



Pierre van der BRUGGEN, Member

Didier COLAU, Senior Investigator
Danièle GODELAINE, Senior Investigator
Nathalie DEMOTTE, Postdoctoral Fellow
Sabrina OTTAVIANI, Research Associate
(until November 2009)
Claude WILDMANN, Research Associate
Violaine FRANÇOIS, Graduate Student
Emilie GAUTHY, Graduate Student
Grégoire WIEËRS, Graduate Student
Débora PICCOLO, Research Assistant
Vinh HA THI, Technician
Laurie VANBIERVLIIET, Technician (from
November 2009)

REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

The identification of tumor-specific antigens recognized by T lymphocytes on human cancer cells has elicited numerous vaccination trials of cancer patients with defined tumor antigens. These treatments have induced T cell responses but have shown a low clinical efficacy in tumor-bearing melanoma patients. We believe that progress depends on unraveling the different blockages for efficient tumor destruction. The analysis of the T cell responses of melanoma patients vaccinated against tumor antigens has led us to consider the possibility that the limiting factor for therapeutic success is not the intensity of the anti-vaccine response but the degree of anergy presented by intratumoral lymphocytes. We aim at a better understanding of dysfunctions of the immune system in tumors and more precisely T lymphocyte dysfunctions.

PREVIOUS WORK IN OUR GROUP: IDENTIFICATION OF TUMOR ANTIGENS RECOGNIZED BY T CELLS

In the 1970s it became clear that T lymphocytes, a subset of the white blood cells, were the major effectors of tumor rejection in mice. In the 1980s, human anti-tumor cytolytic T lymphocytes (CTL) were isolated in vitro from the blood lymphocytes of cancer patients, mainly those who had melanoma. Most of these CTL were specific, i.e. they did not kill non-tumor cells. This suggested that they target a marker, or antigen, which is expressed exclusively on tumor cells. We started to study the anti-tumor

CTL response of a metastatic melanoma patient and contributed to the definition of several distinct tumor antigens recognized by autologous CTL. In the early 1990s, we identified the gene coding for one of these antigens, and defined the antigenic peptide (1). This was the first description of a gene, MAGE-A1, coding for a human tumor antigen recognized by T lymphocytes.

Genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They are therefore referred to as “cancer-germline genes”. They encode tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients (2). A large

set of additional cancer-germline genes have now been identified by different approaches, including purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens. The identification of a larger set of antigenic peptides, which are presented by HLA class I and class II molecules and recognized on tumors by T lymphocytes, could be important for therapeutic vaccination trials of cancer patients and serve as tools for a reliable monitoring of the immune response of vaccinated patients (3-4). To that purpose, we have used various approaches that we have loosely named “reverse immunology”, because they use gene sequences as starting point (5).

Human tumor antigens recognized by CD4⁺ or CD8⁺ T cells are being defined at a regular pace worldwide. Together with colleagues at the de Duve Institute, we read the new publications and incorporate the newly defined antigens in a database accessible at <http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>.

A MECHANISM CAUSING ANERGY OF CD8⁺ T LYMPHOCYTES

The identification of specific tumor antigens recognized by T lymphocytes on human cancer cells has elicited numerous clinical trials involving vaccination of tumor-bearing cancer patients with defined tumor antigens. These treatments have shown a low clinical efficacy. Among metastatic melanoma patients, about 5% show a complete or partial clinical response following vaccination, whereas an additional 10% show some evidence of tumor regression without clear clinical benefit. We believe that progress depends on unraveling the different blockages for efficient tumor destruction.

The tumors of the patients about to receive the vaccine, already contain T cells directed against tumor antigens. Presumably these T cells are exhausted and this impaired function is maintained by immunosuppressive factors present in the tumor. The T cell response observed in some vaccinated patients reinforce an hypothesis proposed by Thierry Boon and Pierre Coulie: anti-vaccine CTL are not the effectors that kill the tumor cells but their arrival at the tumor site containing exhausted anti-tumor CTL, generates conditions allowing the reawakening of the exhausted CTL and/or activation of new anti-tumor CTL clones, some of them contributing directly to tumor destruction (2, 6). Accordingly, the difference between the responding and the non-responding vaccinated patients is not the intensity of their direct T cell response to the vaccine but the intensity of the immunosuppression inside the tumor. It is therefore important to know which immunosuppressive mechanisms operate in human tumors.

