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High DGK-α and Disabled MAPK Pathways Cause Dysfunction of Human Tumor-Infiltrating CD8+ T Cells That Is Reversible by Pharmacologic Intervention

Petra U. Prinz,* Anna N. Mendler,* Ilias Masouris,* Leopold Durner,† Ralph Oberneder,† and Elfriede Noessner*

CD8+ tumor-infiltrating T cells (CD8-TILs) are found in many types of tumors including human renal cell carcinoma. However, tumor rejection rarely occurs, suggesting limited functional activity in the tumor microenvironment. In this study, we document that CD8-TILs are unresponsive to CD3 stimulation, showing neither lytic activity, nor lytic granule exocytosis, nor IFN-γ production. Mechanistically, no deficits in TCR proximal signaling molecules (lymphocyte-specific protein tyrosine kinase, phospholipase Cγ) were identified. In contrast, distal TCR signaling was suppressed, as T cells of TILs showed strongly reduced steady-state phosphorylation of the MAPK ERK and were unable to increase phosphorylation of ERK and JNK as well as AKT and AKT client proteins (IκB, GSK3) after stimulation. These deficits were tumor-specific as they were not observed in CD8+ T cells infiltrating non-tumor kidney areas (CD8+ non-tumor kidney-infiltrating lymphocytes; CD8-NILs). Diacylglycerol kinase-α (DGK-α) was more highly expressed in CD8-TILs compared with that in CD8-NILs, and its inhibition improved ERK phosphorylation and lytic granule exocytosis. Cultivation of TILs in low-dose IL-2 reduced DGK-α protein levels, increased steady-state phosphorylation of ERK, improved stimulation-induced phosphorylation of ERK and AKT, and allowed more CD8-TILs to degranulate and to produce IFN-γ. Additionally, the protein level of the AKT client molecule p27kip, an inhibitory cell cycle protein, was reduced, whereas cyclin E, which promotes G1–S phase transition, was increased. These results indicate that the tumor-infiltrated deficits of TILs are reversible. DGK-α inhibition and provision of IL-2 signals could be strategies to recruit the natural CD8+ T cells to the anti-tumor response and may help prevent inactivation of adoptively transferred T cells thereby improving therapeutic efficacy. *The Journal of Immunology, 2012, 188: 5990–6000.

CD8+ T lymphocytes are potent cytotoxic effector cells that have the capacity to eliminate cells displaying foreign peptide–MHC (pMHC) complexes on their cell surface. Most tumor cells express pMHCs that can trigger lytic effector function leading to their elimination in vitro. In vivo, however, tumors develop, and rejection of established tumors rarely occurs. Among human tumors, renal cell carcinoma (RCC) displays clinical and experimental features indicating that it can be targeted by immune effector cells (1). Clinical evidence includes the observations of spontaneous remission and response to immunotherapy with long-lasting regression in some patients (2, 3). Histologically, RCC has a large immune cell infiltrate of CD8+ T lymphocytes with some expressing TCRs that enable the T cells to recognize and kill tumor cells in vitro (4–7). Despite the local presence of putative tumor-reactive cytotoxic T cells, RCCs are generally not rejected indicating deficits in the cytotoxic response at the tumor site.

Various evasion mechanisms have been acknowledged ranging from ignorance to active suppression (8–10). The most common ones observed in many tumors, such as lack of infiltration with cytotoxic lymphocytes or loss of MHC class I, do not explain the immune escape of human RCC satisfactorily: RCC tissues are generally strongly infiltrated by various types of immune cells that are considered to be associated with an effective immune response, notably dendritic cells, NK cells, and CD8+ T lymphocytes expressing MHC class I-restricted tumor-reactive TCRs (5–7, 11, 12). Moreover, renal tumors express MHC class I and class II molecules on the cell surface, and T cell epitopes are eluted from tumor tissues (13, 14), indicating good Ag processing and presentation in situ. However, earlier studies analyzing NK cells (11) and T cells of RCC showed that infiltrating cytotoxic lymphocytes are functionally inactive but are able to gain function when cultured ex vivo (4, 5, 15), suggesting that inhibitory mechanisms act at the tumor site.

Inhibition of CTL function by the tumor milieu has been described in mouse models (16–19). However, little information is available regarding underlying mechanisms. The deficits and the reasons for T cell inactivity can be manifold and may vary depending on the tumor type. A detailed understanding of the mechanisms that inhibit T cell reactivity at the tumor site is essential for a rational approach to improve all types of immunotherapy concepts that rely on the activity of cytotoxic lymphocytes, including vaccination, adoptive T cell therapy, or Ab intervention directed at the rescued exhausted tumor-reactive T lymphocytes.

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; CI, confidence interval; DAG, diacylglycerol; DGK, diacylglycerol kinase; GMP-17, granule membrane protein of 17 kDa; HS, human serum; LCK, lymphocyte-specific protein tyrosine kinase; LCMV, lymphocytic choriomeningitis virus; MFI, median fluorescence intensity; NIL, non-tumor kidney-infiltrating lymphocyte; NKC, non-tumor kidney cortices; PA, phosphatidic acid; PLCγ, phospholipase Cγ; PMA/I, PMA/ionomycin; pMHC, peptide–MHC; PPV, positive predictive value; RCC, renal cell carcinoma; TIL, tumor-infiltrating lymphocyte.

