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*Research Article*

# JAK3/STAT5/6 Pathway Alterations Are Associated with Immune Deviation in CD8$^+$ T Cells in Renal Cell Carcinoma Patients

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To investigate the molecular mechanisms underlying altered T cell response in renal cell carcinoma (RCC) patients, we compared autologous and allogeneic CD8$^+$ T cell responses against RCC line from RCC patients and their HLA-matched donors, using mixed lymphocyte/tumor cell cultures (MLTCs). In addition, we analyzed the expression of molecules associated with cell cycle regulation. Autologous MLTC responder CD8$^+$ T cells showed cytotoxic activity against RCC cell lines; however the analysis of the distribution of CD8$^+$ T-cell subsets revealed that allogenic counterparts mediate superior antitumor efficacy. In RCC patients, a decreased proliferative response to tumor, associated with defects in JAK3/STAT5/6 expression that led to increased p27KIP1 expression and alterations in the cell cycle, was observed. These data define a molecular pathway involved in cell cycle regulation that is associated with the dysfunction of tumor-specific CD8$^+$ effector cells. If validated, this may define a therapeutic target in the setting of patients with RCC.

## 1. Introduction

Cancer is associated with immune deficiency but the biological mechanisms underlying immune failure remain poorly-defined. T cells are key elements in effective cancer immunity in RCC, a disease that has proven refractory to conventional treatment modalities, such as chemotherapy and radiotherapy [1]. On the contrary, RCC patients have been reported to be responsive to immunotherapeutic approaches establishing the concept that RCC is an immunogenic tumor [2, 3]. Given the perceived importance of CD8$^+$ T cells in mediating antitumor immunity, and that Th1-skewed CD4$^+$ Th cells are required to support durable CD8$^+$ T-cell immunity [4, 5], it becomes particularly important to understand how dysfunctional or partially functional CD8$^+$ T cells arise and what molecular mechanisms underlie their activation in patients with cancer. There is considerable interest in gaining a better understanding of the composition of the antitumor CD8$^+$ T cell population, with regard to its various functional subsets. Sallusto et al. [6] used CCR7 expression status to define T cell memory subsets. Central memory CD8$^+$ T cells ($T_{CM}$) express CCR7, a chemokine receptor required for trafficking to secondary lymphoid organs. In contrast, this marker is significantly downregulated on effector memory CD8$^+$ T cells ($T_{EM}$). $T_{CM}$ have been shown to mediate superior anti-tumor activity compared with $T_{EM}$ [7, 8]. There is a third T cell memory subset, terminally differentiated CD8$^+$ $T_{EM}$ cells ($T_{EMRA}$), that express CD45RA but lack CCR7 expression. Notably, $T_{CM}$ have a higher proliferative potential and greater resistance to apoptosis, whereas $T_{EM}$/$T_{EMRA}$ have a skewed TCR repertoire and are characterized by a "senescent" replication history [9, 10]. Furthermore, the results of gene expression analyses have revealed that $T_{CM}$ are characterized by high basal and

cytokine-induced STAT5/6 phosphorylation, reflecting their capacity for enhanced self-renewal versus effector memory cells, in particular, $T_{EMRA}$ [11].

The JAK3/STAT5/6 pathway is a crucial signal transduction component for many growth factor receptors and it has been shown to be necessary for the downregulation of several genes that inhibit the cell cycle. This pathway regulates the expression of cyclin-dependent kinase (Cdk) inhibitors p27, the hyperphosphorylation of the retinoblastoma protein (Rb) gene product, and the release of active E2F in T cell lines [12]. In vivo studies have provided evidence of the role of $p27^{KIP1}$, a cell cycle inhibitory protein, in T cell proliferation [13, 14]. High $p27^{KIP1}$ levels were correlated with a decreased proliferative response to IL-4 and IL-12 in STAT6 deficient T cells [15, 16]. Therefore, the characterization of cell cycle regulatory proteins and the delineation of aberrations in $CD8^+$ T cell function are important to understand in the context of tumor development and progression.

Inhibition of T-cell proliferation is an important aspect of immunosuppression as observed in cancer patients, and it represents a mechanism adopted by tumors to evade immune rejection [17]. Various studies have led to the identification of a pathway controlling the progression of cells from quiescence, through G1, and into S phase involving the activation of G1 cyclin-dependent kinases (cdk), inactivation of Rb and related proteins [18]. The transcription factors of the E2F family and Id (inhibitor of DNA binding) protein family play a central role in the regulation of cell growth [19]. In particular, E2F4 and Id2 are essential for Rb function [20] and are involved in independent functions that are orchestrated in order to allow for cell cycle progression. Id2 and E2F4 may compete for Rb binding; hence, Id2 may function via the release of restraining pocket proteins on E2F transcription. Deregulation of E2Fs transcriptional control contributes to oncogenic transformation in vitro and the development of metastasis in vivo [21].

In this paper, we have investigated RCC-specific $CD8^+$ T cell response in RCC patients and HLA-matched normal donors that were generated in mixed lymphocyte/tumor cell cultures (MLTCs) in order to define molecular pathways associated with immune deviation in $CD8^+$ T cell compartment in RCC patients. However, the generation of autologous RCC-reactive T cells requires the availability of established RCC cell lines, that can only be obtained from a minority of patients. Thus, we established tumor cell lines from the primary RCC tissue of patients undergoing nephrectomy. In such patients as well as HLA-matched normal donors, we studied the phenotypic and functional profile of autologous and allogeneic RCC-specific $CD8^+$ T cells. In particular, we focused on the integrity of the JAK3/STAT5/6 signaling pathway and its relationship with cell cycle regulatory proteins, such as E2F4 and Id2, in order to better understand relevant mechanisms of dysfunction associated with $CD8^+$ T cells isolated from RCC patients. T cells from RCC patients displayed decreased proliferative responses to tumor antigens, which was linked to defects in JAK3/STAT5/6 expression (and to increased p27KIP1 expression and altered cell cycle progression/proliferation). Interestingly, we observed a significant correlation between RCC $CD8^+$ T subset phenotypes and such signaling defects, providing a surrogate index for clinical immune deviation that might be employed in the monitoring of patient response to immunotherapy.

## 2. Material and Methods

*2.1. Cell Lines.* Cell lines were generated from primary kidney tissue explants of 13 patients with RCC. Freshly isolated tumor tissue was minced, then digested using an enzymatic cocktail as described previously [22]. Corresponding normal renal cell lines (proximal tubular epithelial cells, PTEC) were established from tumor-distal normal kidney tissue and cultured in complete medium. Epstein-Barr virus- (EBV-) transformed lymphoblastoid cell lines (LCL) were also generated from patient RCC1 PBMC (RCC1-LCL) and healthy donor-2 (donor-2-LCL) using the B95.8 (type 1) virus isolate. Allogeneic phytohemagglutinin- (PHA-) activated T cell blasts (PHA-B) from donor-2 and donor-3 (donor-2-PHA-B, donor-3-PHA-B) were prepared by stimulating PBMC in media containing 2 $\mu$g/mL PHA (Sigma) and IL-2 (250 IU/mL, Proleukin, Chiron, Emeryville, CA).

*2.2. Isolation of PBMC and HLA Typing.* Peripheral blood mononuclear cells (PBMCs) were obtained from RCC patients and HLA-matched healthy donors after written consent, under an IRB-approved protocol. All patients had histological confirmed RCC, clear cell subtype, and had not received preoperative therapy. To determine the HLA-class I genotype of donor PBMCs and RCC lines, individual alleles were amplified from genomic DNA with A/B/C allele-specific primers and were then completely sequenced (Table 1, Dr. B. Favoino, Laboratory of typing tissue).

PBMCs were isolated at the interface of Ficoll-Hypaque density gradients centrifugation (Sigma Chemical Co., St Louis, MO), per the manufacturer's instructions, washed twice in PBS 3 (Invitrogen-Life Technologies, Italy) and used in mixed lymphocyte/tumor cell cultures as described below.

