Preclinical Evaluation of Adoptive Cell Therapy for Patients with Metastatic Renal Cell Carcinoma

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Abstract. Background: Interleukin-2 (IL-2) shows encouraging clinical results in metastatic renal cell carcinoma (RCC) patients, but is limited by substantial toxicity. Cell-based therapy holds a promise, but past attempts in RCC were unsuccessful. Advances in tumor-infiltrating lymphocytes (TIL) generation technology and modified clinical protocols recently yielded a 50% response in refractory melanoma patients. Materials and Methods: RCC-derived TIL and tumor cells were processed by current protocols from tumor specimens in a clean laboratory. The expanded TIL were characterized and tested in functional assays. Results: The TIL cultures were efficiently generated and massively expanded. Virtually all the expanded cells were T-cells, but a considerable variability in the CD4/CD8 ratio and a frequent CD4–CD8– phenotype were observed. The TIL exerted cytotoxic or IFNγ-release activities against autologous targets in major histocompatibility (MHC) class I-restricted and -independent functional patterns. The functional results were superior to former technologies. Conclusion: Recent developments in TIL generation technology and clinical patient conditioning protocols indicate that the TIL-based approach for RCC could be revisited.

Renal cell carcinoma (RCC) is the most common renal tumor with an incidence of about 54,390 cases per year in the US alone (1). Twenty to 30% of patients who present with localized disease will develop metastasis following radical nephrectomy, with a 10-year disease-free survival rate of 50% (2-3). Around 30% of RCC patients present with metastatic disease (2-3). Metastatic RCC is an aggressive, chemotherapy-resistant, malignancy with a 5-year survival rate of around 10% (4-5). Recently, two new drugs have been approved by the Food and Drug Administration (FDA) for the treatment of metastatic RCC, sorafenib and temsirolimus, and bevacizumab (Avastin) has shown some clinical benefit as well (5). Like melanoma, RCC is commonly considered as an immunogenic tumor, mainly due to the occurrence of spontaneous regressions (6) and rich intratumoral lymphocytic infiltration (7) with activated phenotype (8). Collectively, a natural immune response develops in most RCC patients, but for various reasons fails to control tumor growth. Indeed, many immunoinhibitory mechanisms have been described in RCC (9-16). Nevertheless, partial or complete response to cytokine therapy with interleukin-2 (IL-2) or IFNα has been observed in 20% of cases (17-18). Comparable response rates and survival have also been observed with regimens of high-dose continuous i.v. infusion (CIV) (19). IFNα activates NK cells and innate immunity, and promotes cell-mediated immune response by increasing dendritic cell cross-priming of T-cells (20). IL-2 has a pivotal role in the stimulation of cytotoxic lymphocytes, re-activation of inhibited lymphocytes (16), overcoming programmed death ligand (PD-L) inhibition of T-cells (21) and increasing proliferation and survival of tumor specific cytotoxic T lymphocytes (22). These observations and others imply that RCC growth could be controlled by immunological intervention, with substantial emphasis on IL-2. Despite considerable efforts, only limited progress has been achieved in treating metastatic RCC patients with immunotherapy since the advent of IL-2 (23). Indeed, only IL-2 can induce a durable complete response or cure in a portion of the patients, while all other modalities, including newly approved...
drugs, only prolong overall survival without achieving a cure (5). However, the success of high dose IL-2 treatments is limited by toxic effects and can thus hardly be improved.

Adoptive cellular immunotherapy has been successfully implemented as a modality for the treatment of some human carcinomas for almost two decades, with considerable progress achieved especially in melanoma (24-26). Cell-based adoptive cell transfer (ACT) regimens might provide a promising and versatile therapeutic platform for RCC. Previous clinical trials with tumor-infiltrating lymphocytes (TILs) in RCC did not yield substantial benefit (27,28), however, in addition to patient pretreatment, conditioning and concurrent treatment protocols, the TIL generation technology has evolved substantially over the past decade. Adoptive cell transfer protocols require a substantial number of cells, usually around 10^10 per infusion (24-25, 29). Rapid numerical expansion is required to achieve these amounts of cells in short time. Currently, an initial short growing phase of TIL stimulated by a high concentration of IL-2 (6,000U/ml) is followed by a second rapid expansion phase that includes a high concentration of IL-2, T-cell receptor (TCR) stimulation with the agonistic antibody OKT3 and allogeneic stimulation with irradiated feeder lymphocytes derived from 3 different allogeneic healthy donors (24, 30). In contrast, in a 1995 RCC study, TIL cells were generated over a long culture time with a low concentration of IL-2 (100U/ml) and initial activation with anti-CD3 stimulation (27), and only low killing activity against autologous RCC tumor cells was observed (27, 31).