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The functional quality of T effector lymphocytes, including lytic activity and cytokine secretion, is governed by a signaling cascade initiated after recognition of pMHC by the anticipatory TCR (20). Upon TCR ligation, lymphocyte-specific protein tyrosine kinase (LCK) phosphorylates CD3ζ recruiting ZAP70 followed by activation of linker for activation of T cells and of phospholipase Cγ (PLCγ). Subsequently, different distal signaling pathways are initiated whereby the activation of ERKs (p42/p44) is critical for the initiation of lytic granule exocytosis (20–22). Activation of the PI3K–AKT pathway supports effector cell function, survival, and cell cycle (23).

In this study, we document that CD8+ T cells of human RCC display specific deficits in the distal TCR signaling cascade related to the suppression of IFN-γ production and lytic granule exocytosis, which is a prerequisite step for lytic function. Notably, signaling alterations were reversible, and functional activity could be restored by pharmacologic inhibition of diacylglycerol kinase-α (DGK-α) or ex vivo culture with IL-2. The identification of key molecules related to functional inhibition and the recognition that deficits are reversible indicate that the anti-tumor activity of CD8+ T cells in the tumor environment can be enhanced with appropriate intervention strategies.

Materials and Methods

Patients and healthy blood donors

Tissue samples of histologically diagnosed clear cell renal cell carcinoma (n = 24) and of non-tumor kidney cortices (NKC, n = 14) of the tumor-bearing kidney were obtained from untreated patients who underwent surgery at the Urological Department of the Ludwig-Maximilians-University Munich or the Urologische Klinik Dr. Castrignius Planegg (Munich, Germany). Detailed patient characteristics are shown in Table I. Spleen tissue was obtained from a patient who underwent surgery for gastic cancer (Experimental Surgery Department, Ludwig-Maximilians-University Munich). PBMCs or PBLs (CD14-depleted PBMCs) were obtained from healthy donors (n = 20). Tissue and blood collection were approved by the ethics committee, and patients/donors consented to the donation.

Abs and cell lines used for stimulation of T cell function

Abs are listed in Table II. P815 and M-T301 cells were cultured in RPMI 1640 supplemented with 10% FCS, 1 mM l-glutamine, 1 mM sodium pyruvate, and 1 mM nonessential amino acids (all Invitrogen). M-T301 is a murine hybridoma cell line expressing surface-bound Ig (isotype IgG1; kindly provided by P.E. Rieber, Institute of Immunology, Technical University of Dresden, Dresden, Germany) (24) and was used in redirected cytotoxicity assays. P815 is a mouse mastocytoma cell line with surface Fe-receptor. Before use in T cell stimulation, P815 cells were incubated with anti-CD3 Ab (OKT3; 10 μg/1 × 106 cells; in-house gift from E. Kremmer) for 30 min at room temperature, then washed and used in the CD107-mobilization assay.

Preparation of tissue suspensions and in vitro cultivation

Tissue suspensions of tumors and of non-tumor kidney cortices were prepared from fresh postoperative material. Briefly, tissues were mechanically minced into small pieces and washed extensively with HBSS (Invitrogen) to remove contaminating blood lymphocytes. Intratumoral leukocytes were recovered from the tissue after two enzymatic digestions using collagenase IA (0.5 mg/ml) and DNase I type IV (0.19 mg/ml) (all Sigma-Aldrich) with an intermittent step of 5 mmol/l EDTA (Invitrogen) in HBSS (without Ca2+ and Mg2+). All incubations were performed for 30 min at room temperature. Suspensions were passed through a 40-μm filter, frozen, and stored at −80°C until use. For analysis, tissue suspensions were thawed and analyzed immediately without being cultured. Where indicated, cell suspensions were cultured for 48 h at 2 × 106 lymphocytes per well in a 24-well plate in AIM-V (Invitrogen) supplemented with 10% human serum (HS; in-house production) and low-dose IL-2 (50 U/ml; Cercnonova).

Ex vivo redirected cell-mediated lysis

Tissue suspensions of RCCs or spleen and PBLs were used as effector cells directly after thawing without exposure to culture medium or cytokines. Because tissue suspensions varied in their content of CD3+ lymphocytes, the results from flow cytometry were used to calculate the ratio of CD3+ cells to target cells (M-T301) ranging from 20:1 to 1.25:1. 51Cr-labeled M-T301 cells were used as targets at a constant cell number of 2000 cells per well in 96-well V-bottom plates. Experiments were performed with duplicate measurements of four-step titrations of effector cells. In parallel wells, target cells were incubated without T cells to determine the spontaneous release of 51Cr. Supernatants were harvested after 7 h and transferred to counting plates (PerkinElmer) for cpm measurements. The maximum cpm was determined by directly transferring labeled target cells to the counting for cpm measurements. The percent of specific lysis was calculated as follows: percent specific lysis = (experimental cpm – spontaneous cpm) / (maximal cpm – spontaneous cpm) × 100.

