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## HUMAN TUMOR IMMUNOLOGY

*Tumor-specific antigens, such as those encoded by the MAGE genes, have been used to vaccinate melanoma patients with detectable disease. About 20 % of the vaccinated patients display a tumor regression, a frequency that appears well above the level reported for spontaneous melanoma regressions. Nevertheless, the treatment fails in most patients, and this can probably only be improved by a better understanding of the anti-tumor immune responses of the patients and of the mechanisms of tumor resistance to immune attack.*

*Along this line, a first objective is to assess the immunogenicity of tumor vaccines and to compare various vaccine modalities. We have developed very sensitive methods for the detection of anti-tumor T lymphocytes, and now apply them to patients included in cancer vaccination trials conducted by Dr. Jean-François Baurain at the Cliniques Universitaires St Luc and by the clinical team of the Ludwig Institute (1, 2, 3).*

*A second objective is to understand the mechanism of the tumor regressions that occasionally occur in vaccinated patients. The detailed analysis of one such patient indicated that, surprisingly, the anti-vaccine T lymphocytes are largely outnumbered by other tumor anti-T cells, which recognize tumor-specific antigens different from the vaccine antigens. These anti-tumor T cells represent most of the T cells present in a regressing tumor, and they probably play a major role in the rejection process (4, 5). Why these anti-tumor T cells become activated following vaccination with antigens that they do not recognize is not clear.*

*That local immunosuppression could be involved in preventing tumor rejection is compatible with the observation that the anti-tumor T cells mentioned above are systematically present in tumors already before vaccination. Considering that suppressive or so-called regulatory T cells are recognized as important attenuators of immune responses, we have initiated an analysis of their role in the vaccinated patients. We have also started to explore the functional status of the T lymphocytes that are infiltrating melanoma tumors, with an in situ genetic approach.*

## ANTI-VACCINE T CELL RESPONSES IN MELANOMA PATIENTS VACCINATED WITH DEFINED TUMOR-SPECIFIC ANTIGENS

*G. Hames, V. Corbière, P.G. Coulie, in collaboration with A.-M. Feyens and J.-F. Baurain, Department of Medical Oncology, Cliniques Universitaires St Luc, and N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.*

We focused on the analysis of CD8 T cell responses to antigenic peptides presented by HLA-A1 or A2 molecules. Several small clinical trials have been performed with the MAGE-A3 antigenic peptide EVDPIGHLY, presented by HLA-A1 (7). Table 1 presents a summary of the results obtained in patients who showed evidence of tumor regression and in patients who did not, after vaccination with either ALVAC-MAGE, a recombinant poxvirus containing a minigene encoding the MAGE-3.A1 peptide, or dendritic cells loaded with the peptide (G. Schuler, Erlangen and K. Thielemans, Vrije Universiteit van Brussel), or peptide MAGE-3.A1 alone. The observed correlation between CTL responses and tumor regression supports the notion that the tumor regressions

are caused by the vaccines (3). We then analyzed melanoma patients vaccinated with 8 antigenic peptides, all encoded by genes displaying a tumor-specific pattern of expression and all presented by HLA-A2 molecules. The peptides were co-administered with the immunological adjuvant CpG7909, a ligand for Toll-like receptor 9. Monitoring the frequencies of blood T cells against each individual peptide indicated a hierarchy in the immunogenicity of these peptides, with proportions of patients with a detectable T cell response ranging from 0% for several peptides to 50% for one peptide. As expected, the adjuvant participates in the immunogenicity of these peptidic vaccines, as T cell responses were much rarer in patients who received the same set up of 8 peptides without adjuvant. We also compared CpG7909 with another immunological adjuvant that can be used in humans : Montanide ISA51, a clinical grade incomplete Freund's adjuvant. The results were similar.

Despite the higher immunogenicity of the CpG + 8 peptides combination, as compared to peptide MAGE-3.A1 alone, we have not observed a significant difference in the clinical results obtained in the two groups of vaccinated patients. This suggests that the main limitation to the clinical efficacy of the MAGE vaccines is not their immunogenicity.

Vaccination mode	CTL response in patients with	
	evidence of tumor regression	no evidence of tumor regression
ALVAC-MAGE	3/4	1/11
Dendritic cells + peptide MAGE-3.A1	3/3	0/3
Peptide MAGE-3.A1 alone	1/7	0/13
	7/14	1/27

Table 1. Summary of anti-MAGE-3.A1 CTL responses in vaccinated melanoma patients.