Neither the current technology for the generation of tumor reactive TIL cultures nor the modified clinical adjuncts have ever been tested in the setting of RCC. Recently, we have established clinical grade capabilities of generating melanoma-derived TIL cultures with current technology in clean laboratories (29) and have already successfully treated 20 refractory metastatic melanoma patients (manuscript submitted). The results of pre-clinical evaluation of this technology in the RCC setting are reported here. The establishment of tumor cells and TIL cultures, as well as the performance of complete dry runs of massive expansion under Good Manufacturing Practice (GMP) conditions to ensure protocol compatibility, was tested. Further immune phenotyping and functional characterization of the established TIL cells were performed. The experimental implementation of this technology for the treatment of RCC patients is discussed.

Materials and Methods

Patients. Patients with diagnosed advanced RCC undergoing radical nephrectomy as part of standard care were included. Tumor specimens were sent for pathology and concurrently to the laboratory for the generation of TIL and tumor cell cultures. All the patients willingly gave their informed consent. The study was approved by the Institutional Review Board (approval 3881/2005) and performed according to the declaration of Helsinki.

Processing of tumors. The methods adopted were precisely as formerly reported (25, 29). The tissue was placed following resection in sterile saline in the operating room and transferred to the laboratory. A trained pathologist performed gross pathological examination, including size measurements and viability, as well as obtaining tissue for final histopathological diagnosis. The remaining tumor tissue was further subjected to various tumor processing methods, including: a) fragmentation of the tumor to 1-2 mm^3 in size and culturing each fragment in a different well of 24-well tissue culture plates (Nunc, Rehovot Israel); b) tissue remnant culture (TRC); c) fine-needle aspiration (FNA) and d) enzymatic digestion of minced tissue by incubation for 3 hours at room temperature. The tumor samples from b) c) and d) were each seeded in a single flask. All the work was performed with clinically compatible reagents and in accordance with good laboratory practice with microbial monitoring laboratory.

Culturing of tumor cells. The cultures designated for the development of RCC were maintained in RPMI-1640 (Cambrex Bioscience, Walkersville, Maryland, USA), supplemented with 10% heat inactivated FCS (Gibco, Minneapolis MN, USA), 100 U/ml Pen-Strep (Cambrex Bioscience), 2 mM L-glutamine (Cambrex Bioscience), 1 mM sodium pyruvate (Cambrex Bioscience) and 1 mM non-essential amino acids (Cambrex Bioscience).

Culturing and rapid expansion protocols of TIL. The methods adopted were precisely as formerly reported (25, 29). Each TIL culture obtained by any of the above methods was maintained independently in RPMI-1640 (Cambrex Bioscience) supplemented with 10% heat inactivated human serum (Valley, Winchester, VA, USA), 100 U/ml penicillin-streptomycin (Cambrex Bioscience), 50 μM 2-mercaptopethanol (Invitrogen, San Diego, CA, USA), 25 mmol/l HEPES pH 7.2 (Cambrex Bioscience), 50 μg/ml gentamycin (Cambrex Bioscience) and 6,000 IU/ml IL-2 (Chiron, Suresnes, France). The cell density was maintained between 0.5x10^5-2x10^6/ml. When sufficient TIL numbers were available, functional assays and massive expansion were performed. Massive expansion was performed by stimulation with the anti-CD3 antibody OKT3 (30 ng/ml), IL-2 (6,000 IU/ml) and irradiated feeder cells (5000 rad, 200:1 ratio between feeder cells and TIL). The feeder cells were allogeneic peripheral blood mononuclear cells derived from at least three healthy donors who were negative for HIV, and hepatitis B and C. The process was halted and the cells were harvested after two weeks. Massive expansion in large scale differs from small scale in the quantities involved, but this process entails additional technical challenges, especially under clean laboratory conditions. These include mainly the cultivation of cells in large volumes and the harvesting and preparation of the cells for infusion. We have recently developed a novel method for harvesting of TIL cells from the culture medium by utilizing pheresis machines (32). Full large scale production protocols were employed for the massive numerical expansion of 2 TIL cultures.