*2.3. Isolation of $CD8^+$ T-Effector Cells.* $CD8^+$ T cells were isolated from the PBMC of autologous and allogeneic normal, healthy donors by positive selection using specific MACS magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) on MiniMACS columns, per the manufacturer's protocol and subsequently assessed for their functional and phenotypic profiles.

*2.4. Mixed Lymphocyte/Tumor Cell Cultures (MLTCs).* PBMCs from RCC patients or HLA-matched healthy donors were cocultured in 24-well plates (Costar, Corning, USA) at $10^6$ cells/well with irradiated RCC stimulator cells ($10^5$ cells/well) in 2 mL of AIM-V medium (Life Technologies, Invitrogen, Italy) supplemented with 10% heat-inactivated pooled human serum (Sigma (medium $M^b$)). Recombinant human IL-2 was added on day 3 at a final concentration of 250 IU/mL (Proleukin, Chiron, and Emeryville, CA). Responder lymphocytes ($10^6$ cells/well) were restimulated weekly with $10^5$ irradiated tumor cells in

Table 1: HLA-class I genotyping of RCC patients and healthy donors.

| | HLA-A | | HLA-B | | HLA-Cw | |
|---|---|---|---|---|---|---|
| RCC1 | 0301 | 2402 | 0702 | 0801 | 0702 | 0702 |
| Donor 1a | 0301 | 2402 | 0702 | 0801 | 0702 | 0702 |
| Donor 1b | 0301 | 2402 | 0702 | 0801 | 0702 | 0702 |
| RCC2 | 0301 | 2402 | 1806 | 3506 | 0401 | 0702 |
| Donor 2 | 0301 | 2402 | 1806 | 3506 | 0401 | 0702 |
| RCC3 | 2402 | 3302 | 3506 | 6506 | 0401 | 0702 |
| Donor 3 | 2402 | 3302 | 3506 | 6506 | 0401 | 0702 |
| RCC4 | 0301 | 2402 | 1806 | 3506 | 0401 | 0702 |
| Donor 4 | 0301 | 2402 | 1806 | 3506 | 0401 | 0702 |
| RCC5 | 0201 | 28 | 3506 | 5104 | 0401 | 0702 |
| Donor 5 | 0201 | 28 | 3506 | 5104 | 0401 | 0702 |

IL-2-containing medium $M^b$ for 2 weeks before $CD8^+$ T cells isolation. $CD8^+$ T cell isolation was performed using immunomagnetic $CD8^+$ beads (Miltenyi Biotec, Milan, Italy) and positively isolated T cells cultured for an additional 2 weeks. On day 35 of culture, $CD8^+$ T cells responders were used as effector cells in functional and molecular analyses.

*2.5. IFN-γ ELISPOT Assay.* $CD8^+$ T cell responders were assessed for their ability to secrete IFN-$\gamma$ in response to specific stimulation using ELISPOT assays (Mabtech, Mariemont, OH), as previously described [23]. Determinations were performed in triplicate and spots were enumerated using an automatic plate reader (Zeiss-Kontron, Jena, Germany). In some cases, the HLA-restriction of T cell recognition was determined by addition of blocking antibodies (final concentration = 100 $\mu$g/mL) to replicate ELISPOT wells. Antibodies included; W6/32, an anti-HLA class I, $\alpha$-gap A3, an anti-HLA-A3, B1.23.2, an anti-HLA-B and HLA-CA24, an anti-HLA-A24, L243, an anti-HLA class II-DR. IgG2a isotype-matched control Abs were used as negative controls.

*2.6. $^{51}$Chromium-Release Assay.* Responder $CD8^+$ T cells were evaluated for their ability to kill target cells including patient-derived RCC cell lines, PHA-blast or EBV-cells and K562 in standard 4 h $^{51}$Cr-release assays [23].

*2.7. Phenotypic Analysis.* T cells were washed and resuspended in FACS buffer (phosphate-buffered saline pH 7.2, 0.2% bovine serum albumin, and 0.02% sodium azide) containing 3% human serum and incubated with fluorochrome-conjugated mAbs for 15 minutes at 4°C, then washed with the same buffer before the performance of 6-color flow cytometry. For the surface staining of T cells, fluorescently labeled mAbs were used: PE-CD3, APC-CD8, FITC-CD45RA, PerCP-CD28, (all BD-9Pharmingen), Pe-Cy-7-CCR7 (R&D Systems), and APC-Cy-7-CD27. Flow data were acquired using a FACSortTM (Becton Dickinson) flow cytometer and analyzed using WinMDI version 2.8 software.

*2.8. Cell Cycle Analysis.* T cells were fixed in 70% ethanol for 24 hours, prior to being treated with 50 mg of RNase A/mL at 37°C for 30 minutes After resuspension in 0.5 mL of propidium iodide solution (50 pg/mL propidium iodide, 0.1% sodium citrate, and 0.1% Nonidet P-40) and a 30-minutes incubation in the dark at 4°C**,** the cellular DNA content was analyzed by flow cytometry using WinMDI version 2.8 software.

*2.8.1. Confocal Analysis.* The expression of STAT5, STAT6, p-$27^{KIP1}$, E2F4, and Id2 in T cells was analyzed by confocal fluorescence microscopy using specific Abs (Santa Cruz Biotechnology, DBA, Italy). $CD8^+$ T cells were allowed to adhere to poly-L-lysine-coated slides (Sigma-Aldrich, Italy) for 1 hour on ice, followed by fixation in 3.7% paraformaldehyde. After washing three times with PBS (pH 7.4), and being blocked with BSA 1% and PBS, cells were stained with anti-pSTAT5 Ab, anti-pSTAT6 Ab, anti-E2F4 Ab, anti-p27KIP1 Ab and anti-ID2 Ab. After washing, T cells were than incubated with fluorescently labeled secondary Abs: Alexa Fluor FITC- and TRITC-conjugated anti-Ig (Molecular Probes, Eugene, OR). Stained cells were then washed four times with PBS, air-dried, and mounted using Gel/Mount (Biomedia, Foster City, CA). Samples were analyzed using a laser scanning Leica TCS SP2 confocal microscope (Leica, Wetzlar, Germany) equipped with argon krypton (488 nm), green-neon (543 nm), and helium-neon (633 nm) lasers. The expression levels of pSTAT5/6, p-$27^{KIP1}$, E2F4, and Id2 were measured in at least 15 high-power fields (HPFs; 63×)/section by two independent observers blinded to the origin of the slides. The final count was the mean of the two measures ± standard deviation (SD). In no case the interobserver variability exceeds 20%.

*2.8.2. Statistical Analysis.* Data are reported as mean ± standard deviation (SD) and analyzed for differences using an unpaired Student's *t*-test. Statview software package (SAS Institute Inc. Cary, NC, USA; 5, 0 version) was used for all analyses. $P$ values $< .05$ were considered statistically significant.

## 3. Results and Discussion

*3.1. Selective Expansion of Autologous and Allogenic Antitumor CD8⁺ T Cells by MLTC.* In our initial set of experiments, we attempted to establish primary RCC lines from 13 patients who underwent radical or partial nephrectomy for unilateral renal cell carcinoma. However, given limited quantities of available tissue, we were only able to develop 5 RCC lines which exhibited with the appropriate morphology, histopathology, and immunohistochemical characteristics of (clear-cell) RCC. Autologous and HLA-matched allogeneic PBMCs isolated from normal, healthy donors (Table 1) were used as responder cells to these tumor cells in MLTC established in the presence of high dose rhIL-2. After 3 weeks of culture, responder cells were harvested and MACS-purified CD8⁺ T cells were restimulated in an identical fashion (to the primary induction) for additional 2 weeks. On day 35 of culture, CD8⁺ T cells were evaluated for specific anti-RCC reactivity using IFN-$\gamma$ ELISPOT assays and $^{51}$Cr-release assays.

