The objective of the present study was to evaluate the cytotoxic immune response mediated by the TCRγ/δ T cells population in clear cell metastatic renal cell carcinoma.
TCRγ/δ T cell represent 1-10% of peripheral T lymphocytes in human and the majority express a Vγ9Vδ2 TCR rearrangement. They recognize low molecular weight molecules derived from bacterial extracts (phosphoantigen, aminophosphates, alkylamines) without MHC restriction and do not require antigen presentation. They mostly express a memory phenotype (CD45RO, CD95+) and produce IFN-γ and TNF-α in response to mycobacteries or undefined antigen present on tumor cells, indicating their crucial role in the innate and adaptative immune response. In the present study, we evaluate their capacities to lyse primary renal tumor cells and characterize the mechanisms involved in their lytic function.

Key words: renal cell cancer, TCRγ/δ T cells, immune response, cell lysis.

Evaluation of TCR γ/δ T cells in the lysis of renal tumor cells

Ocena funkcji limfocytów T γ/δ w lizie komórek raka nerki

Emilie Viey, Gaelle Fromont, Salem Chouaib, Bernard Escudier, Anne Caignard

INSERM Institut Gustave Roussy, Paris, France

The objective of the present study was to evaluate the cytotoxic immune response mediated by the TCRγ/δ T cells population in clear cell metastatic renal cell carcinoma.

TCRγ/δ T cell represent 1-10% of peripheral T lymphocytes in human and the majority express a Vγ9Vδ2 TCR rearrangement. They recognize low molecular weight molecules derived from bacterial extracts (phosphoantigen, aminophosphates, alkylamines) without MHC restriction and do not require antigen presentation. They mostly express a memory phenotype (CD45RO, CD95+) and produce IFN-γ and TNF-α in response to mycobacteries or undefined antigen present on tumor cells, indicating their crucial role in the innate and adaptative immune response. In the present study, we evaluate their capacities to lyse primary renal tumor cells and characterize the mechanisms involved in their lytic function.

Several reports have outlined that renal cell carcinoma represent an indication of choice for the use of TCRγ/δ T cells in oncology as they are present at the tumor site and they exert a lytic activity towards tumor renal cell lines *in vitro* (Choudhary et al., *J Immunol* 1995; Kobayashi et al., *Cancer Immunol Immunother* 2001).

However, these results were obtained from long term established cell lines and with CTL clones as effectors. In addition the mechanisms regulating the tumor cell lysis by TCRγ/δ T cells were not determined.

Material and method

From a series of 15 mRCC patients, the following cell lines were derived. Primary tumor cells derived from enzymatic digestion of a tumor fragment, designed as TC (Tumor cells. Normal renal cells (NC) were obtained using the same protocol from a fragment of renal parenchyma harvested at distance from the tumor.

PBMC were obtained upon centrifugation on Hypaque Ficoll from peripheral blood samples post nephrectomy. From PBMC, TCRγ/δ T cells were amplified using Phosphostim TM and IL-2. Phosphostim is a synthetic phosphoantigen that activate Vγ9Vδ2T cells and in presence of IL-2 induce their proliferation (Belmant et al., *JBC*, 2001). The active molecule ifs the BrHPP (Bromo Halohydrin Pyrophosphate). PBMC from healthy donors were used as control in parallel experiments. Following 2-3 weeks of *in vitro* culture, Vγ9Vδ2 T cell culture comprising 75-90% of Vδ2 T cells were obtained. Phenotypic analysis of amplified Vδ2 CTL were performed using different activation markers by flow cytometry. The lytic capacities were determined using classical chromium release assay with different cell lines: Daudi and Raji lymphoma cell lines as specific and resistant targets respectively. In addition TC and NC as well as RCC6, an established tumor cell line were used as targets.

Results

PHOSPHOSTIM™ stimulation induces the expansion of peripheral Vδ2 T cells from MRCC patients

Peripheral percentages of γ/δ T cells in both donors and MRCC patients were <5% of PBMC in our studies series. Following activation (14 days) with

Celem niniejszego badania była ocena odpowiedzi cytotoksycznej, w której pośredniczą limfocyty T posiadające receptor TCRγ/δ w przerzutowym raku jasnokomórkowym nerki.
Limfocyty Tγδ stanowią 1–10 proc. obwodowej populacji limfocytów T u człowieka i w większości charakteryzują się obecnością wariantu Vγ9Vδ2 receptora T. Komórki te rozpoznają cząsteczki o niskim ciężarze molekularnym pochodzące z bakterii (fosfoantygeny, aminofosforany, alkilaminy) bez potrzeby prezentacji ich w kontekście MHC. Limfocyty Tγδ w większości posiadają fenotyp charakterystyczny dla komórek pamięci (CD45RO, CD95+) i produkują IFN-γ i TNF-α w odpowiedzi na mikobakterie lub niezidentyfikowane antygeny obecne na komórkach nowotworowych, demonstrując swoją kluczową rolę zarówno we wrodzonej, jak i nabytej odpowiedzi immunologicznej. W niniejszej pracy oceniono zdolność limfocytów Tγδ do niszczenia pierwotnych komórek raka nerki i scharakteryzowano mechanizmy warunkujące funkcje lityczne tych limfocytów.