Rationale for using CD3 ligation for TCR stimulation instead of natural pMHC ligands

The natural ligands triggering T cell responses are pMHC complexes (pMHC ligands) presented on cell surfaces (i.e., tumor cells). In the system studied here, biological specifics of the human material preclude stimulation via natural pMHCs. But moreover, stimulation via CD3, which is closest to the physiologic pMHC stimulation, is advantageous if the T cell intrinsic response competence is to be analyzed. Concerning the biological specifics, tumor-infiltrating lymphocytes (TILs) from tissues of different patients were analyzed. Patients were not HLA typed, and each patient’s tumor-associated antigenic repertoire is unknown. Therefore, the TILs analyzed will exhibit different HLA restrictions and will have different Ag specificity. In the specific situation of RCC, tumor-associated Ags shared among a high percentage of patients are not known (7), precluding the use of one selected pMHC for stimulation if TILs of a larger patient group were to be analyzed. Primary tumor cell cultures corresponding to the respective patient’s TILs are not an appropriate source for stimulation because their MHC and/or Ag presentation may be variable (including loss thereof), and inhibitory ligands may be present (19). Thus, primary tumor cells may provide insufficient T cell stimulation signals, thus a T cell may not respond, even if it has no intrinsic response deficits. Using the same anti-CD3-coated cells or beads for stimulation eliminates variations in signal intensities from different pMHC ligands and/or different cellular backgrounds and excludes the potential presence of inhibitory ligands thereby permitting the T cell intrinsic responsiveness to be addressed. Yet, the signal strength provided by CD3 ligation could be stronger than that of natural pMHC ligands overriding potentially present deficits in TILs. Thus, if a T cell response is observed after CD3 ligation, deficits in response to pMHC could nevertheless exist, and in situ T cell response could still be inhibited by inhibitory receptor. However, if no T cell response is observed despite strong CD3 stimulation in the absence of inhibitory signals, it strongly suggests that T cells have an intrinsic deficit in their response capacity.
Data acquisition was done with LSR II (BD Pharmingen), and data were analyzed using FlowJo (Tree Star). The lymphocyte population within the TILs was selected based on forward scatter/side scatter characteristics using PBMCs of healthy individuals as reference. Selection of lymphocytes was followed by exclusion of 7-AAD<sup>+</sup> (dead) cells and duplets. Then, CD45-expressing cells were selected and CD14<sup>+</sup> myeloid cells and CD19<sup>+</sup> B cells excluded (Supplemental Fig. 1A). CD8<sup>+</sup> T cells were selected by gating on live CD45<sup>+</sup>CD14<sup>-</sup>CD19<sup>-</sup> lymphocytes. The percentage of perforin<sup>+</sup> and granzyme B<sup>+</sup> or CD107<sup>+</sup> cells within the gated CD8<sup>+</sup> lymphocytes was determined using an internal population negative for the analyzed marker as reference or isotype controls, respectively. In case of surface expression, the percentage or MFI was referenced to the gated CD8<sup>+</sup> T cells, thus eliminating skewing due to different absolute frequencies in the different tissue samples.

**Phosphoprotein multiplex**

CD8<sup>+</sup> cells of TILs and PBMCs were positively selected using magnetic bead separation and the MACS System (Miltenyi) following the manufacturer’s protocol. After stimulation (8 min) with PMA/I (50/500 ng/ml) and PLCy (5 min) or with PMA/I (50/500 ng/ml; Sigma-Aldrich) to detect phosphorylation of ERK and AKT (8 min) or left unstimulated for control. Where indicated, TILs were preincubated with DGK-<i>a</i> inhibitor I (50 μM; Sigma-Aldrich) or left untreated for 3 h and subsequently stimulated with PMA/I (50/500 ng/ml) in the presence or absence of DGK-<i>a</i> inhibitor I. Reaction was stopped by adding an equal volume of Cytofix buffer (BD Biosciences) for 15 min at 37˚C. After permeabilization (BD Bioscience Permabuffer III, 30 min, 4˚C), cells were stained with Abs against surface molecules (anti-CD45-PE–Cy7, anti-CD3-Pacific blue) and phosphorylated intracellular signaling proteins. T cells among cell suspensions were selected by gating on CD45<sup>+</sup>7-AAD<sup>+</sup>CD8<sup>+</sup> T cells. Expression levels of phosphorylated proteins were determined as the MFI of gated T cells.

**Multiparameter immunoﬂuorescence and confocal microscopy**

PBLS from healthy donors were immobilized on poly-L-lysine–coated 8-well slides (Erie Scientific Company), fixed in 100% ice-cold acetone, blocked with 2% BSA in PBS, and then incubated with primary Ab combinations followed by corresponding combinations of secondary species-specific fluorescent-labeled Abs. Secondary Abs showed no cross-reactivity. All Abs were diluted in 12.5% HS in PBS and incubated at room temperature. The following Ab combinations were used: mouse anti-human granule protein molecule of 17 kDa (GMP-17) (TIA-1, IgG1; Immunotech), mouse anti-human perforin (6G9, IgG2b; BD Pharmingen), and rabbit anti-human CD8 (Sigma-Aldrich) followed by secondary Abs anti-mouse IgG2b–A488, anti-mouse IgG1–A568, and anti-rabbit Cy5 (Invitrogen). After fixation (4% paraformaldehyde) and nuclear staining with DAPI (Sigma-Aldrich), slides were mounted with Vectashield (Vector Laboratories). Fluorescence images were captured with a laser scanning microscope (TCS SP AOBH; Leica Microsystems, Wetzlar, Germany) using an HCX PL APO 63×/1.40 oil immersion objective lens, pinhole Airy disk × 1024 pixel image format, four-frame averaging, and zoom 5.1. Sequential recording was applied to avoid fluorescence spillover, and z-stacks were scanned to detect fluorescence across different planes of the visual field. Image editing and analysis was applied using Leica LAS AF software.