Quality and sterility tests. The total viable cell number was determined by microscopic cell count and trypan blue exclusion. Microbiological tests were performed during rapid expansion
protocol (REP) by membrane filtration and washing of the culture medium followed by 14-day incubation of the filter on bacterial growth media and on the end product by direct inoculation of a diluted product sample to bacterial growth media followed by 14-day incubation. Specific PCR for the detection of the mycoplasma genome with a chromogenic endotoxin assay which utilizes a modified limulus amoebocyte lysate and a synthetic color-producing substrate were also conducted.

Cytotoxicity and IFNγ-release assays. The killing assays were performed in 96-U shaped microplates (Nunc) following a standard 5h $^{35}$S-Met release protocol, as previously described (33). With the IFNγ-release assay TIL cultures are usually tested for reactivity against autologous tumor cells (25, 29-30). Therefore, the reactivity of TIL cultures could be tested only when cognate tumor cell cultures were established. IFNγ release was determined by a Sandwich ELISA Duo Set (R&D Systems, Minneapolis MN, USA) in 100 μl of supernatant harvested from each 96-U well, or from overnight co-incubation in 96-F wells, performed according to the manufacturer's instructions. The RCC-derived TIL cultures unresponsive both in IFNγ secretion and killing assays were tested in redirected killing assays, in which the T-cell receptor (TCR) is directly engaged, resulting in non-antigen restricted elimination of pre-labeled P815 murine target cells (34). Specifically, these cells express a receptor that binds the constant portion of immunoglobulins (Fc) of mouse anti human antibodies. Therefore, anti-human TCR antibodies, such as OKT3, pre-incubated for 1 hour with P815 cells at a concentration of 1 μg/ml are bound via the Fc portion. After washing of excess free antibody and co-incubation with human effector CD8+ T-cells, TCR is engaged by the antibody, resulting in T-cell-mediated elimination of the P815 cells, which is not antigen restricted.

Antibodies and cell lines. The following monoclonal antibodies were used in this work: anti-CD3 OKT3 (Janssen-Cilag, Australia), anti CD3-PE (DAKO, Glustrop, Denmark), anti-CD8-fluorescein isothiocyanate (FITC) (DAKO), anti-CD4-phycocerythrin/Cy5 (DAKO), anti-von Hippel Lindau (VHL) (BD, San Diego, CA, USA), anti-carcinomembronic antigen cell adhesion molecule-1 (CEACAM1) NCS (kind gift from Dr Lopez-Botet, Universitat Pompeu Fabra, Barcelona, Spain) and anti-human leukocyte antigen-G (HLA-G) MEM-G/4 (ABR-Fisher, USA). The following cell lines were used in this work: 526mel, 624mel, P815, and Chinese hamster ovary (CHO) cells transfected either with empty vector or with human VHL (kind gift of Dr. Michal Safran, Sheba Medical Center, Tel Hashomer, Israel).

Flow cytometry. The cells were harvested, washed and re-suspended in FACS buffer (PBS x1, 0.05% NaN3, 0.5% bovine serum albumin (BSA)) at a density of 2x10^6/ml. Then, 2x10^5 cells were distributed in 100 μl per well were distributed in 96-U microplates (Nunc) and placed on ice. The various antibodies of interest were added in an additional 100 μl per well. The cells and antibodies were incubated on ice under dark conditions for 30 minutes and then centrifuged (350 xg, 5 minutes). The supernatants were discarded, the cells washed and re-suspended in 200 μl FACS buffer and then transferred into FACS tubes. The analysis was performed with a FACS-Calibur instrument and the CellQuest© software (BD Biosciences, Rockville, MD, USA).