Słowa kluczowe: rak jasnokomórkowy nerki, limfocyty Tγδ, odpowiedź immunologiczna, liza komórek.

Phophostim and IL-2, 11/15 cultures derived MRCC patients exhibited a pourcentage Vδ2 T cells comprised between 73.3 and 96.4% with an amplification rate >200 (patients 3,5-9 et 11-15). These patients were designed as «responders» to the expansion test. In the other patients, designed as non responders, the pourcentage of Vδ2 T cells was <20%, and there were not included in functional studies (patients 1,2,4 et 10). In control, Vδ2 T cells were amplified from donors PBMC (Table 1.).

Phenotypic analysis of Vδ2 T cells amplified from patients MRCC patients (Table 2)

Amplified Vδ2 T cells were phenotyped at Day 14. The total Vδ2T cell population present a memory phenotype (CD45RO+), an activated state (CD69+, HLA-DR+). Compared to Vδ2 T cell cultured derived from donors, a significative increased expression of CD8 mean fluorescence intensity was observed (532±192 in MRCC versus 297±27 in donors). These cells express

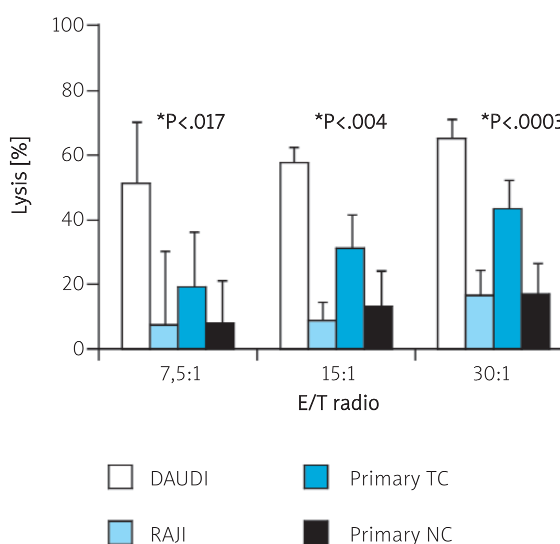

Fig. 1A. Mean lytic activity of Vδ2 T cells amplified from 10 MRCC patients PBMC towards different targets. P values between primary TC and NC at different E/T ratio were determined by Student T test

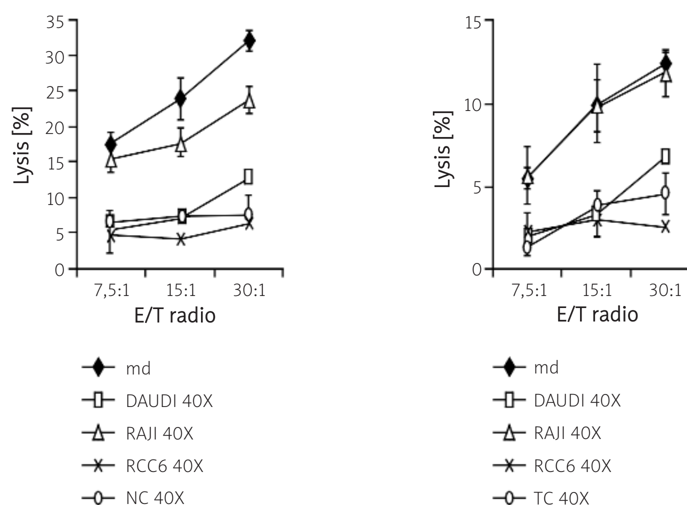

Fig. 1B. Cold target competition experiments with amplified Vδ2 T cells from MRCC PBMCs. The lytic capacities of the Vδ2T cells towards TC (left panel) and NC (right panel) were determined in presence of a 40 fold excess of cold targets

Table 1. Clinical and hematological characteristics of MRCC patients (n=15)

