room temperature for 3 hours. 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 (Valley Biomedical) with 10% dimethyl sulfoxide (DMSO) and frozen at −70°C. Tumor lines from patients with RCC were established and maintained in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies) including 10% FBS (Life Technologies), 10% tryptose phosphate (Sigma), 1× insulin-transferrin selenium (Life Technologies), and 1× serum pyruvate (Life Technologies).

For T-cell phenotypic analyses, fresh tumor digests were thawed, resuspended in RPMI-1640 with 10% human serum, and cultured overnight at 37°C in 5% CO2. Nonadherent 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 melanomas lines were obtained from Surgery Branch Laboratories (National Cancer Institute, Bethesda, MD), 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 coculture assays, respectively.

Antibodies
For immunophenotypic analyses, monoclonal antibodies (MAB) 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 M6P9); 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; 0.03–10 μg/mL) or controls were plated onto a 96-well flat-bottom plate that was precoated with anti-mouse CD8 alpha or PBS alone (Ancell; 10 μg/mL) overnight at 4°C. The plate was blocked with PBS containing 1% bovine serum albumin (Sigma) for 30 minutes at room temperature, and washed with PBS 3 times before adding T cells.

Peripheral blood lymphocytes (PBL) from patients with RCC were thawed and cultured in RPMI-1640 with 10% human serum overnight. Nonadherent cells were harvested the next day, labeled with fluorescence-conjugated antibodies (Abs) against CD3, CD62L, and CD45RO. Cells were incubated for 30 minutes at 4°C, washed with PBS containing 1% bovine serum albumin, and sorted for CD3+CD62L−CD45RO− (naïve) or CD3+CD62L+CD45RO+ [central memory (CM)] 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 Biosciences). After sorting, cells were plated at 2 × 105 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
Effecter memory (EM; CD3+CD62L−CD45RO−), 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 carried out by the SMARTer RACE cDNA Amplification Kit (Clontech) following the manufacturer’s instructions. The RACE cDNAs (~800 bp) were obtained with primers complementary to the constant region of TCRα or β chain and then inserted into the pCR2.1 vector by TA cloning (Life Technologies). Primers for the TCRα or β chain were synthesized by Life Technologies, and their sequences were 5’-GCCACAGCAGTTGCTCTTGAAAGCC or 5’-CAGGCAGTATCTGGAGTCATTGAG, respectively. After TA cloning, 96 colonies were picked from each 5’ RACE product of both TCRα and β 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 TCRα chains and β chains were cloned into the pMSGV1 plasmid, which is a derivative of the murine stem cell virus-based splice-gag vector (pMSGV), as described in previous publications with some modifications (25). Briefly, full-length TCRα and β chain cDNAs were amplified by PCR using the pairs appropriate to corresponding sequences of each TCRα chain and β 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 3 μg of plasmid RD114 using Lipofectamine 2000 (Life Technologies; 60 μL). 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 (50 ng/mL) and IL-2 (300 IU/mL) for 2 days before transduction was carried out. The stimulated cells were added to 24-well plates initially coated with Retro-Nection (Takara) and subsequently precoated with retrovirus by spinoculation (2,000 × g, 32°C, 2 hours) at 5 × 10^5/mL. The plates were then centrifuged at 1,000 × g for 10 minutes 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 between 0.5 and 1 × 10^6 cells/mL. Transduction efficiency was determined by analyzing Vβ expression of retrovirally transduced cells if the antibody was available.

Determination of CD70 expression

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 TCR α and β chains from fresh TIL. The most overrepresented TCRs were then reexpressed in PBL and tested for reactivity with RCC from which the TCRs 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 α or β chains. Approximately, 70 α chain and 70 β 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 shown 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 shown 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 show that T cells that reside in renal tumors are more terminally differentiated.

**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 with 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 (Lin1⁻) population (i.e., nonimmune cells) in renal fresh tumor digests compared with melanoma (Fig. 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 (Fig. 2B; ref. 18), whereas 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 (Supplementary Fig. S1). We further tested the effect of CD70 using anti-CD3 antibody and purified human recombinant CD70 protein coated onto plastic plates. As shown in Fig. 2C and D, naïve human T cells differentiated when they received both a TCR signal and costimulation by CD70 as shown 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.

**Oligoclonal Tumor-Reactive EM T Cells in CD70-Expressing RCC**

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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 naive T cells had a diversified repertoire. Interestingly, the specific oligoclonal TCRs found in EM TIL were never found in either the CM or naive T-cell populations. In total, renal fresh tumor digests from 4 patients with RCC 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 Fig. 4, the diversity and clonotypes of TCR \( \alpha \) (Fig. 4A) and \( \beta \) chains (Fig. 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.