**Statistics**

Nonparametric statistical methods were used (Prism Windows 5.01; GraphPad, La Jolla, CA). For comparison of two unmatched groups, two-sided Mann–Whitney U test was applied. Comparison of one parameter between more than two groups was performed with Kruskal–Wallis test followed by Dunn’s post hoc test. The p values are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001.

The prediction accuracy of GMP-17 compared with CD107 regarding the identification of perforin<sup>+</sup> cells within the CD3<sup>+</sup> population was evaluated in PBLS by calculating the positive predictive value (PPV) (25) of GMP-17 or CD107 using the equation:

\[
PPV = \frac{\text{sensitivity}(v) \times \text{prevalence}}{\text{sensitivity}(v) \times \text{prevalence} + \text{specificity}(v) \times \text{prevalence}}
\]

where sensitivity is the percentage of GMP-17<sup>+</sup>perforin<sup>+</sup> or CD107<sup>+</sup>perforin<sup>+</sup> cells among CD3<sup>+</sup> cells divided by all perforin<sup>+</sup> cells among CD3<sup>+</sup> cells; specificity is the percentage of GMP-17<sup>+</sup>perforin<sup>+</sup> or CD107<sup>+</sup>perforin<sup>+</sup> cells among CD3<sup>+</sup> cells divided by all perforin<sup>+</sup> cells among all CD3<sup>+</sup> cells; and prevalence is the mean percentage of perforin<sup>+</sup> cells among all CD3<sup>+</sup> cells of all tested BPL samples.

To define the PPV of CD107 or GMP-17 for perforin prediction, PBLS of healthy donors (n = 11 for GMP-17, n = 5 for CD107) were analyzed by intracellular FACS staining using the Ab combination CD3–Pacific blue, CD4–allophycocyanin–eFluor 780, CD8–V500, perforin–FITC with either GMP-17–Cy5 (in-house labeled) or CD107–PE. The percentages of cells positive or negative for respective markers (outlined later) were calculated using FlowJo.

The prevalence of perforin<sup>+</sup> cells among CD3<sup>+</sup> cells was 13.5% (mean of 16 PBLs of different donors). Among PBLs analyzed for GMP-17 (n = 11), on average 14.1% (range, 4–26%) of CD3<sup>+</sup> cells were perforin<sup>+</sup> and 85.9% (range, 74–96%) were perforin<sup>+</sup> ; 12.4% of CD3<sup>+</sup> cells were GMP-17<sup>+</sup>perforin<sup>+</sup> (range, 3–24%) and on average 79.5% (range, 66–91%) were GMP-17<sup>+</sup>perforin<sup>+</sup> . Among PBLs analyzed for CD107 (n = 5), 12.2% (range, 5–20%) of CD3<sup>+</sup> cells were perforin<sup>+</sup> and 87.4% (range, 78–95%) were perforin<sup>+</sup> ; 12.2% were CD107<sup>+</sup>perforin<sup>+</sup> cells (range, 5–20%) and 0.3% were CD107<sup>+</sup>perforin<sup>+</sup> (note: all cells are CD107<sup>+</sup> ; Supplemental Fig. 2C). The PPV was calculated for each PBL donor individually and thereby the mean PPV with 95% confidence interval (CI) was obtained. Using the percentages from above, the mean PPV for GMP-17 was 65.6% (95% CI: 58%; 72%); and the mean PPV for CD107 was 13.5% (95% CI: 13.5%; 13.5%). Thus, in a PBL population, the frequency of GMP-17 predicts the frequency of perforin<sup>+</sup>CD3<sup>+</sup> cells with an accuracy of 65.5%, whereas the prediction accuracy of CD107 is 13.5%, which is equal to the prevalence of perforin<sup>+</sup> cells. In conclusion, CD107 has no predictive value for perforin expression whereas GMP-17 does.

**Results**

CD8<sup>+</sup> T lymphocytes from RCC tissue are defective in lytic function and granule exocytosis

T cells from RCC tissue (RCC-TILs) (Table I) were tested for lytic activity directly after isolation from the tumor tissue without being cultured in vitro. The functional response capacity was assessed using a CD3-stimulated redirected cytotoxicity assay that closely mimics the physiologic TCR stimulation. Specific TCR stimulation through natural TCR ligand pMHC complexes cannot be performed, as TILs are derived from different patients, and each patient’s HLA constitution and tumor-associated Ag expression are not known (see Materials and Methods). TILs were found to be only marginally lytic, whereas T lymphocytes isolated from spleen the same way, and tested in parallel, showed good specific lysis (Fig. 1A), excluding the tissue dissociation procedure as cause for the lack of cytotoxicity of RCC-TILs. Remarkably, the cytolytic activity of RCC-TILs was even significantly lower compared with that of PBMCs of healthy donors (p = 0.002) or of RCC patients (data not shown), although PBMCs are largely naive T cells that do not contribute to lytic activity.