In a first example of the results obtained, we examined autologous and allogeneic CD8⁺ T cell responses against the RCC1 cell line. Interestingly for this model, we employed 2 allogenic responders: donor 1a (a healthy sibling to the patient) and donor 1b (a healthy unrelated donor). As shown in Figure 1(a), on day 0 (T0) CD8⁺ T cells isolated from RCC1 patient's PBMC displayed an elevated (HLA class I-restricted) reactivity against the autologous RCC1 cell line versus unstimulated CD8⁺ T cells isolated from either of the healthy donor controls (Figures 1(b) and 1(c), resp.), which was statistically significant ($P < .03$). After MLTC, the generated patient's CD8⁺ T cells continued to recognize RCC1 tumor cells, but they failed to react against autologous nonmalignant kidney cells (PTECs) or autologous PHA blasts (Figure 2(a)). T cell reactivity against the RCC1 line was MHC class-I restricted, with mAbs directed against single HLA-A (i.e., HLA-A24) or common HLA-B/-C determinants inhibiting specific T cell recognition of tumor cells by 75.5% and 43.4%, respectively (Figure 2(b)). An analysis of T cells generated from the patient's sibling (i.e., MLTC2) revealed that the responder CD8⁺ T cells recognized the HLA-matched RCC cell line to a statistically greater degree than observed from the patient's own CD8⁺ T cells ($P < .05$), while responding against the K562 LAK/NK target cell line to a moderate level (Figure 2(c)). These CTL recognized the RCC1 cell line in a predominantly class I-restricted manner based on the ability of the anti-A24 and anti-HLA-B/C mAbs to inhibit responses by 52.3% and 70.7%, respectively. Finally, in the case of the HLA-matched, unrelated donor (i.e., MLTC3), we observed the greatest capacity (versus MLTC1 and MLTC2) of CD8⁺ T cells to respond to the RCC1 cell line ($P < .03$; Figure 2(d)). Anticlass I blocking studies identified HLA-A24 (28% inhibition) and HLA-A3 (57.8% inhibition) as the predominant HLA-restricting elements for MLTC3 T cells. In all the three anti-RCC1 T cell systems studied, ELISPOT assays showed that T lymphocytes specifically secreted IFN-$\gamma$ and granzyme B, but not interleukin-5 (IL-5), supporting dominant Type1 T cell responsiveness (data not shown).

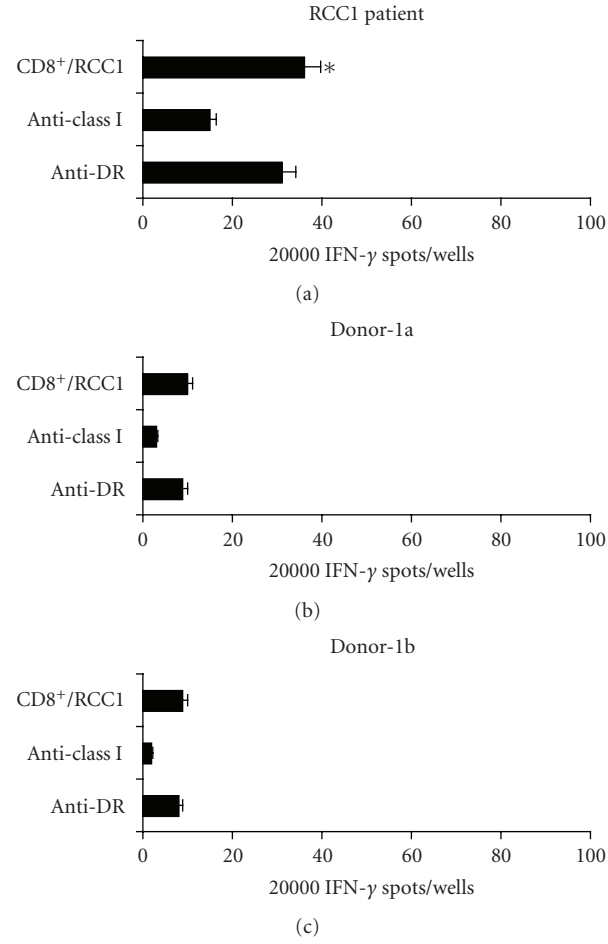



Figure 1: IFN-$\gamma$ Elispot Assay CD8⁺ T cells isolated at day 0. RCC1 cell line as stimulator cells and CD8⁺ T cells isolated from PBMC of RCC1-patient (a) and their allogeneic donor-1a (b) and donor-1b (c) at day 0 were tested in 20 h IFN-$\gamma$-Elispot Assay. HLA restriction of anti-RCC reactivity was determined by adding mAbs specific for total HLA-class I and HLA-DR. Results represent the average (± SD) of triplicate wells and the presented data derive from 1 representative experiment of 5 performed. A paired $t$-test analysis was used to compare the frequencies of autologous versus allogenic specific CD8⁺ T cells stimulated with RCC1 cell line, after subtraction of HLA class I reactivity; $P < .03$ was considered statistically significant (*).

Analysis of cytotoxic CD8⁺ T cell responses using $^{51}$Cr-release assays similarly indicated a hierarchy of MLTC3 (75% lysis at an E/T ratio of 20 : 1) > MLTC2 (55% lysis) > MLTC1 (40% lysis) T cell response against the RCC1 cell line (Figures 3(a)–3(c)). MLTC1 and MLTC2 T cells also mediated moderate cytolysis of K562 cells, but not RCC1-LCL (Figures 3(a) and 3(b)), while MLTC3 T cells recognized RCC1-LCLs (65% lysis at an E/T ratio of 20 : 1) but not K562 cells (Figure 3(c)).

*3.2. Differential Turnover of the CD8⁺ Memory T Cell Subset in RCC Patients after MLTC Stimulation.* Given the perceived importance of CD8⁺ T cells in mediating anti-tumor

