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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 displayed 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.

A first objective is to understand the mechanism of the tumor regressions that occur in a few of the vaccinated patients. Detailed analyses indicated that, surprisingly, the anti-vaccine T lymphocytes are widely 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. We wish to understand why these anti-tumor T cells become activated following vaccination.

A second objective is linked to the observation that the anti-tumor T cells mentioned above are often present in tumors already prior to vaccination. But they appear to be quiescent, as tumor cells co-exist with them without clear signs of immune attack. We are studying various aspects of this co-existence. One is the analysis of lymphocytes infiltrating human melanoma metastases, another is a direct lymphocyte inhibition by melanoma cells in culture, and the last is the analysis of the suppressive or so-called regulatory T cells, which are important attenuators of immune responses.

IMMUNE RESPONSES TO CANCER VACCINE ANTIGENS

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

To understand why a minority of cancer patients vaccinated with defined tumor antigens display an objective tumor regression while most of them do not, it is essential to know whether and how the anti-tumor immune responses of the patients are either primed, amplified, or modified by the vaccinations. We nevertheless felt that it was crucial to know whether or not low-level responses had occurred and whether such cytolytic T lymphocytes (CTL) responses showed a correlation with tumor regression, in order to understand why most patients failed to show any evidence of regression. We therefore developed a sensitive approach based on *in vitro* restimulation of blood lymphocytes with the antigenic peptide over two weeks, followed by labeling with tetramers. To evaluate precursor frequencies, these mixed lymphocyte-peptide cultures were conducted under limiting dilution conditions. Cells that were labeled with the tetramer were cloned, the lytic specificity of the clones was verified, and their diversity was analyzed by T cell receptor (TCR) sequencing (1, 2).

Focusing on CD8 T cell responses to antigenic peptides presented by HLA-A1 or A2 molecules, we observed a surprisingly low levels of anti-vaccine T cell responses in several of the patients who displayed tumor regression after vaccination. Moreover we did not observe the anticipated correlation between the intensities or breadth (proportions of peptides against which a response is observed) of the immune responses and the clinical impact of the vaccinations (3, and unpublished observations). All these results suggest that the main limitation to the clinical efficacy of these therapeutic anti-cancer vaccines is not the intensity of the induced anti-vaccine T cell responses.

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

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

We described previously a low level anti-vaccine CTL response in the blood of a melanoma patient who displayed tumor regression after vaccination with MAGE antigens. With a genetic approach including T-cell receptor (TCR) cDNA libraries, we also found very few anti-vaccine CTL in the regressing metastases. But we found up to 1,000 times more of other TCR sequences, several of which corresponding to CTL clones specific for non-vaccine tumor antigens (4). We then observed another TCR that was enriched in regressing metastases, and that became detectable in blood only after vaccination. We wished to identify the antigen recognized by the T cells bearing this receptor. We used the TCR sequence to detect and clone the desired T cells out of tumor-infiltrating lymphocytes. The resulting CD8+ clone specifically lysed the autologous melanoma cells and displayed HLA-A2 restriction. Its target antigen was encoded by the gene coding for the mitochondrial enzyme caseinolytic protease. The gene bears a mutation in the tumor, resulting in a new antigenic peptide. These results reinforce our hypothesis that T lymphocytes enriched in tumors as compared to blood (5) do recognize tumor-specific antigens, and that the main effectors of tumor rejection in this patient were CTL to non-vaccine tumor-specific antigens. Of these, the mutated antigen described above is the only one against which no T cells could be detected before vaccination. Following vaccination, the spreading of the anti-tumor T cell response to this truly tumor-specific antigen may have contributed decisively to tumor regression.

IN SITU ANALYSIS OF TUMOR-INFILTRATING LYMPHOCYTES

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

Our detailed analyses of melanoma patients vaccinated with tumor-specific antigens clearly indicated that anti-tumor T lymphocytes were already present prior to vaccination, both in blood and in some tumors. It is obvious that there is a seemingly pacific coexistence between tumor cells and tumor-specific T lymphocytes that occurs in many of these cancer patients (6). The reasons for this coexistence may well be the key towards improving the clinical efficacy of cancer vaccines. We are gaining information about human tumor-infiltrating or tumor-associated T cells through an *in situ* analysis. Human tumor samples are processed simultaneously for histological analysis including immunochemical detection of immune cells, for complete gene profiling on a fragment of the tumor, and for laser microdissection on frozen material. Whenever possible a small piece is put into culture to derive a melanoma cell line.

We compared the gene expression profiles of pre-vaccine cutaneous metastases from melanoma patients who showed either complete tumor regression or no regression following vaccination with tumor antigens. We observed no relevant difference between the two groups. But we noticed the presence of a specific inflammatory signature, quite variable between samples, and independent of the clinical evolution of the patients. It comprises T cell and macrophage markers. The T cell signature includes activation markers, IFN γ target genes, and the IFN γ transcript itself. Using immunohistology on adjacent tumor sections, we established that this inflammatory signature correlates with the degree of immune cell infiltration in these tumors. Thus melanoma metastases host various degrees of active Th1 inflammation, and we conclude that the immu-

nosuppressive environment in these tumors does not result in a complete inhibition of T cell activation.