To define the effector status of TILs and PBMCs, intracellular flow cytometry and confocal imaging were performed with Abs against CD107a/b (LAMP1/LAMP2), GMP-17, and perforin (Table II) to test for the presence of lytic granules and lytic effector molecules. GMP-17 is a protein that has been localized to the membrane of lytic granules by electron microscopy (26, 27). GMP-
Table I. Patient demographics and tissue characteristics

<table>
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<tr>
<th>Tumor size</th>
<th>No. of Patients (n = 24)</th>
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<tbody>
<tr>
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<tr>
<td>pT2</td>
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<tr>
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<tr>
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<td>21</td>
</tr>
<tr>
<td>pN1</td>
<td>1</td>
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<tr>
<td>pN2</td>
<td>2</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
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<tr>
<td>pM0</td>
<td>20</td>
</tr>
<tr>
<td>pM1</td>
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<td>G2 (moderate)</td>
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<td>G3 (poor)</td>
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<td>Male</td>
<td>7</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
</tr>
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</table>

17 was found to distinguish a CD8⁺ T cell subset in PBMCs that composed on average 34% of CD8⁺ T cells of PBMCs (range, 26–53%) (Fig. 1B–D) and coexpressed perforin to a large extent (median, 53%; range, 44–68%) (Fig. 1C, 1D, Supplemental Fig. 2A). CD4⁺ T cells of PBMCs were negative for GMP-17 (median, 2%; range, 1–5%) (Supplemental Fig. 2B) and perforin. In contrast, intracellular staining with CD107 Abs identified all cells as positive [all leukocytes, including CD4⁺ (Supplemental Fig. 2C) and CD8⁺ T cells, and also tumor cells (data not shown)], which is consistent with CD107 recognizing the common lysosomal markers LAMP1/LAMP2, which are not specific to lytic granules. The presence of GMP-17 strongly predicted coexpression of perforin in T cells of PBMCs (PPV = 65.5%), whereas CD107 had no predictive value (PPV = 13.5%) (see Materials and Methods). Analysis of TILs revealed that nearly all CD8⁺ T cells were GMP-17⁺ thus fitted with lytic granules (Fig. 1B). The frequency of perforin among CD8-TILs was similar to that of CD8-PBMCs (median TILs, 31%; range, 11–54%; median PBMCs, 31%; range, 20–54%), and the frequency of granzyme B was higher in CD8-TILs than in CD8-PBMCs (median TILs, 30%; range, 16–48%; median PBMCs, 14%; range, 3–27%) (Fig. 1E, 1F) Thus, as TILs are nearly all GMP-17⁺, the lytic granules in CD8-TILs are not fully equipped with lytic proteins (Supplemental Fig. 2A). This, however, cannot fully explain why the lytic activity of TILs is lower compared with that of PBMCs, as TILs have yet similar percentages of perforin⁺ and even higher percentages of granzyme B⁺ CD8⁺ T cells than PBMCs.

Normal activation of proximal and intermediate signaling molecules in T cells of TILs from RCC tissues

Activation of the TCR signaling cascade is required to yield cytotoxic and cytokine responses. As T cell numbers from RCC or NKC tissues were limited, indicative events of the TCR signaling cascade and key molecules at signaling branches were selected for the analysis of TILs that were not exposed to culture conditions (20). One of the very early events in the TCR signaling cascade, placed immediately proximal to TCR ligation, is the dephosphorylation of LCK at the inhibitory Y505. Lack of dephosphorylation at Y505 has been described to be associated with the unresponsive state of Ag-specific T cells in cancer patients (29). The steady-state phosphorylation level of LCK at Y505 (Supplemental Fig. 3A) as well as the reduction of phosphorylation after CD3 stimulation in T cells of TILs of RCC (T-TILs) was comparable to that of T cells of PBMCs (T-PBMCs) (Fig. 2A), although both T cell groups showed different functional performance. T cells of NKC (T-NKC) analyzed in parallel were too few to yield reliable values (data not shown). PLCγ is one of the most important intermediate signal transducers, which hydrolyzes phosphatidylinositol-4,5-bisphosphate to yield the second messengers diacylglycerol (DAG) and inositol-4,5-triphosphate that link TCR ligation to downstream MAPKs and protein kinase C. Phosphorylation of PLCγ in T-TILs occurred to the same extent as in T-PBMCs or T-NKC (Fig. 2A, Supplemental Fig. 3B). Thus, TCR proximal signaling and intermediate TCR signal transducers were activated in T-TILs and thus cannot account for the observed deficits in CD3-stimulated T-TIL degranulation. Whether stimulation with specific pMHC complexes would uncover TCR proximal deficits cannot be assessed with the available material due to the lack of knowledge of Ags and the patients’ HLA types.

Deficits in the phosphorylation of TCR distal signaling molecules ERK1/2, JNK, and AKT in T cells of TILs

TCR stimulation activates multiple distal pathways including the MAPK pathways ERK and JNK, as well as NF-κB and NFAT pathways, which are additionally controlled through the protein kinase AKT (20). Phosphoflow analysis was performed with TILs freshly isolated from tissue and not exposed to cell culture conditions. Reduced basal ERK phosphorylation levels in T-TILs compared with those in T-NKCs and T-PBMCs of healthy donors was observed (Fig. 2B, Supplemental Fig. 3C), despite similar ERK protein levels in T-TILs and T-NKCs (Fig. 2B). Moreover, after PMA/I stimulation, the MFI of phosphorylated ERK was significantly lower in T-TILs than in the corresponding cells of NKC or PBMCs (Fig. 2B, Supplemental Fig. 3C). Steady-state phosphorylation of AKT in T-TILs was similar to that of T-PBMCs of healthy donors, whereas T-NKCs had elevated basal phosphorylation levels. PMA/I stimulation induced AKT phos-
phosphorylation in T-PBLs of healthy donors but not in T-TILs (Fig. 2B, Supplemental Fig. 3D).