Patient number	Age	Sexe	Grade	TNM	Lymphocytes ($x10^9/L$)	% NK cells day J0	% CD8 cells day J0	% γδ T cells day J0	% γδ T cells day J14	Amplification γδ T cells
1	80	M	III	T3b N0	–	12	13	3.7	12	–
2	55	M	IV	T2 NX	1	ND	20.7	3.5	15.3	1
3	70	M	III	T3b NX	2.3	ND	29.3	1.7	80	–
4	79	F	III	T1b NX	3.6	ND	34.4	1.8	19.5	26
5	46	M	III	T2 NX M1	2.2	17.3	23	3.4	80.8	-
6	43	M	II	T1a NX	–	15	30	4.1	78	250
7	28	M	IV	ND	2.6	30.1	60.8	1.6	73.3	248
8	52	F	ND	ND	1.6	11	25.8	3.4	83	–
9	ND	M	ND	ND	1.4	–	–	2	86	407
10	69	M	ND	ND	0.6	14.7	16.8	0.7	16.7	9
11	57	F	ND	ND	1.3	15.2	25.9	1.7	96.4	578
12	48	F	III	T3a N0	–	8.5	32.5	1.9	87.6	347
13	67	F	II	T1b NX	2.3	14.2	17	2	75	425
14	53	M	III	T2 NX	2.2	–	36.8	0.3	87.1	2787

the activating receptor and no difference between patients and donors derived cultures was observed (mean 85.9%, rate 74.2-99.9% in MRCC patients and mean 88.9%, rate: 67.2-99.9% in donors).

The activation of Vδ2 T cells is controlled by a balance between possitive and negative signals, negative signals that are mediated by the heterodimeric CD94/NKG2-A receptor. This receptor is expressed on identical percentages of Vδ2 T cells in both MRCC patients and donor derived cultures. In addition, 2/11 cultures derived from MRCC patients exhibited an increased percentages of KIR (Killer Immunoglobulin like Receptors positive Vδ2T cells, indicative of a antigen experienced T cell phenotype.

Primary renal tumor cell lysis by Vδ2 T cells from MRCC patients involveTCR and NKG2D receptors

In a first series of experiments we tested the lytic potential of Vδ2 T cells from MRCC patients in autologous setting towards different targets: TC, NC, RCC6, Daudi et Raji cells). Vδ2 T cells kill efficiently lyse RCC6, primary TC and exhibit a low lysis towards the autologous NC. Fig. 1A depicts the mean lytic activity of Vδ2 T cells derived from 10 MRCC patients in autologous settings, showing that the mean lytic activity against TC is increased compare to that against NC ($P<0.05$ at all the E/T ratio).

We then performed cold targets competition experiments by addition of an excess (up to 40 fold) of non radiolabelled targets to determine if the same antigenic moieties is recognised on the different targets. An excess of Daudi cells (but not resistant Raji cells) decreases the lysis of renal tumor cells RCC6 as well as the lysis of TC. The excess of NC also diminishes the lysis of TC , indicating that the Vδ2 T cells recognize a common antigenic structure present on Daudi, TC and NC.

To determine the respective involvement of TCR and NKG2D receptors in the Vδ2 T dell mediated lysis, cytotoxicity assyas were performed in presence of saturating concentrations of specific mAbs. The lysis of primary tumor cells is decreased in presence of anti-TCRγ/δ mAb (inhibition of 63% at E/T ratio of 5/1) Blocking the NKG2D receptor also decreased lysis of TC (inhibition of 38% at E/T ratio of 5/1) indicating the involvement of this receptor in the Vδ2 T cell mediated lysis of renal tumor cells.

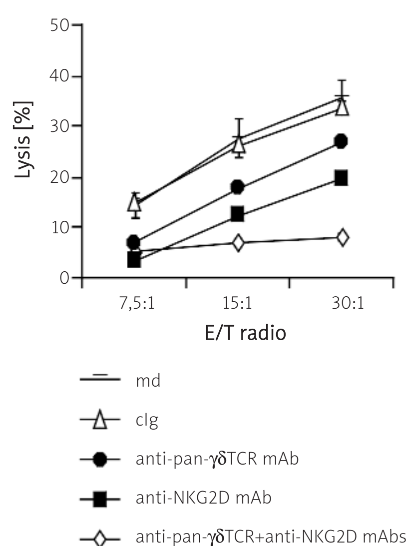

Fig. 1C. TCRγ/δ and NKG2D receptors are invloved in renal tumor ceklls lysis mediated by Vδ2T cells. Vδ2 T cells were incubated with anti-pan-γδTCR and/or anti-NKG2D before addition to the radiolabelled TC targets md, medium; cIg, isotypic control