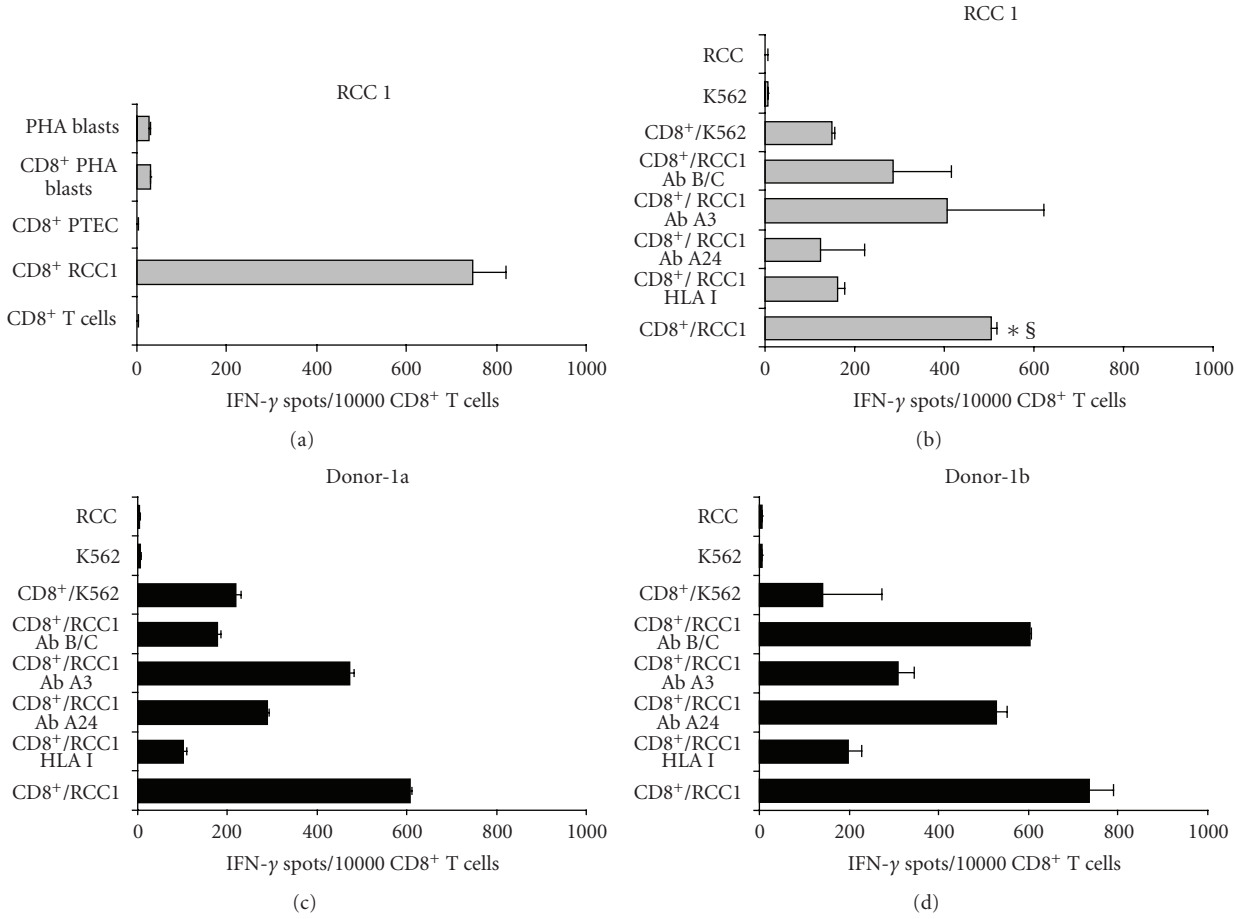



Figure 2: IFN-$\gamma$ ELISPOT analyses of RCC-specific CD8$^+$ T cell responses after MLTC. RCC1 CD8$^+$ T cells stimulated by MLTC were tested for their reactivity against HLA-matched target cells such as autologous RCC, PTEC, and PHA-activated PBMC, (a). In panels (b), (c), and (d), K562 cells served as targets. A paired $t$-test analysis was used to compare the frequencies of autologous responder-CD8$^+$ T cells versus allogenic responder-CD8$^+$ T cells generated from the patient's sibling ($^*P < .05$) and unrelated donor ($^{\S}P < .03$), stimulated with RCC1 line. The HLA restriction of the anti-RCC T cell response was determined by addition of blocking mAbs specific for total HLA-class I, HLA-A24, HLA-A3, or HLA-B/-C alleles. Results represent the average ($\pm$ SD) of triplicate wells and the presented data derive from 1 representative experiment of 5 performed.

immunity, we extended our characterization of autologous and allogenic RCC-specific CD8$^+$ T cells generated from MLTC, using 6-color flow cytometry, focusing on markers of T cell activation, proliferation, and differentiation. In agreement with previous studies [6, 10], four distinct phenotypic subsets of responder CD8$^+$ T cells could be identified using CD45RA and CCR7 as markers. Whereas naive T cells express both CD45RA and CCR7, memory T cells were characterized by the down regulated expression of CD45RA and could be additionally subdivided, based on differential expression of CCR7, into effector memory ($T_{EM}$: CD45RA$^-$, CCR7$^-$) and central memory subsets ($T_{CM}$: CD45RA$^-$, CCR7$^+$). Several recent reports have defined the CD45RA$^+$/CCR7$^-$ T cell subset ($T_{EMRA}$) as *terminally differentiated effector* T cells which lack CD27 and/or CD28 expression and are able to migrate to inflamed tissues, where they may exert vigorous ex vivo effector functions, including target cell lysis [24, 25].

CD8$^+$ T cells were purified from PBMC isolated from 5 RCC patients as well as HLA-matched normal donors and analyzed for their phenotypes on day 0 (T0) and after 5 weeks of MLTC culture (T35; Table 2). Freshly isolated CD8$^+$ T cells from RCC patients displayed a predominant memory phenotype comprised of $T_{EM}$ and $T_{EMRA}$, while similarly isolated CD8$^+$ T cells from healthy donors exhibited a predominantly naïve (i.e., CD45$^+$, CCR7$^+$) phenotype, with lower percentages of $T_{EMRA}$ cells and comparable levels of $T_{CM}$ and $T_{EM}$ cells. As expected, after 35 days of MLTC, the percentage of naïve T cells (versus T0) declined in all subjects, and the percentage of $T_{CM}$ increased, most notably in the normal donor MLTC (59% $\pm$ 4.6) versus the patient MLTC (11 $\pm$ 2.8%).

When taken together, these data are in line with previous reports in which tumor-reactive CD8$^+$ $T_{CM}$ mediate superior anti-tumor efficacy when compared with $T_{EM}$ [7, 26]. In contrast, MLTC stimulation induced higher frequencies of

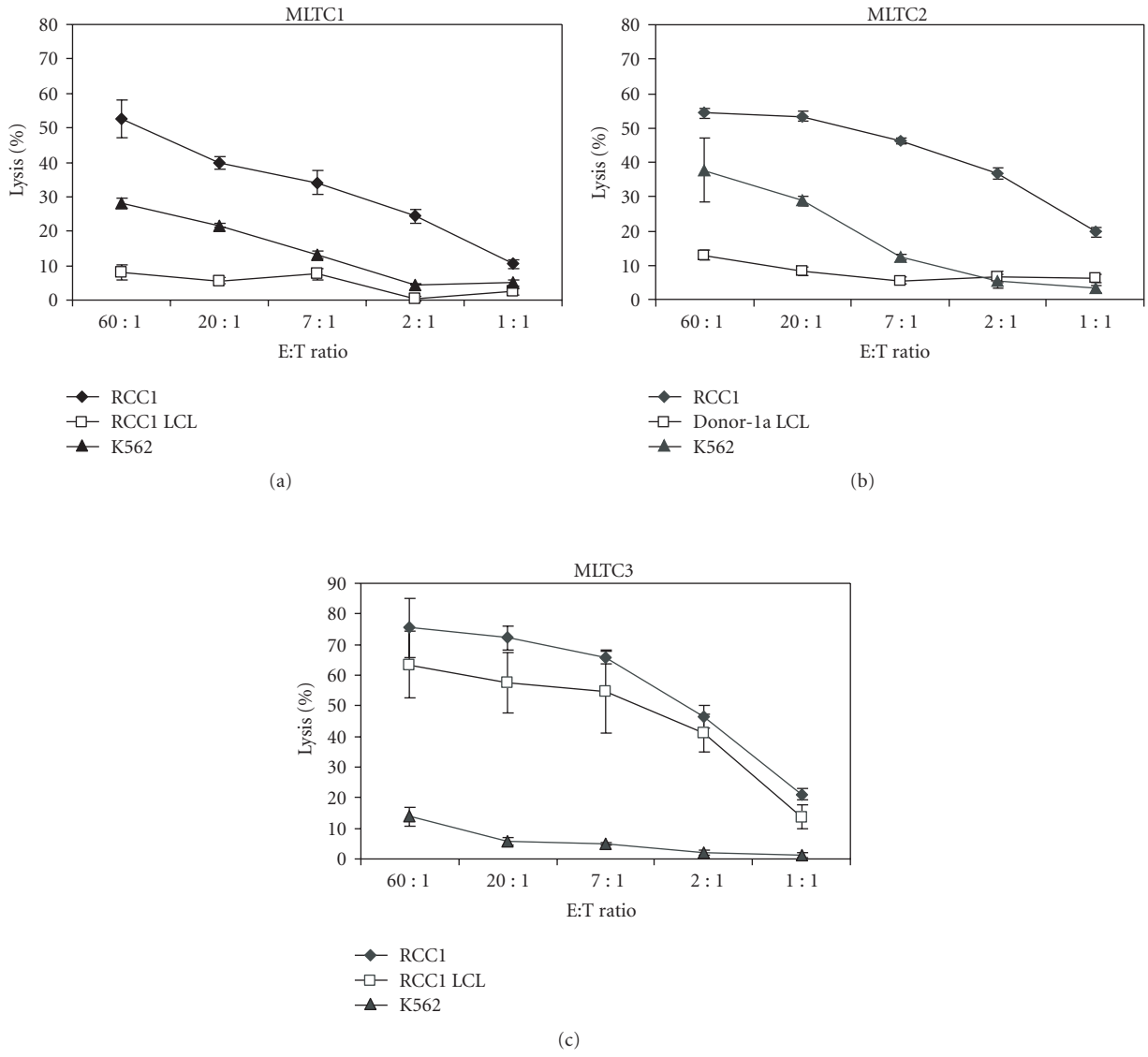
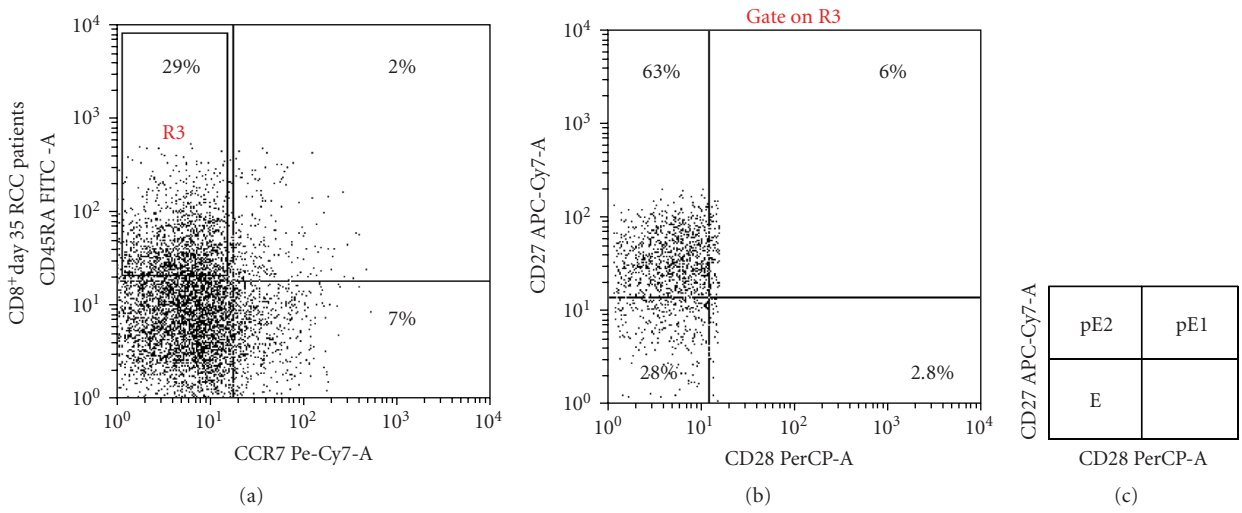