Western blot. The total protein extracts (200 μg from each sample) were resolved by size on 12.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Inc., Keene, NH, USA), which were stained with Ponceau S (Sigma, Rehovot, Israel) to determine equal protein sample loading and transferrence. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris base [pH 7.6], 150 mM NaCl) containing 0.1% Tween-20 (TBS-T) and probed with the anti-VHL antibody (0.5 mg/ml; BD) overnight at 4°C. After washing in TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse 1:10,000; Bio-Rad Laboratories Inc. Hercules, CA, USA) and developed using an enhanced chemiluminescence detection system (ECL detection kit; Amersham Pharmacia Biotech, Arlington Heights, IL, USA) according to the instructions of the manufacturer. The membranes were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY, USA). CHO/VHL cells were used as positive controls.

Results

Processing of tumor specimens for development of tumor and TIL lines. Ten tissue specimens were obtained from surgical resections of renal cell carcinoma tumors. The specimens were from patients in various clinical stages, including stage I (2 patients), stage II (1 patient) stage III (3 patients) and stage IV (4 patients) (Table I). The size of most of the tumors (length of the long axis) ranged between 5.3 and 8.5 cm. All three larger specimens #2, #4 and #5 (Table I) were derived from stage IV patients. There was no clear correlation between the size of the resected tumor and the clinical stage among the rest of the cases. In total, 43 stable tumor cultures were established from 6 different patients (Table I). All the successful attempts were derived from the clear cell RCC subtype, while among the unsuccessful cases, two were of clear cell subtype and the rest were of non-clear cell subtypes, oncocytoma and chromophobe (Table I). There was no clear correlation between the ability to establish tumor cell culture and the disease stage (Table I). Tumor cell cultures were established in 6/10 (60%) cases with the fragmentation method, in 5/7 (71%) with the enzymatic digestion method, in 4/10 (40%) with the FNA method and in 2/10 (20%) with the TRC method. Twelve fragments were generated from each tumor, resulting in a total of thirty-two established tumor cultures derived from 6 of the patients. Importantly, there were no morphological differences between the various tumor cell cultures (data not shown).

TIL cultures were established from all 10 specimens, but the different processing methods had different yields. The fragmentation method yielded established TIL cultures in 10/10 (100%) cases, enzymatic digestion yielded 7/7 (100%), FNA yielded 3/10 (30%) and the TRC method yielded 4/10 (40%) (Table I). There was no correlation between size or disease stage and success in establishing TIL cultures.
Expansion potential of developed TIL culture. All the expansion attempts were successful and the TIL cultures derived from the RCC specimens were expanded by at least 2 orders of magnitude, between 133-fold to 521-fold (Table II). The two expansions using the large scale protocol successfully yielded 776-fold and 815-fold (Table II). All the microbiological and contamination tests were negative (data not shown).

**Phenotypic characterization of developed TIL cultures.** All the expanded TIL cultures were triple stained for CD3, CD4 and CD8. Virtually all the cells were T-cells, as evident by CD3 expression, varying between 90-99% (Table III). There was no consistent CD4 and CD8 expression pattern, as the percentage of CD4+ T-cells varied between 14 to 85.9 percent and of CD8+ T-cells between 12 to 82 percent (Table III). In almost all cases there was an inverse correlation between these cell populations.

Out of the phenotyped TIL cultures, only TIL8-F11 and TIL9-Digest displayed specific killing activity against the autologous tumor cells (Table III). In both these TIL cultures there was a CD8+ predominance, although it was not robust (Table III). Noteworthy, in two of the cultures (TIL7-Digest and TIL8-Digest), a double positive CD4+CD8+ population was observed. The functional significance of these cells is still undefined. In addition, in six of the TIL cultures (TIL7-Digest, TIL8-F11, TIL8-Digest, TIL9-F9, TIL9-Digest and TIL10-Digest) a significant double negative CD4–CD8– T-cell population was identified, in some instances accounting for an unusually high percentage up to 30% of the cells (Table III).