**Functional analysis of oligoclonal EM TCRs**

The presence of overrepresented 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 3 patients with RCC with oligoclonal TCRs in their EM populations were examined and their tumor reactivity was tested. Testing only a total of 8 TCRα chains and 8 TCRβ chains from these 3 patients, tumor-reactive TCRs were identified in 2 of them. Tumor reactivity of TCRs from patient RV was shown in Fig. 5. Nine retroviruses representing all the possible pairing of 3 TCRα chains and 3 β chains, and used to transduce to anti-CD3-stimulated allogeneic PBL. One TCR, consisting of TRAV24 and TRBV24, could confer reactivity against CIITA-transduced, HLA-class II expressing autologous tumor cells (RCC/CIITA) but not parental RCC line (Fig. 5A). This reactivity could be blocked by anti-HLA class II and anti-HLA DR antibodies, but not anti-HLA class I antibody (Fig. 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; Fig. 5C).

Figure 2. CD70 expression on tumors and the effect of purified CD70 on naïve CD8⁺ or CD4⁺ T cells. A, CD70 expression on nonimmune cells in renal fresh tumor digests compared 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 nonimmune 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 patients with RCC, and 10 patients with melanoma were thawed and grown till their confluence. 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 were thawed and cultured in RPMI with 10% human serum 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 x 10⁵/well) were added to the 96-well plate that were precoated with anti-human CD3 (10 μg/mL) and/or purified human CD70 protein (10 μg/mL) and cultured in RPMI + 10% human serum + IL-2 (50 cu/mL) for 5 days. Five 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 (0–10 μg/mL) in the presence or absence of anti-CD3 Ab (10 μg/mL). The experiments in C and D were conducted multiple times and gave similar results.
No tumor recognition was detected when tumor lines did not express HLA class II. In addition, an HLA-DRB1*01/C301/C01 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.

Figure 3. TCR clonotypes in EM, CM, or naïve T cells from renal fresh tumor digests. Fresh tumor digests from patient DS with RCC were thawed and cultured in RPMI + 10% human serum overnight. The nonadherent 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 α chains and β chains in each population were evaluable after 5’RACE using gene-specific primers. Each pile chart shows the composition of TCR α or β chains. Each sector represents a single α or β chain with a unique CDR3.

Figure 4. Frequencies of EM TCR α chains (A) and β chains (B) from fresh tumor digests compared with PBL from patient RV with RCC. Each single bar represents the frequency of a single variable TCR α or β chain. The gray bar within each bar represents the frequency of the dominant single TCR α or β chain with a unique CDR3.
Using the same \( \alpha/\beta \) TCR pairing approach, we also identified a tumor-reactive EM TCR in patient DS with RCC. As shown in Fig. 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 (Fig. 6B), and it recognized 2 HLA-A3-matched renal tumor lines (Fig. 6C), suggesting the reactivity could be HLA-A3 restricted. Because of the availability of the antibody against TRBV13 (V\( ^{b} \)23), we were able to fluorescence-sort V\( ^{b} \)23\(^{+} \) 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 showed 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 malignancies, 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 in 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 costimulation positively modulates T-cell activation, differentiation, and survival, and therefore maintains antigen-specific T-cell responses (27–29). Using CD27-deficient mice, Hendriks and colleagues showed that these mice infected with influenza virus had decreased numbers of effector T cells in the lung. They have also shown that costimulation 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-expressing 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 choriomeningitis...
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Table 1. 5’ RACE of TCR α chain in Vβ23+ EM populations from patient DS with RCC

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Identical variable region

Identical CDR3 region

82% of Vβ23+ EM populations was TRBV13, 88% of which shared the same CDR3 region.

Total sequences analyzed for α chain were 68.

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 reexpressing them in fresh T-cells to allow their characterization.

Several RCC TIL did indeed show individual T-cell clonotypes in their EM populations that constituted up to 19% of all the TCR randomly selected and cloned with 5’ RACE. This clonotypic dominance was not seen in naive or CM TIL from the same patients. The most overrepresented 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 3 patients with 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 showed 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 overrepresented TCR species could be shown to react with autologous tumor. This could be because of 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, whereas 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 that 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 reengineering 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 reexpression in PBLs allows for the further study of RCC TIL and points to a means of using these findings in new adoptive cell therapies.

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 tumour-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 because of 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 that 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 that 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 patients with RCC 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 overrepresented clonotypes in this environment would be those seeing antigens that were dominant or abundant on tumor.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Q.J. Wang, P.F. Robbins, J. Yang
Development of methodology: Y.F. Li, J. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q.J. Wang, Y. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q.J. Wang, J. Yang
Writing, review, and/or revision of the manuscript: Q.J. Wang, K. Hanada, P. F. Robbins, J. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Yang
Study supervision: J. Yang

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