## **TUMOR REGRESSIONS OBSERVED AFTER VACCINATION: A ROLE FOR TUMOR-SPECIFIC CYTOLYTIC T LYMPHOCYTES THAT DO NOT RECOGNIZE THE VACCINE ANTIGENS**

*V. Corbière, T. Connerotte, T. Aerts, C. Muller, P.G. Coulie, in collaboration with C. Lurquin, B. Lethé, Brussels branch of the Ludwig Institute for Cancer Research.*

It is clear from Table 1 that several vaccinated patients displayed tumor regression in the absence of a detectable anti-vaccine CTL response. In addition, even among those vaccinated patients who showed a CTL response, most had a low frequency of anti-MAGE-3.A1 CTL in the blood, ranging between  $10^{-6}$  and  $10^{-5}$  of CD8 T cells. Because such a level of CTL might be insufficient to produce on its own the observed tumor regressions, we examined the possibility that CTL directed against other antigens present on the tumor might contribute to the regression. For seven vaccinated melanoma patients, selected because it had been possible to derive a permanent cell line from their tumor, we estimated the blood frequencies of CTL directed against any antigen present on the tumor cells. For all seven patients, anti-tumor CTL were found at high frequencies, i.e. from  $10^{-4}$  to  $3 \times 10^{-3}$  of the CD8 T cells, in the blood after vaccination. Unexpectedly, they were already present at similar high frequencies before vaccination. The frequency of anti-tumor CTL observed after vaccination was considerably higher than that of the anti-vaccine CTL, ranging from 12 fold to 20,000 fold higher (4). Because T cells directed at other tumor antigens than the vaccine antigen could make an important contribution to the tumor regressions, we felt that it was necessary to define the precise nature of their target antigens. We focused our effort on patient EB81, who had shown complete regression of a large number of cutaneous metastases following vaccination with ALVAC-MAGE. A majority of anti-tumor CTL clones

recognized antigens encoded by MAGE-C2, a cancer-germline gene (4). Others recognized an antigen encoded by gp100, a melanocytic differentiation gene. In conclusion we are facing a paradoxical situation where the melanoma patients that are being vaccinated, have already mounted a high spontaneous response against the types of antigens used in the vaccines. At the time of vaccination this spontaneous T cell response is clearly ineffective in halting tumor progression. To evaluate the potential contribution of the “anti-tumor” T cells to the tumor rejection that occurred following vaccination, we measured the frequency of the anti-vaccine and anti-tumor T cells in metastases of patient EB81. The frequency of anti-MAGE-3.A1 T cells was  $2.5 \times 10^{-6}$  of CD8 T cells in the blood and it was 6-fold higher in a metastasis. An anti-tumor CTL recognizing an antigen encoded by MAGE-C2 showed a considerably higher enrichment: its blood frequency was  $9 \times 10^{-5}$ , and it was about 1,000 times higher in the tumor. Several other anti-tumor T cell clonotypes also had frequencies above 1% and appeared to constitute the majority of the T cells present in metastases (5). These results suggest that the anti-vaccine CTL may not be the principal effectors that kill the bulk of the tumor cells. They may exert their effect mainly by an interaction with the tumor that creates conditions enabling the stimulation of large numbers of CTL directed against other tumor antigens, which then proceed to destroy the tumor cells.

## **FUNCTIONAL ANALYSIS OF TUMOR-SPECIFIC T CELL CLONES**

*T. Connerotte, T. Aerts, P.G. Coulie*

The results summarized above suggested that, at least in some vaccinated patients, a surprisingly low number of anti-MAGE-3.A1 T cells sufficed to trigger a tumor rejection response. Among other possibilities, these rare anti-vaccine T cells could do so as a result of a particularly high affinity for the MAGE-3.A1

antigen, or a very high lytic activity against melanoma cells. We therefore conducted functional analyses on these anti-MAGE-3.A1 T cells. Our first objective was to find explanations for their putative anti-tumor activity in vivo, keeping in mind their low frequencies in the vaccinated patients. Our second objective was to examine whether different vaccination modalities with the same antigen resulted in different functions being exerted by the anti-vaccine T cells. The very low number of anti-MAGE-3.A1 T cells in most of our vaccinated patients prevents robust ex vivo functional analyses, and we resorted on analysing a representative collection of 16 anti-MAGE-3.A1 CTL clones, derived from 9 vaccinated melanoma patients who showed tumor regression following vaccination. The CTL clones were screened for their lytic activity, functional avidity, cytokine secretion and gene expression profiles. The functional avidities of these CTL clones were surprisingly low, suggesting that high avidity was not part of the putative capability of these CTL to trigger tumor rejection. Most anti-MAGE-3.A1 CTL clones obtained after vaccination with dendritic cells, but not with peptide or recombinant ALVAC poxviruses, produced IL-10 (9). Transcript profiling confirmed this result and indicated that about 20 genes, including *CD40L*, *prostaglandin D2 synthase*, *granzyme K* and *granzyme H*, were highly differentially expressed between the anti-MAGE-3.A1 CTL clones derived from patients vaccinated with either peptide-ALVAC or peptide-pulsed DC. These results indicate that the modality of vaccination with a tumor-specific antigen influences the differentiation pathway of the anti-vaccine CD8 T cells, which may have an impact on their capacity to trigger a tumor rejection response.