**Reactivity of developed TIL cultures.** Specimens 4-6 and 8-10 (Table I) from which tumor cells were established were tested. A notable IFNγ release was measured in three TIL cultures (a single TIL culture derived from patient #4 and two TIL cultures derived from patient #6) out of a total of 30 tested (Table IV). The same TIL cultures were concomitantly tested for killing activity against the same autologous tumor cells. Surprisingly, the TIL cultures that were positive for IFNγ secretion did not display any killing activity.

### Table I. Renal cell carcinoma biopsies processed to establish tumor lines and TIL for potential adoptive therapy.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Tumor type (TNM)</th>
<th>Tumor size</th>
<th>Processing method</th>
<th>Established tumor lines</th>
<th>Established TIL</th>
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<tbody>
<tr>
<td>2</td>
<td>RCC1 T4N0M1</td>
<td>13.0</td>
<td>Fragments</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>RCC1 T3aN0M0</td>
<td>6.5</td>
<td>Fragments</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>RCC1 T3aN1M1</td>
<td>12.0</td>
<td>Fragments</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>RCC1,2 T4N1M1</td>
<td>27.0</td>
<td>Fragments</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>RCC1,3 T3aN0M1</td>
<td>7.5</td>
<td>Fragments</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>RCC4 T3aN0M0</td>
<td>6.0</td>
<td>Fragments</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>RCC1 T2N0M0</td>
<td>6.5</td>
<td>Fragments</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>RCC1 T3bN0M0</td>
<td>8.5</td>
<td>Fragments</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>RCC1 T1aN0M0</td>
<td>5.3</td>
<td>Fragments</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>RCC5 T1bN0M0</td>
<td>5.5</td>
<td>Fragments</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>43</td>
</tr>
</tbody>
</table>

RCC: Renal cell carcinoma; 1clear cell type RCC; 2RCC with sarcomatoid characteristics; 3cystic RCC; 4oncocytoma; 5chromophobe. Tumor size (length of long axis, centimeters) was measured in pathology. TRC: tissue remnant culture; FNA: fine needle aspiration; TIL: tumor infiltrating lymphocytes.

### Table II. Results of rapid expansion.

<table>
<thead>
<tr>
<th>Patient</th>
<th>TIL</th>
<th>Fold Expansion</th>
<th>Scale</th>
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<tbody>
<tr>
<td>6</td>
<td>Digest</td>
<td>776</td>
<td>Large</td>
</tr>
<tr>
<td>5</td>
<td>F12</td>
<td>815</td>
<td>Large</td>
</tr>
<tr>
<td>8</td>
<td>F7</td>
<td>515</td>
<td>Small</td>
</tr>
<tr>
<td>9</td>
<td>F7</td>
<td>446</td>
<td>Small</td>
</tr>
<tr>
<td>10</td>
<td>F4</td>
<td>420</td>
<td>Small</td>
</tr>
<tr>
<td>11</td>
<td>Digest</td>
<td>133</td>
<td>Small</td>
</tr>
</tbody>
</table>

Fold expansion of TILs from different patients following a 14-day process of rapid expansion. F: fragment; FNA: fine-needle aspiration; Digest: enzymatic digestion, tumor processing method.
activity (Table IV), whereas some of the IFNγ-negative TIL cultures displayed a marked direct cytotoxic activity (Table IV). Altogether, five additional TIL cultures displayed cytotoxic activity against autologous tumor cells (Table IV). The autologous target cells were confirmed as RCC cells by testing for the VHL protein. The VHL protein was undetectable in the tumor cells derived from specimens 6, 8, 9 and 10 by Western blotting (Figure 1A), thus confirming them as RCC cells (35). VHL was successfully detected in the positive CHO/VHL control cells (Figure 1A). All the developed RCC cell cultures were positively stained for MHC class I expression (Figure 1B), which ruled out lack of recognition due to a simple MHC class I down-regulation, which is frequently observed during the tumor formation process (reviewed in 36). No staining was observed for CEACAM1 or HLA-G (Figure 1B).