Figure 3: Cytotoxicity assay of RCC1-specific CTL at day 35 of the MLTC. Specific lysis was determined in a 4-hour $^{51}$Cr-release assay using CD8$^+$ T cells collected from d 35 MLTC established from patient RCC1 (a) or HLA-matched normal donors (the patient's sibling (b) and an unrelated individual (c). T cells were used at varying effector-to-target (E:T) cell ratios against target cells including RCC1, K562, and RCC1-LCL. Data are presented as percentages of specific lysis ± SD.

Table 2: Flow cytometry analysis of CD45RA/CCR7 expression on CD8$^+$ T cells at day 0 and after 35 days of MLTC. Differential expression of the CD45RA, CCR7 cell surface molecules was analyzed on CD8$^+$ T cells at day 0 and after extended MLTC stimulation. CD8$^+$T cells were separated into four subsets ($T_N$, $T_{CM}$,$T_{EM}$,$T_{EMRA}$) based on differential expression of the CD45RA and CCR7 markers. Results are the mean (+/− SD) of values obtained from 5 RCC patients and their corresponding HLA-matched normal donors.

| | CD8$^+$ Naive CD45RA$^+$CCR7$^+$ | | $T_{CM}$ CD45RA$^-$CCR7$^+$ | | $T_{EM}$ CD45RA$^-$CCR7$^-$ | | $T_{EMRA}$ CD45RA$^+$CCR7$^-$ | |
|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 35 | Day 0 | Day 35 | Day 0 | Day 35 | Day 0 | Day 35 |
| RCC patients | 30.6 ± 4.0 | 4.4 ± 1.6 | 8.2 ± 2.3 | 11 ± 2.8 | 14 ± 3.8 | 52.6 ± 6.7 | 46 ± 5.3 | 30.6 ± 4.2 |
| Healthy donors | 49 ± 3.1 | 3.1 ± 1.6 | 10.8 ± 5.3 | 59 ± 4.6 | 20.2 ± 3.9 | 32.8 ± 7.2 | 17.4 ± 3.6 | 4.4 ± 1.8 |
| *P* value | .021 | .139 | .019 | .008 | .034 | .003 | .0017 | .0006 |




| | pE1 CD27+ CD28+ | | pE2 CD27+ CD28− | | E CD27− CD28− | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 35 | Day 0 | Day 35 | Day 0 | Day 35 |
| RCC patients | 20 ± 2.3 | 13 ± 4 | 19 ± 3.8 | 62 ± 4.1 | 61 ± 5.3 | 25 ± 2.5 |
| *P* value | .018 | | .001 | | .005 | |

(d)

FIGURE 4: Characterization of the $T_{EMRA}$ population in RCC patients. Based on CD28/CD27 expression, $CD8^+$T cells were separated into three distinct populations: pE1 ($RA^+CCR7^-27^+28^+$), pE2 ($RA^+CCR7^-27^+28^-$), and E ($RA^+CCR7^-27^-28$- ). Results are depicted for patients RCC2 but are representative of all 5 RCC patients evaluated.

$CD8^+$ $T_{EM}$ (and predominantly $T_{EMRA}$) from RCC patients versus normal donors. Given this difference, we further characterized the $T_{EMRA}$ subpopulation of $CD8^+$ T cells generated from RCC patients versus normal donors with regard to comparative levels of expression of the CD27 and CD28 markers (Figure 4). In accordance with the model proposed by Rufer et al. [10], we identified three functionally distinct $CD8^+$ $T_{EMRA}$ ($RA^+CCR7^-$) subsets in RCC patients: pE1 ($27^+28^+$), and pE2 ($27^+28^-$), and E ($27^-28^-$) that have been defined "early", "intermediate," and "late" phenotypes, respectively, based on a proposed linear pathway of differentiation.

Our data suggest that activated $CD8^+$ T cells isolated from RCC patients were more likely to be in a state of intermediate differentiation (pE2) when compared to normal donor $CD8^+$ T cells. Indeed, we noted a profound increase in T cell expression of CD27 after MLTC stimulation (from (T0) 19% ± 3.8 to (T35) 62% ± 4.1). Given previous reports that the level of CD27 expressed by $CD8^+$ T cells correlates inversely with the ability of these cells to mediate cytotoxic functions [27], our results intimate that the reduced cytotoxic activity observed for $CD8^+$ T lymphocytes in RCC patients may be due to their lack of terminal (functional) differentiation.

*3.3. Cell Cycle Dysfunction in Activated $CD8^+$ T Cells from RCC Patients.* To further investigate the relationship between cell cycling and T cell differentiation status, we evaluated the expression pattern of cell cycle proteins involved in the G1/S transition in $CD8^+$ T cells isolated from RCC patients vs. normal donors on day 35 of MLTC. It has been well established that initiation of T lymphocytes-mediated immune responses involves two cellular processes: entry into the cell cycle (G0 → G1) for clonal proliferation and coordinated changes in surface and secreted molecules that mediate effector functions, associated with the differentiation of T lymphocytes into an effector or memory subpopulations. As shown in Figure 5, activated patient $CD8^+$ T cells were mainly present in the G0/G1 phase (85.7 ± 2.08 vs. 63.3 ± 5.5) and less in the S phase (1.41 ± 0.18 vs. 12 ± 4.5) when compared with $CD8^+$ T cells generated in normal donor MLTC. A statistically significant negative correlation was noted between phases G1 and S ($r = -0.95$, $P < .05$) in the patients' T cells, suggesting that in RCC patients there was an arrest at this cell cycle transition point (Figure 6).