Using phosphoprotein multiplex assay, the data of phosphoflow were confirmed and extended. CD8^+ T cells isolated by MACS separation were used. PMA/I stimulation induced much lower phosphorylation of ERK and JNK in CD8^-TILs than in CD8^-PBLs (Fig. 2C). Moreover, IκB and GSK3, which are client proteins downstream of AKT and regulate the transcription factors NF-κB and NFAT, respectively, were analyzed. It was observed that phosphorylation of IκB and GSK3 was reduced in CD8^-TILs compared with that in CD8^-PBLs consistent with the observed poor activation of AKT in CD8^-TILs.

CD8^+ T cells of TILs have high DGK-α levels, and diacylglycerol kinase inhibition improves ERK phosphorylation and degranulation

DGK-α catabolizes DAG to phosphatidic acid (PA) thereby reducing its availability for transmitting TCR ligation-initiated signals to downstream mediators, like ERK and protein kinase C. CD8^-TILs, which showed reduced ERK phosphorylation, had
higher levels of DGK-α (Fig. 3A) compared with CD8-NILs of the same kidney, which did not have reduced ERK phosphorylation. Pharmacologic inhibition of DGK-α activity during CD3 stimulation significantly increased the degranulation capacity of CD8-TILs, but to a lesser extent for CD8-NILs (Fig. 3B), suggesting higher DGK-α activity in TILs as the underlying cause for the reduced degranulation activity. Degranulation requires sustained ERK activation (21, 22). As DGK-α inhibition improved degranulation of CD8-TILs, its effect on ERK phosphorylation was analyzed. It was observed that treatment of TILs with DGK inhibitor I increased the basal and, moreover, the PMA/I stimulation-induced levels of phosphorylated ERK in T-TILs (Fig. 3C). It may seem contradictory to reveal deficits associated with altered DAG metabolism using the DAG analogue PMA for stimulation as it compensates low levels of DAG and, thus, may conceal potentially existing deficits. In fact, the relative increase of PMAI-stimulated ERK phosphorylation (determined as the \( \frac{p}{o} \)-fold change between stimulated and unstimulated T cells) was not markedly different between PBMCs, untreated TILs, or DGK inhibitor-treated TILs. However, DGK inhibition of TILs (without PMA exposure) increased the basal level of phosphorylated ERK, and this led, after the same PMA/I-induced relative increase, to higher end levels of ERK phosphorylation (determined as the absolute MFI value). Together with the observation that DGK-inhibited TILs showed improved degranulation, we assume that not only the relative increase of ERK phosphorylation is relevant but also the amount and strength of the stimulation-induced end phosphorylation of ERK, which is consistent with published results identifying ERK phosphorylation level as calibrator of TCR stimulation threshold (30).

Whether TILs have additional deficits in the relative increase in ERK phosphorylation cannot be assessed using PMA/I stimulation. CD3 stimulation, which could reveal such deficits, was insufficient (in our hands) to induce ERK or AKT phosphorylation in any T cell type, including CTL clones, which did not show functional deficits (data not shown).

Effect of ex vivo IL-2 cultivation on the functional capacity of CD8+ T cells of TILs

IL-2 is an activator of T effector cells and is used in immunotherapy of metastatic RCC leading to tumor regression and even complete responses in some patients (2, 3). Ex vivo cultivation of tissue suspensions of RCCs in low-dose IL-2 led to reduced expression levels of DGK-α protein in CD8-TILs (Fig. 4A) and enhanced steady-state phosphorylation levels of ERK and AKT (Fig. 4B). Additionally, PMAI stimulation increased end phosphorylation levels of ERK and AKT resulting in MFIs much higher than in uncultured T-TILs (Fig. 4B) and comparable to MFIs observed in T-PBLs and T-NILs (Fig. 2B). Note that the relative increase was similar in uncultured and cultured TILs. Therefore, the increased

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**Table II. Abs used in this study**

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<td>Molecular Probes, Invitrogen</td>
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<td>Anti-rabbit</td>
<td>Cy5</td>
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<td>Molecular Probes, Invitrogen</td>
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</table>

* A, Alexa Fluor.
basal levels of phosphorylation of ERK and AKT seem causal for the enhanced end levels after stimulation. Consistent with stronger activation of AKT, changes in downstream AKT client proteins were observed. These included reduced protein levels of p27kip, an inhibitory cell cycle protein, and increased levels of cyclin E, which promotes transition from G1 to S phase, in CD8+ T cells of IL-2–cultured TILs (Fig. 4C, Supplemental Fig. 3E).

Concomitant with the improvements in the signaling pathways, IL-2–cultivated tissue suspensions of RCCs showed on average 5-fold more degranulating CD8-TILs during CD3 stimulation than CD8+ T cells of uncultured TILs (Fig. 4D). In addition, IL-2 cultivation increased the frequency of perforin+ (median, 79%; range, 52–96%) and granzyme B+ (median, 76%; range, 50–99%) CD8-TILs (Fig. 4E). Together with improved degranulation capacity, it is to be expected that the lytic activity of TILs would also increase, which could not be tested due to limited TIL resources, but has been shown previously (4, 5, 11, 15).