Effector machinery of RCC-derived TIL cells. Figures 2A and 2B show that all the TIL cultures derived from a representative patient, #5, did not exhibit significant killing or IFNγ-release activities. Importantly, however, forced engagement of the TCR with OKT3 mAb resulted in robust killing activity against the P815 cells (Figure 2C). Similar results were observed with additional TIL cultures that were non-reactive to autologous tumor cells (data not shown). IFNγ secretion was also concomitantly induced in this experimental setup (data not shown).

Specificity of developed TIL cultures. Finally, cognate tumor reactive TIL cultures were further tested for specificity by co-incubation with autologous or allogeneic RCC cells, melanoma cells or alone. TIL4-F6, TIL6-F12 and TIL6-Digest were tested in IFNγ-release assays, while TIL8-F11 and TIL9-Digest were tested in killing assays. TIL4-F6, TIL6-F12 and TIL6-Digest displayed specific reactivity against autologous tumor cells only, as there was no release of IFNγ in the presence of allogeneic RCC or melanoma cells (Figure 3A). However, a high spontaneous release of IFNγ was observed by TIL6-F12 and TIL6-Digest, but not by TIL4-F6, even in the absence of any target cells (Figure 3A). TIL8-F11 and TIL9-Digest displayed strong cytotoxic activity against cognate autologous tumor cells, but also against allogeneic RCC cells (Figure 3B). A shared HLA-A24 allele between patients #8 and #9 could explain the reciprocal TIL alloreactivity (data not shown). The measurable killing activity observed against allogeneic melanoma cells further argues for a lack of strict specificity (Figure 3B).

Discussion

The substantial differences between previous and current technologies in efficiently generating potent TIL cells may be of considerable importance. For example, it has recently been shown that stimulation of CD8* T-cells with high concentrations of IL-2 (3,000 U/ml) markedly and
directly enhanced CD27-CD70 interactions, which facilitated activation and proliferation (37). Long culturing frequently results in cell “exhaustion”, and sometimes in the clinical deterioration of patients while waiting for preparation of the TILs. Decreased telomerase activity and shortening of telomere length has been associated with cell exhaustion (38). Furthermore, it has been suggested that decreased telomerase activity and length of telomeres in the TILs transferred to a patient, are directly correlated with decreased persistence and potentially with a worse outcome (39). Hence, shortening the expansion phase is important and could contribute to the success of the treatment.

The present results showed that tumor cell cultures could be developed in the majority of the cases (60%), especially in the clear cell type (6 out of 8). It is suggested that this technology should focus on that malignancy, especially since 90% of metastatic renal malignancies are clear cell type RCC. Furthermore, TIL cultures were developed from all the patients and representative cultures were successfully massively expanded by 500-fold on average.

The fragmentation and enzymatic digestion were superior for the establishment of both tumor cell and TIL cultures and should therefore be the methods of choice. The expansion results were similar to those previously reported for the production of TIL cultures form melanoma specimens (29). Therefore, the TIL cultures developed from the RCC specimens were expanded to sufficient numbers for clinical therapy. All work was performed in adherence to GMP standards in clean labs, with all microbiological tests negative. Phenotypic analysis showed that the developed TIL cultures were comprised of T-cells (Table III). However, while most TIL cultures developed from melanoma specimens are predominantly CD8+ (30), a substantial percentage of CD4+ T-cells were observed here. CD8+ predominance has been reported before in TILs derived from RCC specimens (27, 40). Although the antitumor effect is presumed to be mediated through the CD8+ cells, the CD4+ cells may have beneficial effect (30). Indeed, one of our TIL-treated melanoma patient, who exhibited a complete response, was treated with TIL cultures that almost exclusively comprised of CD4+ T-cells.
(unpublished results). Of note, a relatively high frequency of cells with CD4−CD8− double negative phenotype was observed (Table III). It has previously been proposed that double negative T-cells might have immunoregulatory (suppressive) functions both in vitro and in vivo in a variety of physiological and pathological contexts (41), but their properties are still not completely understood. In general, the low effector functions observed in the in vitro functional assays with the TILs derived from patients 8, 9 and 10, and the higher effector functions observed with TILs derived from patient 6, which contained minute quantities of these cells (Table IV and Figure 3) supported this suggestion. An exception was noted, however, with the TIL9-Digest, which was comprised of 30% double negative T-cells, but still displayed significant cytotoxic activity (Table IV and Figure 3).