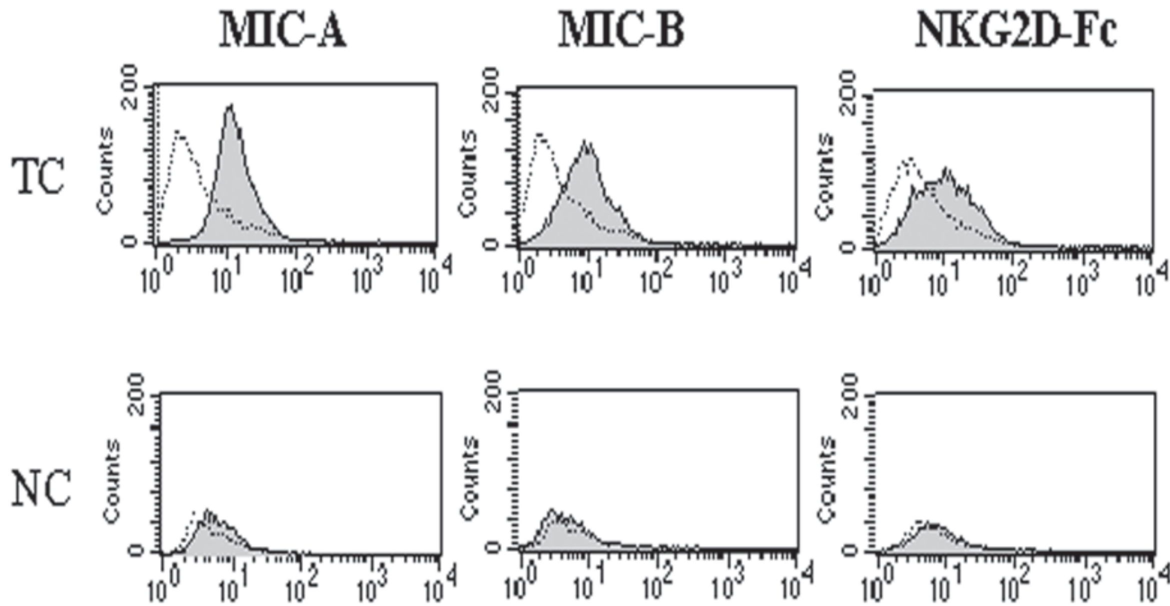

Fig. 1D. MICA/B and ULPB molecules expression on primary TC and NC. Open profiles are isotypic controls

Preferential expression of NKG2D ligands on primary renal tumor cells

NKG2D ligands such as MICA/B, ULBP1,2,3 proteins are induced following a cellular stress and are often overexpressed by transformed tumor cells. By flow cytometry, we have assessed the expression of these molecules on TC versus NC, using specific mAbs and a NKG2D fusion protein. We show that primary tumor cells overexpressed NKG2D ligands compared to normal counterparts.

This results are in agrements with the role of NKG2D receptor in the preferential lysis of renal tumor cells by activated Vδ2 T cells.

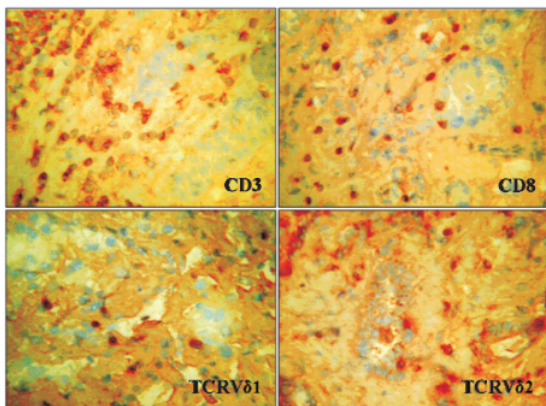

Fig. 2. Vδ2 T cell infiltrate renal tumors where they constitue major componant of the CD3 T cells infiltrates représentent la composante majoritaire des cellules Tγδ infiltrant les tumeurs rénales. Immunohistochemical analysis of serial slides of a frozen tumor fragment: positive cells ate stained in brown

Vδ2T cells infiltrate renal tumors

Serial immunohistologic slides of frozen tumor samples from 7 MRCC patients were analysed for the *in situ* detection of Vδ2 et Vδ1 T cells. Tumors were selected upon hematoxyline-safranine coloration for intense infiltrates. Fig. 2 show the immune infiltrate of a representative tumor. Numerous γ/δ T cells are detected in close contact with the tumor cells and it shows that the Vδ2 T cell subset is a major component in the tumor.

Conclusions

The elevated percentages of patients able to respond to Phosphostim *in vitro*, the activation status of the amplified Vδ2 T cells and their lytic capacities towards renal tumor cells constitute strong arguments for the clinical development of such compound. While the involvement of Vδ1 T cells in the control of tumor growth was evidenced in experimental models (*Mitropoulos et al., Clin Exp Immunol 1994; Girardi et al., Science 2001*), the role of Vδ2 T cells is nor yet proven.

It was recently showed that the treatment of patients with a multiple myeloma by aminobisphosphonates may induce objectives clinical responses. Interestingly, *in vivo* responses were correlated with *in vitro* expansion of cytolytic Vδ2 T cells from the corresponding patients PBMCs. These clinical results and the present studies are encouraging observations for the development of clinical trials using g/d T cells for MRCC patients. In parallel, *in vitro* studies will be performed to determine the migration capacities of such effectors by phenotypic and functional analyses of chemokines receptors expressed by these activated CTL.