**Discussion**

Despite the local presence of putative tumor-reactive cytotoxic T cells (5–7), RCC tumors are generally not rejected indicating deficits in the cytotoxic response at the tumor site. This study was performed to gain an understanding on a mechanistic level as to why CD8+ T cells infiltrating human RCC are not proficient to achieve tumor growth control. Cytotoxicity and production of IFN-γ are the most important effector activities required for tumor rejection (31–33). We report in this study that tumor-infiltrating CD8+ T cells of RCC tested directly ex vivo without being cultured lack cytotoxic function and are unable to respond to CD3 stimulation with lytic granule exocytosis as well as IFN-γ secretion, whereas CD8+ T cells isolated from non-tumor tissues were responsive. Thereby, they exhibit similar impairments to NK cells.
from RCC tissues, which were previously shown to lack cytotoxic activity (11).

Different forms of functional unresponsiveness have been described, including T cell anergy and functional exhaustion. The anergic state is classically assigned to CD4+ T cells (34, 35) whereby the unresponsive state is induced by means of TCR stimulation in the absence of costimulation or nonphysiologically where the unresponsive state was assigned to the failure to dephosphorylate the inhibitory Y505 of LCK during CD3 stimulation (29). The unresponsive state of the herein studied CD8-TILs of RCC differed, displaying uncompromised dephosphorylation of LCK at the inhibitory Y505. In a mouse model of adenocarcinoma, it was noted that nonfunctional TILs showed defects in proximal TCR signal activation (19). In TILs of RCC, we did not find evidence for deficits in TCR proximal signaling (ZAP70, LCK, PLCγ). However, because biological specifics of the human material (see Materials and Methods) restricted the analysis to the use of CD3 stimulation, it cannot be excluded that proximal deficits might exist, if TILs were to be stimulated with natural pMHC ligands. Whereas CD3 stimulation revealed no deficits at the proximal side of TCR signaling, CD8-TILs had severe deficits in the activation of TCR distal MAPK pathways, ERK and JNK, as well as in the activation of Akt and Akt client proteins, IκB and GSK3. The altered transmission of TCR signals was caused by the tumor microenvironment, as they were not observed in functionally active CD8+ T cells from non-tumor kidney areas or the peripheral circulation.

DGKs are physiological inhibitors of TCR signaling and cellular function (41). This results from DGK-α-catalyzing DAG to PA thereby reducing DAG levels, which leads to termination of ras signaling and failure to activate downstream MAPK ERK. ERK activation has been linked to granule exocytosis (21, 22), and DAG was found to be required for MTOC polarization (42) which is a required step for granule exocytosis. Thus, the high level of DGK-α observed in CD8-TILs could lead to a shortage in DAG causing the observed defects in activating the ras-MEK1/2/ERK pathway and impeding the degranulation and lytic response of CD8-TILs of RCC. The importance of DGK-α and the phosphorylation level of ERK for lytic granule exocytosis of CD8-TILs of RCC were substantiated by the observation that addition of a DGK-α inhibitor enhanced ERK phosphorylation and improved the degranulation of CD8-TILs.

DAG has, moreover, been shown to activate mTOR signaling and Akt through the ras-MEK1/2/ERK pathway (43). Thus, the deficit in the Akt pathway that was observed in CD8-TILs may also be linked to the high DGK-α levels and limited DAG availability causing poor ERK activation. Deficits in Akt activation and subsequently the observed poor phosphorylation of IκB, which is required for its degradation, will restrict NF-κB-meditated gene transcription, including the transcription of Th1 cytokines and survival factors (20). In parallel, the failure to provide the inactivating phosphorylation of GSK3 via Akt, which is required for NFAI nuclear localization, will impede NFAI-mediated transcription. Maintaining GSK3 in its unphosphorylated active state will additionally have pleiotropic consequences, suppressing Th1 cytokine production and survival, as well as causing cell cycle arrest via stabilization of the cell cycle inhibitory protein p27kip (44).

Collectively, multiple different pathways were found altered in CD8+ T cells infiltrating RCC explaining the lack of effector activity and indicating transcriptional reprogramming and cell cycle arrest of CD8+ T cells in the tumor environment. The identified features of CD8-TILs of RCC, including high DGK-α levels, defective ERK and JNK activation, and reversibility of functional
suppression by IL-2, indicate a relationship to the “peptide-induced” anergic state described for CD4+ T cells (34–36, 45).

Although high level of DGK is recognized as a pivotal feature of the anergic signature distinguishing it from exhaustion, DGK regulation is incompletely understood. Transcriptional upregulation by TCR stimulation, however, has been described (46). Thus, an in vivo scenario can be envisioned where persistence of Ag and perpetual TCR stimulation cause upregulation of DGK-α in CD8-TILs thereby terminating the DAG-ras-MEK-ERK–controlled and AKT-controlled pathways, which would be required for effector activity, proliferation, and survival. However, as high DGK-α is not part of the molecular signature of exhausted T cells that develop in settings of chronic viral infection (38, 39), unceasing Ag stimulation seems not sufficient to induce the TIL unresponsive state.

In addition to persistent Ag presentation, other environmental factors may contribute to the anergic signature of TILs, such as TGF-β causing observed high levels of p27kip. Alternatively or additionally, high p27kip levels may be a consequence of the poor activation of AKT maintaining FOXO activity, which is a transcription factor for p27kip, and/or by maintaining active GSK3, which stabilizes p27kip (44).