Human CD8 tumor-infiltrating T lymphocytes show impaired IFN- γ secretion

Human CD8 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. They were unable to secrete INF- γ or other cytokines after non-specific stimulation with anti-CD3 and anti-CD28 antibodies (7). TCR were observed to be distant from CD8 on the cell surface of TIL, whereas TCR and CD8 co-localized on blood T lymphocytes (Figure 1).

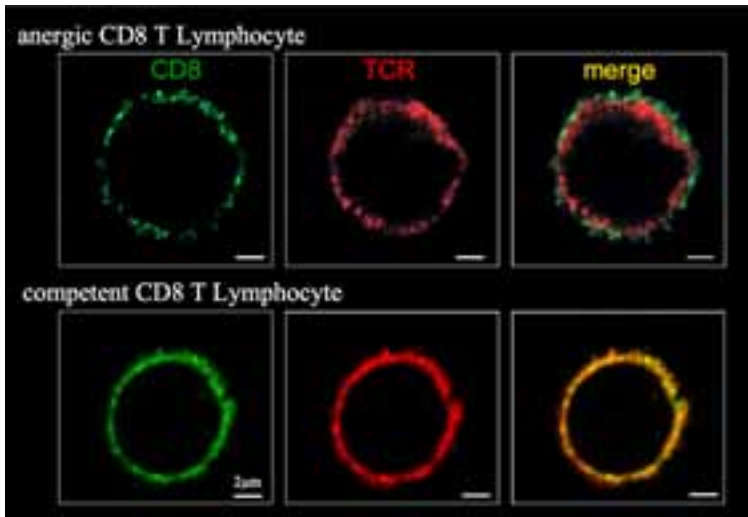


Figure 1. TCR and CD8 do not co-localize on recently stimulated CTL without effector functions.

Why do galectin-3 ligands improve human TIL function?

We have attributed the decreased IFN- γ secretion to a reduced mobility of T cell receptors upon trapping in a lattice of glycoproteins clustered by extracellular galectin-3. Indeed, we have shown that treatment of TIL with N-acetyllactosamine (LacNAc), a galectin-competitor ligand, restored this secretion (Figure 2). Our working hypothesis is that TIL have been stimulated by antigen recently, and that the resulting activation of T cells could modify the expression of enzymes of the N-glycosylation pathway, as shown for murine T cells. The re-

cently activated TIL, compared to resting T cells, could thus express surface glycoproteins decorated with a set of glycans that are either more numerous or better ligands for galectin-3. Galectin-3 is an abundant lectin in many solid tumors and carcinomatous ascites, and can thus bind to surface glycoproteins of TIL and form lattices that would thereby reduce TCR mobility. This could explain the impaired function of TIL. The release of galectin-3 by soluble competitor ligands would restore TCR mobility and boost IFN-g secretion by TIL. We recently strengthened this hypothesis by showing that CD8⁺ TIL treated with an anti-galectin-3 antibody, which could disorganize lattice formation, had an increased IFN- γ secretion.

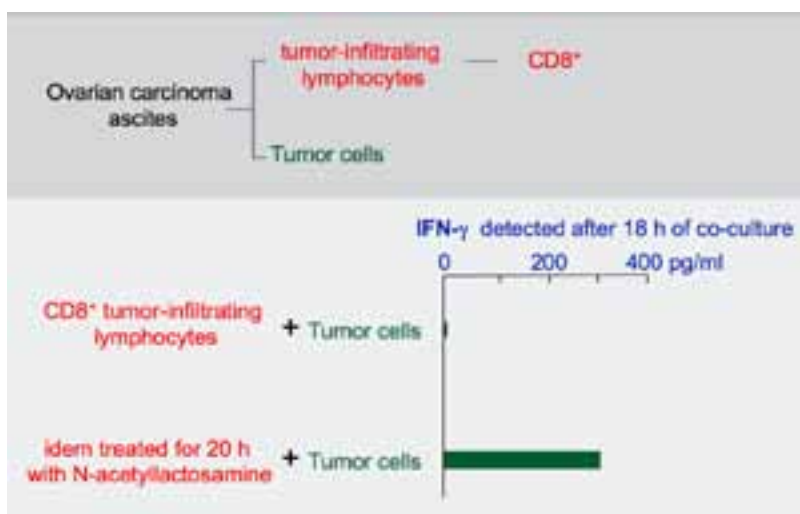


Figure 2. Treatment of tumor-infiltrating lymphocytes with a galectin ligand reverses anergy.