*3.4. Association between Cell Division and Tumor Responsiveness in Activated RCC $CD8^+$ T Cells.* To better understand

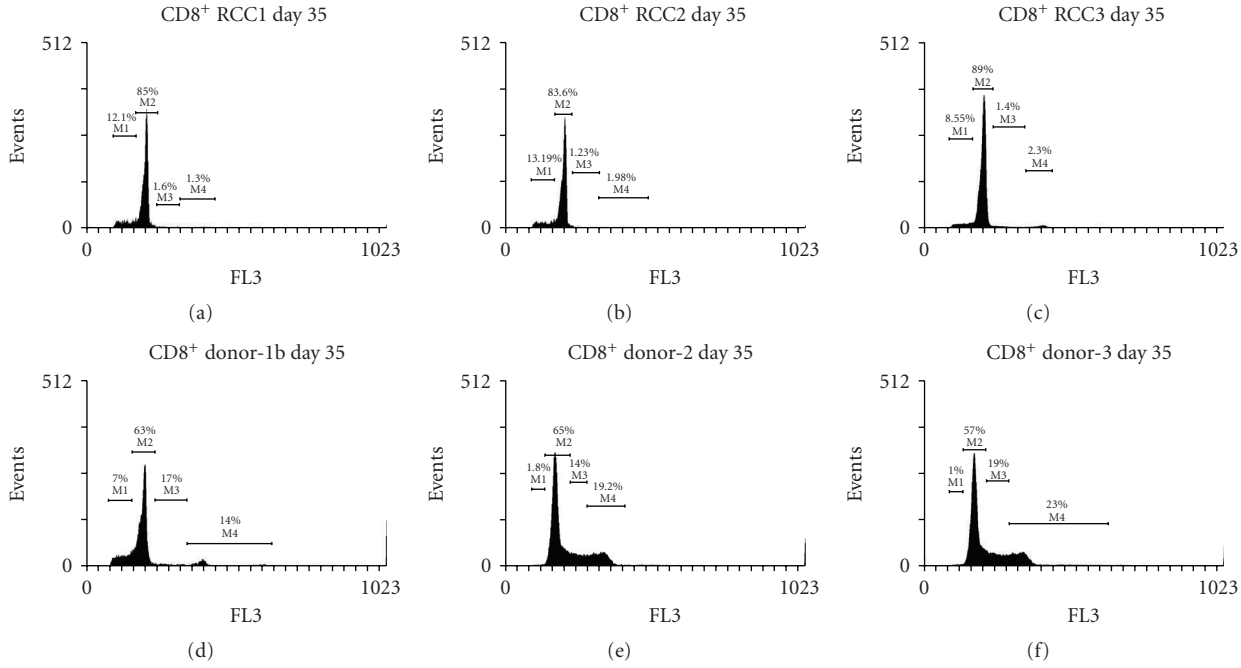



Figure 5: Cell cycle analysis of responder-CD8$^+$ T cells after MLTC. Cellular DNA content was analyzed in three RCC patients and their HLA-matched normal donors by flow cytometry, with data analyzed on WinMDI version 2.8 software.

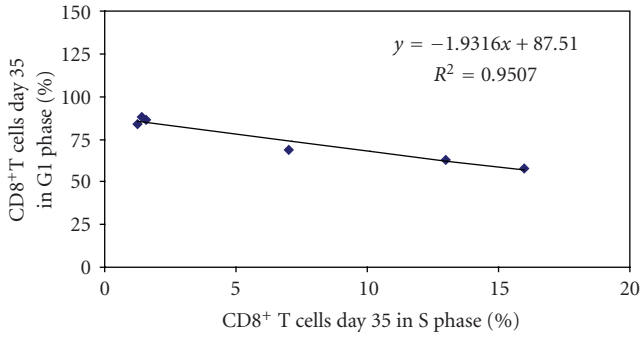



Figure 6: Alterations in the cell cycle distribution of patient versus norma donor anti-tumor CD8$^+$ T cells. A statistically significant, negative correlation was observed between G1 and S percentages in 3 RCC patients.

the association between T cell cycling and anti-tumor tumor responsiveness, we assessed day 0 and day 35 MLTC CD8$^+$ T cells for their expression of $p27^{KIP1}$ and STAT5/6, proteins known to be required for normal T cell proliferation [15, 28]. IL2-mediated downregulation of $p27^{KIP1}$ is crucial for the normal progression of CD8$^+$ T cells from the G1 phase to the S phase of the cell cycle. Activation of the JAK3/STAT5/6 pathway is required for $p27^{KIP1}$ downregulation, hyperphosphorylation of RB, and release of active E2F in T cells. When we analyzed STAT5 expression (Figure 7(a)), we found that IL-2-induced STAT5 phosphorylation was readily detectable in normal T cells (64.5 ± 5.3 /HPF and 71 ± 6.1/HPF resp.) but resulted markedly reduced in RCC patients (22.1 ± 3.3/HPF, $P < .001$). Interestingly, as displayed in Figure 7(b), we observed that STAT6 expression decreased in patients (40.2 ± 4.6/HPF), but not normal donors (57.3 ± 6.8/HPF and 60.8 ± 1.7/HPF resp., $P < .0032$) MLTC CD8$^+$ T cells.

This suggests that, despite the presence of high doses of rhIL-2, the inability of patient CD8$^+$ T cells to proliferate in MLTC likely results from defects in the JAK3/STAT5/6 signaling pathway. To test this hypothesis, we evaluated $p27^{KIP1}$ expression status in MLTC CD8$^+$ T cells developed from RCC patients vs. normal donors. We observed that T cell expression of $p27^{KIP1}$ was increased in T cells from patients (17.5 ± 3.2/HPF) vs. normal, healthy donors (8 ± 1.55/HPF and 10.2 ± 0.37/HPF, $P = .0046$ resp.) (Figure 7(b)).

These data suggest that the integrity of the JAK3/STAT5/6 pathway is likely linked to T cell division and that activated T cells that fail to divide upon appropriate stimulation present with operational defects in this pathway.

In order to confirm that G1/S regulatory defects were present in MLTC CD8$^+$ T cells developed from RCC patients, we next investigated these cells for expression of two key factors in the control of the $G_1$–S-phase transition, E2F4 and Id2, which might confer a proliferative advantage to tumor cells in vivo [21, 29]. As shown in Figure 8, E2F4 expression was reduced in MLTC CD8$^+$ T cells from patients (11.2 ± 3.2/HPF) vs. normal donors (14.71 ± 1.0.28/HPF and 16.6 ± 2.8/HPF, $P = .0018$ resp.). We also found that CD8$^+$ T cells from patients expressed lower levels of Id2 vs. normal donor T cells (28.7 ± 4.3/HPF vs. 56.9 ± 8.4/HPF and 34.8 ± 6.5/HPF, $P = .037$, resp.), confirming an arrest in the G0 cell cycle phase among patient T cells (Figure 9).