## TUMOR INFILTRATING LYMPHOCYTES

*A. Cipponi, C. Muller, G. Hames, P.G. Coulie, in collaboration with N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.*

As indicated above, our analysis of the complete T-cell response that some melanoma patients mount against their own tumor indicated that spontaneous responses occurred prior to any kind of vaccination, and demonstrated that some of these tumor-specific T cells could be present in the tumors. The reasons for this seemingly pacific co-existence of tumor cells and tumor-specific T lymphocytes remain unclear. It is known from histological analyses that some melanoma tumors are infiltrated by T cells, and for primary tumors this infiltration is correlated with a better clinical prognosis. But the reason for the T cell infiltration of only some tumors is unknown. Over the last year, we have set up and combined several methods to analyze melanoma-infiltrating T cells in situ. Our objective is to be able to characterize the activation status and T cell Receptor (TCR) repertoire of these T cells and to compare these results with the exact localization of the T cells within the tumor. Nicolas van Baren observed that, very often, the so-called tumor-infiltrating lymphocytes are actually clustered around the tumor nodules. In some tumors they are present both in these margins and within the nodules. We would like to know whether these different types of T cells are activated, if this is the case what is their proportion and exact localization, and ultimately whether or not they are tumor-specific and whether or not they are immunosuppressed. The methodological approach that we follow comprises laser microdissection of small numbers ( $\pm 100$ ) of cells, guided by histochemistry. The very low amount of starting material imposes a preliminary amplification of the cDNA prior to real-time PCR analysis of many genes of interest, or to complete gene expression profiling. To this end we have adapted methods proposed for the amplification of genetic material from single cells.

## CLONAL ANALYSIS OF REGULATORY T CELLS FROM CANCER PATIENTS

S. Lucas, J. Stockis, M. Panagiotakopoulos, T. Aerts, P.G. Coulie

Regulatory T cells, or  $T_{\text{regs}}$ , are a subset of  $CD4^+$  lymphocytes specialized in the suppression of immune responses. Their existence was initially revealed by their ability to prevent the development of auto-immune diseases in mouse models. Transcription factor *FOXP3* is specifically expressed in murine  $T_{\text{regs}}$  and is indispensable for their differentiation, maintenance and function. *FOXP3* is also highly expressed in human  $CD4^+CD25^+$  T cells with suppressor function. However, in contrast to mouse cells, *FOXP3* is also expressed in other activated human T cells. In mice,  $T_{\text{regs}}$  were shown to contribute to cancer progression by inhibiting anti-tumor immune responses. It has long been proposed that  $T_{\text{regs}}$  could play a negative role in cancer patients, but this has remained difficult to verify due to the lack of a  $T_{\text{reg}}$ -specific marker, and to an incomplete understanding of their suppressive function. Our long term objective is to develop tools to test whether anti-tumor immune responses in cancer patients are under the negative influence of  $T_{\text{reg}}$  cells.

We succeeded in obtaining stable human Treg clones, namely clones that expressed high surface CD25 at rest, were anergic in vitro, and suppressed the proliferation of co-cultured  $CD4^+$  cells. *FOXP3* mRNA and protein were high in these clones, but were also detected in CTL and in non-suppressive  $CD4^+$  Thelper clones. In contrast, as was previously described for polyclonal T cells, demethylation of a conserved region of *FOXP3* intron 1 was only observed in the Treg clones. We used a set of clones defined by this stable epigenetic mark to gain insight into human  $T_{\text{reg}}$  cell function. Microarray analysis of  $T_{\text{reg}}$  and  $T_{\text{helper}}$  clones indicated that the transcriptional profile that is specific of activated  $T_{\text{reg}}$  clones includes a TGF $\beta$  signature (10). A TGF $\beta$  signature was also dis-

played by a  $T_{\text{helper}}$  clone “suppressed” by a  $T_{\text{reg}}$  clone. Finally, activation of  $T_{\text{reg}}$  but not Thelper clones resulted in the cleavage of the inactive pro-TGF $\beta$  precursor protein into mature TGF $\beta$ . Altogether, these results provide a rigorous demonstration that a hallmark of activated human  $T_{\text{reg}}$  cells is to produce bioactive TGF $\beta$  which has autocrine and paracrine actions on neighboring T cells (10).

We are currently attempting to identify the mechanisms by which activated  $T_{\text{regs}}$  cleave the pro-TGF $\beta$  precursor. We will also analyze the consequences of TGF $\beta$  signaling on the effector function of human  $T_{\text{helper}}$  or cytolytic T cells. Finally, we will try to define the mechanisms of resistance to TGF $\beta$  that characterizes some  $T_{\text{helper}}$  lymphocytes. The latter aspect seems to us of a particular interest. Indeed, we made the unexpected observation that some human lymphocyte populations are resistant to the cytostatic effect of TGF $\beta$ , in contrast to what is observed with other types of lymphocytes and as it is generally described in the literature. The lymphocytes that we analyze are derived from non-leukemic patients, implying that resistance is not a consequence of tumoral transformation. We observed that sensitive and resistant lymphocytes express similar levels of the TGF $\beta$  receptor and phosphorylate SMAD factors to comparable levels. However, TGF $\beta$  signal transduction is interrupted in resistant lymphocytes, which do not induce nor repress genes that are regulated by TGF $\beta$  in sensitive lymphocytes. We will further dissect the molecular causes of resistance to the cytostatic effects of TGF $\beta$  in human T lymphocytes.

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