HUMAN REGULATORY T CELLS AND TGF β

S. Lucas, J. Stockis, C. Huygens, N. Remy, M. Panagiotakopoulos, P.G. Coulie.

Regulatory T cells, or Tregs, are a subset of CD4 $^+$ lymphocytes specialized in the suppression of immune responses. They are required to prevent the development of auto-immune diseases, but in mice they were also shown to contribute to cancer progression by inhibiting anti-tumor immune responses. Tregs could play a negative role in cancer patients, but this has remained difficult to verify due to the lack of a Treg-specific marker in humans, as well as to an incomplete understanding of the mechanisms underlying their suppressive function.

Our objective is twofold: develop tools to quantify Tregs in human tissues, and identify mechanisms important for their suppressive function which could be specifically targeted to improve the efficiency of cancer vaccines.

Our previous work lead to the obtention of stable human Treg clones, representing long-term cultures of pure lymphocyte populations available for repeated analysis (7). A stable epigenetic mark unambiguously distinguished human Treg clones from non regulatory CD4 $^+$ (Thelper) or CD8 $^+$ (cytolytic) clones: a conserved region in intron 1 of gene *FOXP3*, encoding a transcription factor indispensable for the development and function of Tregs, was found demethylated in Treg clones only. We set up a methylation-specific real-time PCR assay to quantify demethylated *FOXP3* sequences, indicative of the presence of Treg cells. We are currently using this assay to monitor frequencies of Tregs in peripheral blood. In collaboration with laboratories from Italy, The Netherlands and Germany, we measured

circulating Treg frequencies in patients who received tumor vaccines in combination with different potentially Treg depleting strategies. None of the strategies tested up to now (i.e. low dose Cyclophosphamide, Ontak or Daclizumab) induced a significant decrease in Treg frequencies in a majority of patients. We will attempt to develop our assay to measure Treg frequencies in tumor samples.

T cell receptor (TCR) stimulation is required for the suppressive function of Tregs. We used expression microarrays to identify functional features that are unique to stimulated Treg clones, by comparison to stimulated Thelper clones. This analysis revealed that a hallmark of stimulated human Treg clones is to produce the active form of TGF- β , a cytokine with well-known immunosuppressive actions. We are currently attempting to identify the mechanisms by which human Tregs can produce active TGF- β .

mature TGF- β , binds to GARP, a transmembrane protein containing leucine rich repeats which is present on the surface of stimulated Treg clones but not on Th clones (8). Membrane localization of latent TGF- β mediated by binding to GARP may be necessary for the ability of Tregs to activate TGF- β upon TCR stimulation. As illustrated in the figure below, a model by which activated Tregs would accumulate latent TGF- β on their surface and release its active form in close proximity to their target represents an interesting intermediate between the release of a soluble active TGF- β in the environment, and that of a Treg acting by direct contact with its target. If this model proves to be relevant, it will be important to elucidate the precise mechanism which produces active TGF- β at the surface of Tregs. Our results imply that binding to the GARP receptor is not sufficient, as lentiviral mediated expression of GARP in human Th cells induces binding of latent TGF- β to the cell surface, but does not

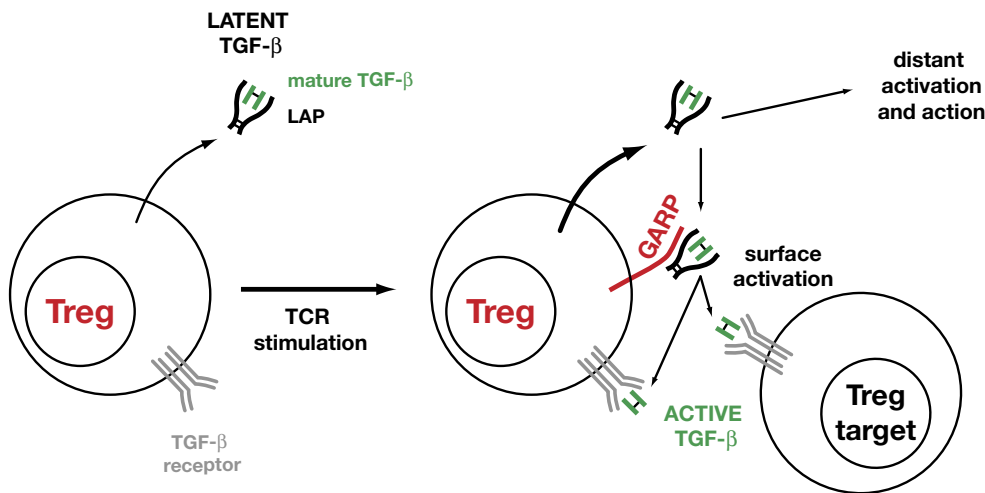


Figure 1. Possible model for TGF- β production by human Treg clones.

Many cell types, including Treg and Thelper clones, produce the latent, inactive form of TGF- β . In latent TGF- β , the mature TGF- β protein is bound to the Latency Associated Peptide, LAP, and is thereby prevented from binding to the TGF- β receptor. We recently showed that latent TGF- β , i.e. both LAP and

result in the production of active TGF- β upon stimulation of these Th cells. We are currently trying to identify additional proteins that interact with GARP, and could represent the missing link for the activation of TGF- β by human Tregs.

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