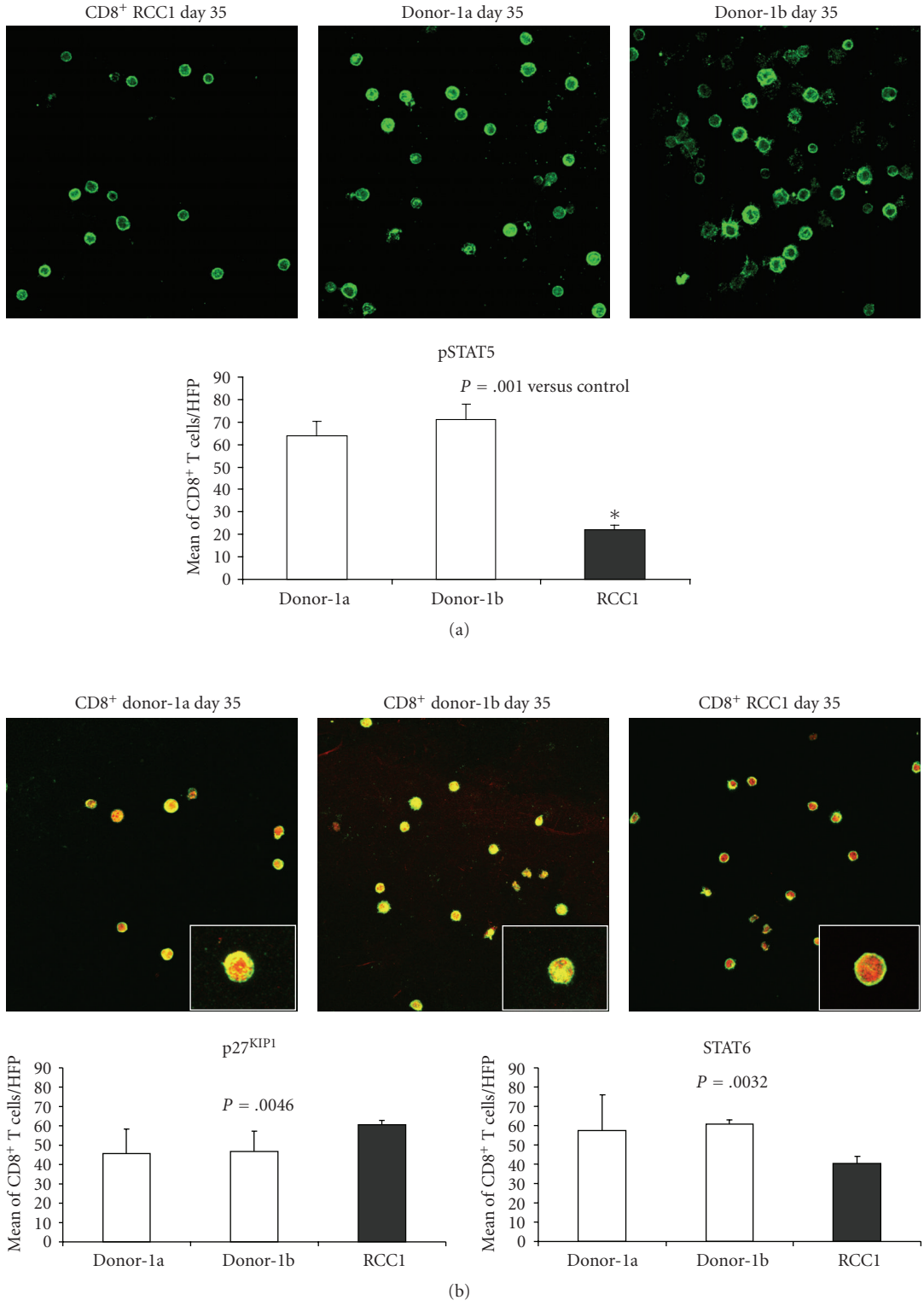



Figure 7: Altered expression of pSTAT5, pSTAT6, and $p27^{KIP1}$ in RCC patient $CD8^+$ T cells. (a) Distribution of pSTAT5 evaluated by confocal microscopy on $CD8^+$ T cells of RCC patients and their HLA-matched normal donors, using anti-STAT5 (green) antibodies. (b) The distribution of p-$p27^{KIP1}$ and pSTAT6 were evaluated by confocal microscopy on $CD8^+$ T cells of RCC patients and their HLA-matched normal donors, using anti-p-$p27^{KIP1}$ (red) anti-STAT6 (green) antibodies. $p27^{KIP1}$/STAT6 interaction results in a yellow staining in the merge. Quantitative analysis of high-power fields (HPFs) in (a) and (b) was carried out by confocal microscopy software (Leica, TCS-SP2). The results were expressed as mean values ± SD. A $P$-value < .05 was considered statistically significant. Data are from 1 of 5 experiments that all gave similar results.

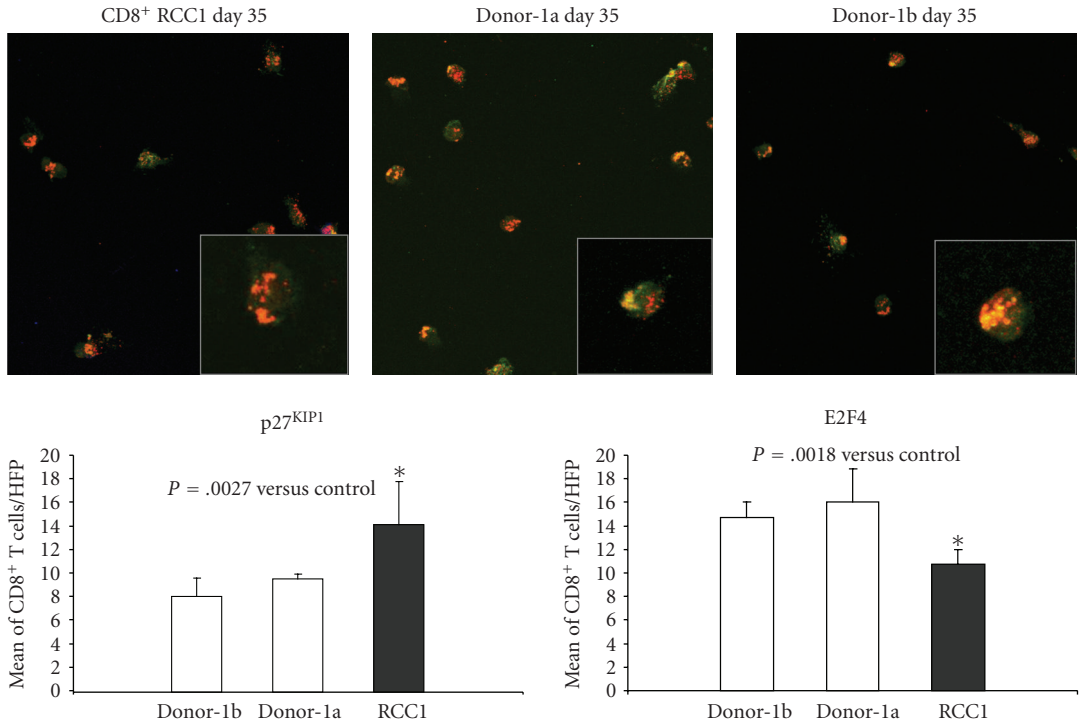



FIGURE 8: Altered expression of E2F4 in RCC patient $CD8^+$ T cells. $CD8^+$ T cells were surface-labeled with anti-p-$p27^{KIP1}$ (red) and anti-E2F4 (green) mAbs and the performance of confocal microscopy analysis. Quantitative analysis of HPF was carried out by confocal microscopy software (Leica, TCS-SP2; Student's $t$-test, $P < .05$). Data are from 1 of 5 experiments, that all gave comparable results.