Functional analyses of TIL cultures developed from RCC revealed that most were “inert” and did not display killing or IFNγ secretion activities. The “inert” non-functional TIL cells were not defective or anergized, as TCR stimulation redirected killing activity (Figure 2). Hence, other mechanisms may play a role, such as suppressive cell populations, or improper recognition of autologous RCC cells. No significant down-regulation of MHC class I was observed on the tumor cells, which concurred with previous observations (42, 43). Although not observed in the present study, it has been reported that tumor-protective proteins such as CEACAM1 (44) and HLA-G (9) can be found on some RCC specimens. The CEACAM1 protein protects tumor cells by directly inhibiting effector lymphocytes such as NK cells (34, 45), T-cells (46-47) and TIL cells (16). HLA-G is recognized by inhibitory receptors such as leukocyte inhibitory receptor-1 (LIR1) (48). “Inert” TIL cultures are routinely identified among TIL cultures developed from melanoma specimens (26, 29). Out of the 30 TIL cultures tested, three displayed measurable IFNγ-secretion activity and another five displayed killing activity (Table IV). This frequency was similar to the tumor-reactive TIL cultures derived from melanoma (29). There were no TIL cultures that displayed simultaneous killing and IFNγ-secretion activities. This observation implies that these activities may be differentially regulated, as has been formerly suggested (33). Thus, the implementation of both tests enhances the chances of the identification of functional tumor-reactive TIL cultures. Cut-off values should be set to determine which TIL cultures are suitable for clinical use.

Some of the RCC-derived TIL cultures were unresponsive to both IFNγ-secretion and killing assays, but demonstrated robust killing activity in the redirected killing assay. Thus they could have had intact effector capacities, but were either actively inhibited, or had lost the ability to recognize and interact with the tumor cells. Additionally, some of the reactive TILs were not
exclusively specific to autologous tumor cells. TIL cultures exerting killing activity clearly reacted against autologous RCC tumor cells, but non-specific killing of allogeneic RCC tumor cells and even of melanoma cells was observed. This killing pattern has been previously described in RCC-derived TIL cultures and was mainly ascribed to NK or CD3+ lymphokine activated killer (LAK) cells (49). Non-specific effector functions are sometimes observed with melanoma-derived TILs as well (29). These results highlight the importance of specificity test, as most of the developed reactive TIL cultures were actually non-specific and could potentially cause undesired effects in a clinical setting. However, the phenotype of all tested TIL cultures in the present study almost exclusively comprised of CD3+ T-cells (Table III). Interestingly, the IFNγ-secreting TIL cultures were specific to the autologous tumor cells and did not react with the presence of allogeneic RCC or melanoma cells (Figure 3). Yet, some of these TIL cultures spontaneously secreted some amounts of IFNγ in the absence of any tumor cell. The clinical relevance of this non-MHC class I restricted killing activity is not entirely determined.

In summary, the successful melanoma protocol was demonstrated to be adaptable for the development of TILs from RCC specimens. TILs were able to be developed, activated and expanded to ACT compatible numbers under full GMP conditions, and tumor-reactive TIL cultures were generated and identified. A suggested schematic flow chart is presented in Figure 4. Furthermore, it was implied that reactive TILs might be selected with two concurrent functional assays, cytotoxicity and IFNγ-release, as these functions do not necessarily overlap and both are required for the elimination of tumors. Comparison with the in vitro functional results observed in a historic clinical trial demonstrated superior results with the current technology. The cut-off values for suitable clinical use should be determined. However, there may be a potential problem with decreased specificity, which could potentially lead to undesired effects of auto-reactivity. Nevertheless, even past treatments with peripheral blood derived LAK cells with broad reactivity did not cause severe autoimmune manifestations. The enhancement of specificity remains an important objective for further research. In conclusion, TILs generated with the current technology adopted from melanoma studies could provide an improved cell-based therapeutic platform for ACT in RCC. This platform could be further improved by other approaches, such as genetic modifications of TCR or co-stimulating molecules.

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