Repression of degranulation and cytotoxicity as well as IFN-γ production was evident when CD8+ T effector cells of TILs were stimulated within the unseparated cell mixture of tumor tissue suspension. Thus, the suppression could also be caused by the presence of regulatory cells, which are part of the RCC infiltrate (9, 47) and/or of tumor cells expressing or secreting inhibitory factors (19). However, the recovery of degranulation by adding DGK-α inhibitor to the tissue suspension without removing any cell type suggests that the repression is, at least in part, caused by a T cell intrinsic mechanism. Moreover, the TCR signaling deficits that were observed in T cells stimulated in tumor tissue suspension were also observed when purified CD8-TILs were stimulated, supporting the notion that the CD8-TIL deficits are not imparted by bystander regulatory cells.

Our results not only identified a signature related to functional deficits of CD8-TILs but also unraveled target molecules for intervention strategies. Among them are DGK inhibition and IL-2 application. Systemic IL-2 administration has a long history in the treatment of RCC yielding partial and sometimes complete responses in a subgroup of patients (2, 3). Documented in this study is its potent capacity to restore the cytokine secretion and cytotoxic response of in vivo-repressed CD8-TILs at very low dose. Restoration of function occurred concomitantly with reduction of DGK-α levels and normalization of key signaling pathways. Additionally, decrease in p27kip and increase in cyclin E suggest that IL-2 culture enables CD8-TILs to move toward cell cycle progress, which is considered important to overcome anergy.

IL-2 targets multiple pathways related to CTL function that were found to be altered in CD8-TILs. It has been shown that IL-2...
signaling through JAK3 and mTOR inhibit expression of anergy-inducing genes (i.e., DGK-α, Ikaros, Cbl-b) (45). DAG-dependent strong and sustained ERK phosphorylation is required for CTL degranulation (21), and ERK phosphorylation levels were shown to control the magnitude of TCR-triggered response (30), thus linking DGK activity to CTL functional response via ERK. By reducing DGK in CD8-TILs, IL-2 may allow the observed increase in steady-state phosphorylation of ERK, which then results in higher end levels of phosphorylated ERK after stimulation. Moreover, IL-2 signaling through STAT5 activates AKT, which in turn stimulates downstream transcriptional programs of NFAT and NF-kB, cell cycle progress, and translation. IL-2-treated TILs show signs of an activated AKT pathway, including reduced cell cycle inhibitor p27kip and increased cyclin E, increased perforin and NF-κB, cell cycle progress, and translation. IL-2–treated TILs show signs of an activated AKT pathway, including reduced cell cycle division together with DGK transcriptional inhibition. Treatment of TILs with proteasome inhibitors during IL-2 culture could help resolve this issue.

The observed results clearly demonstrate that IL-2 has the capacity to influence positively several pathways in CD8-TILs allowing functional suppression to be overcome ex vivo. In human cancer therapy, systemic IL-2 has shown some success, yet effects are in general not very predictable and limited to a small subgroup of patients (2, 3). This might seem at odds with the strong and consistently observed positive effects that IL-2 exerted in our ex vivo treatment of TILs. However, the results have to be seen in the different architectural contexts of the ex vivo versus in situ setting. Whereas in vitro, IL-2 was exogenously added to tissue suspensions, allowing unrestricted access to TILs, IL-2 systemically given in vivo has to penetrate into the solid mass of human carcinoma to gain access to TILs for signal initiation. Clinical efficacy of IL-2 is likely limited by poor penetration leading to spatial separation of IL-2 from TILs, and severe toxicity of systemic IL-2 precludes increasing the IL-2 dose needed for sufficient tissue saturation. Consistent with this scenario are clinical observations that only IL-2 regimens at the highest tolerable dose have the potential of clinical benefit. Moreover, experimental mouse models using intratumoral delivery of IL-2 show activation of TILs in the tumor (49, 50), supporting our results that IL-2 can have positive effects in situ if access to TILs in the tissue is enabled.

The experimental setup as performed, adding IL-2 to the complete tissue cell suspension, does not allow us to distinguish whether IL-2 exerts its effect by acting on the CD8-TILs directly or indirectly via other cell types (such as NK cells) that are present in the mixture and are also activated during the in vitro culture (data not shown). Notably, however, using this setup, it is demonstrated that IL-2 can overcome CTL suppression despite the presence of potentially inhibitory cellular components of the tumor milieu, such as regulatory T cells or tumor cells.

All types of immunotherapy, including systemic cytokine infusion, vaccination, or adoptive T cell therapy, show some positive effects in subgroups of patients. However, overall clinical efficacy remains unsatisfactory. Effector phase inhibition of cytotoxic lymphocytes in the tumor milieu is one mechanism that will limit efficacy. Our studies identified DGK-α as a potential new target that may help improve CD8+ T effector function. This proposition is supported by findings in a DGK-knockout mouse model describing enhanced CD8+ T cell function and anti-tumor activity when DAG metabolism was decreased (51). Notable in our system, DGK-α inhibition was able to improve CD8+ T cell function even in the continuous presence of the cellular tumor milieu with all its potentially inhibitory components. Adjunct pharmacological targeting of DGK-α may thus have the potential to improve clinical efficacy of current cancer immunotherapy.

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Disclosures
The authors have no financial conflicts of interest.

References