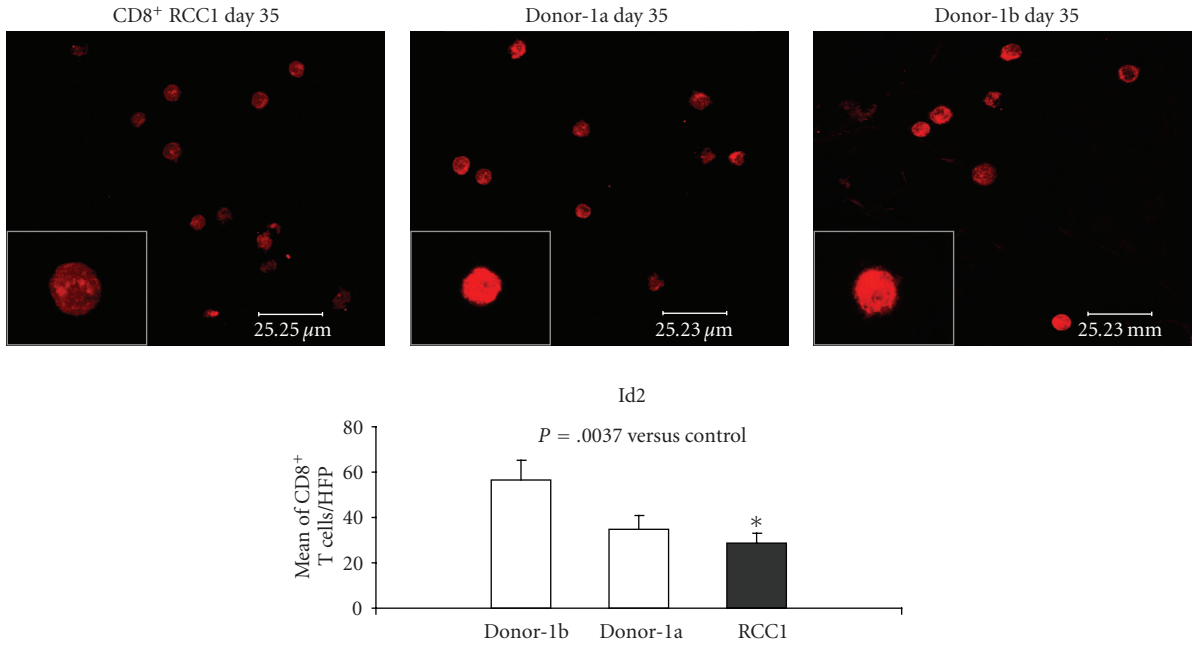



FIGURE 9: Altered expression of Id2 in RCC patient $CD8^+$ T cells. $CD8^+$ T cells were surface-labeled with anti-Id2 (red) mAb and the performance of confocal microscopy analysis. Quantitative analysis of HPF was carried out by confocal microscopy software (Leica, TCS-SP2; Student's $t$-test, $P < .05$). Data are from 1 of 5 experiments that all gave similar results.

## 4. Conclusions

The experimental approaches used in our study provide valuable tools to assist in the elucidation of cellular mechanisms underlying the altered activation state of T cells in patients with cancer. We used RCC as a model cancer to explore this question, as it is well documented that patients with active, disseminated disease are typically characterized by "functionally inappropriate", dysfunctional, or proapoptotic immune cells that are impotent in mediating clinically beneficial outcomes in vivo [30, 31].

We confirmed that it is possible to generate and expand tumor-reactive $CD8^+$ T cells in vitro from the peripheral blood of RCC patients as well as normal donors [32]. Normal donor RCC-specific $CD8^+$ T cells appeared to recognize a diverse array of antigens and displayed superior anti-tumor activity when compared with patient-derived $CD8^+$ T cells [23]. Normal donor $CD8^+$ T cells developed in MLTC cultures also demonstrated significant reactivity against K562 target cells (in contrast to patient MLTC-derived $CD8^+$ T cells), supporting the conclusion that these allogenic MLTC-$CD8^+$ T cells were capable of mediating HLA class I-restricted as well as HLA-unrestricted cytotoxicity.

A corollary analysis of the phenotypes of allogenic and autologous MLTC-$CD8^+$ T cells predicated on a recently proposed pathway of T-cell lineage differentiation [6] revealed that the majority of freshly isolated $CD8^+$ T cells from RCC patients display a memory phenotype characterized by very low frequencies of $CD45RA^-$, $CCR7^+T_{CM}$ cells, high frequencies of $CD45RA^-$, $CCR7^-T_{EM}$, and terminally differentiated $CD45RA^+$, $CCR7^-T_{EMRA}$. Interestingly, we noted that after MLTC expansion, patient $CD8^+$T cells are mainly composed of antigen-experienced $T_{EM}$ and $T_{EMRA}$ and are deficient in $T_{CM}$. In sharp contrast, MLTC-$CD8^+$ T cells developed from normal donors contain high frequencies of $T_{CM}$ population and far lower percentages of both $T_{EM}$ and $T_{EMRA}$ cells. These data are particularly important as tumor-reactive $CD8^+$ $T_{CM}$ have been previously suggested to provide superior anti-tumor protection vs. $T_{EM}$ [7, 26]. Furthermore, it has been shown that $T_{CM}$ exhibit greater expansion potential in concert with enhanced resistance to apoptosis, whereas $T_{EM}/T_{EMRA}$ have a skewed TCR repertoire and proliferate poorly in response to cognate antigen [9]. Importantly, our study revealed that patients $CD8^+$ $T_{EMRA}$ expanded in MLTC are more likely to exhibit an intermediate state of differentiation (i.e., $CD27^+$, $CD28^-$), capable of mediating only partial effector functions and proliferative capacity. Normally, $CD8^+$ T cell differentiation correlates with a progressive loss in expression of the CCR7, CD28, and CD27 markers and increased cytolytic capacity [33]. Hence, our results suggest that although $CD8^+$ T cells in RCC patients are capable of reacting against tumor cells, their effector functions and longevity are suboptimal due to the absence of $T_{CM}$ cells and their incomplete state of differentiation. These findings have important implications for cancer immunotherapy approaches [34], including DC-based vaccines, and in this regard recently we have demonstrated that dendritic cells (DCs) generated using IFN-$\alpha$ and loaded with tumor antigens are capable of "revitalizing" or expanding a therapeutically preferred cohort of anti-tumor $CD8^+$ T cells ($T_{CM}$) from the PBMC of RCC patients [35].

Other groups have investigated the profiles of human $CD8^+$ $T_{CM}, T_{EM}, T_{EMRA}$ using gene expression microarrays, showing that $T_{CM}$ are characterized by a higher level of basal and cytokine-induced STAT5/6 phosphorylation, reflecting their superior capacity for self-renewal vs. effector memory cells, and in particular, $T_{EMRA}$ [11]. Interestingly, we observed a down regulation of both STAT5/6 molecules in patient $CD8^+$ T cells, in association with their inferior proliferative capacity, which is consistent with their known dysfunction in vivo. These data suggest a role for STAT pathway in the post activation survival of T cells and provide new insights into the nature of a late (cell cycle) proliferation block in the T cell compartment as a result of STAT6 deficiency. Indeed, in $CD8^+$ T cells analyzed from RCC patients, we observed a down regulation in JAK3/STAT5/6 expression and corollary defects in cell cycle (G1/S) progression and cell proliferation. These results establish the crucial role of the JAK3/STAT5/6 signaling pathway in normal $CD8^+$ T cell proliferation/differentiation, consistent with a recent report by Jin et al. [36].

T cell activation and proliferation require several appropriate signals to complete transitions through the cell cycle, from the G1 to the S phase. Interestingly, activated $CD8^+$ T cells from RCC patients, which exhibit reduced proliferative responses against their own tumors, express increased levels of $p27^{KIP1}$, which is known to lead to G0 phase cell cycle arrest and a slowed/arrested progression to G1/S transition. These results are consistent with those reported by Zhang et al. [28] where $p27^{KIP1}$ was found to inhibit antigen-driven $T_{EM}$ expansion in vivo. Our report adds to a very limited literature in identifying a G1/S cell cycle regulatory defect in T cells isolated from cancer patients, and it provides a better understanding of mechanism(s) underlying tumor-induced immune deviation that occurs during cancer progression.

One of the key events during the G1 phase is activation of the transcription factor E2F4 [37]. E2F4 is a crucial switch in the control of homeostasis and tumorigenesis [21] and, together with Id2, serves as a critical regulator of cell cycle progression. Specifically, Id2 has been suggested to antagonize the regulation of the cell cycle by tumor suppressor proteins [38]. Our analyses revealed a dramatic reduction in E2F4 and Id2 expression in $CD8^+$T cells from RCC patients vs. healthy donors after MLTC. These data provide evidence for the importance of Id2 in enforcing "normal" cell cycle progression in tumor-reactive T cells harvested from RCC patients.

In conclusion, we have shown for the first time that RCC patients exhibit a functionally suboptimal anti-tumor $CD8^+$ T cell repertoire based on defects in (the JAK3/STAT5/6) intracellular signaling pathway that leads to T cell arrest in the G0/G1 phase of the cell cycle and prevention of their terminal differentiation. Furthermore, our data support the conclusion that $CD8^+$ T cell overexpression of p27KIP1 and/or loss of Id2 expression are characteristics of dysfunctional T cells in RCC patients, making these potential therapeutic targets in the setting of patients with RCC.

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