Towards a clinical trial combining vaccination and galectin-binding polysaccharides

These observations indicate that ex vivo human tumor-infiltrating lymphocytes can recover their effector functions with galectin-competitor ligands and suggest that treatment of cancer patients with galectin-competitor ligands could correct the anergy of TIL. It is possible that peptide vaccination combined with local injection of a galectin-competitor ligand will be more effective at producing tumor regression than vaccination alone. Galectin competitor ligands, e.g. disaccharides lactose and LacNAc, are rapidly eliminated in urine, preventing their use in vivo. Other compounds that could block interactions between galectin-3 and glycoproteins are under development by several groups. We found that a plant-derived polysaccharide, which is in clinical development, detached galectin-3 from TIL and boosted their IFN- γ secretion. Importantly, we observed that not only CD8⁺ TIL but also CD4⁺ TIL that were treated with this polysaccharide secreted more IFN- γ upon ex vivo re-stimulation. We therefore intend to pursue clinical trials involving the use of these polysaccharides in combination with anti-tumoral vaccination.

DETECTION OF ANTI-MAGE-A3 REGULATORY T CELLS IN VACCINATED MELANOMA PATIENTS

The presence of regulatory T cells, which have the ability to dampen immune responses, could participate in an immunosuppressive environment in tumors. We have analyzed the blood T cells from 14 vaccinated melanoma patients who carried the HLA-DP4 allele and whose tumor expressed MAGE-A3 (8). The vaccines involved various antigens present on

melanoma cells and all contained the MAGE-A3₂₄₃₋₂₅₈ peptide presented to T cells by HLA-DP4. The vaccines were either a mix of peptides without adjuvant, autologous mature dendritic cells loaded with peptides, or a MAGE-A3 protein mixed with adjuvant and combined with peptides. Our approach, outlined in Figure 3, involved the ex vivo selection of CD4⁺ T cells that were labeled by DP4 MAGE-A3 tetramer and amplified under clonal conditions. A total of 197 tetramer⁺ stable clones were isolated from 10 out of the 14 patients. Each of the 197 tetramer⁺ CD4⁺ T cell clones recognized the MAGE-A3.DP4 antigen. Anti-MAGE-A3.DP4 cells were found in 1 out of 2 patients injected with a mix of peptides without adjuvant, in 3 out of 6 patients injected with peptide-loaded dendritic cells, and in each of the 6 patients injected with a MAGE-A3 protein mixed with adjuvant and peptides. The frequencies in the blood samples collected after at least four vaccines ranged from 2×10^{-6} to 2×10^{-3} among the CD4⁺ blood T lymphocytes. We found no correlation between the frequencies of anti-MAGE-A3.DP4 T cells and the clinical evolution of the patients, but the very small number of patients and the diversity of the vaccines preclude any conclusion.

Interestingly, 12 out of 197 clones expressed CD25 in resting state. This CD4⁺CD25⁺ phenotype was evocative of T cells with suppressive activity, known as regulatory T cells. Because the antigen recognized by our CD4⁺ T cell clones was known, we designed a suppression assay where the potential regulatory T cell clone and an indicator T cell clone are each stimulated with their specific antigen presented by irradiated EBV-B cells. These twelve CD25⁺ clones had a high capacity to suppress in vitro the proliferation of another T cell clone. Eleven of them had a high FOXP3 expression at rest and an unmethylated *FOXP3* gene. They secrete upon stimulation no IFN- γ , IL-2, IL-4, IL-5 or IL-10, but they produce active TGF- β . Their suppressive activity in vitro seems partly attributable to their secretion of active TGF- β . These regulatory T cell clones represent about 5% of the anti-MAGE-A3.DP4 T

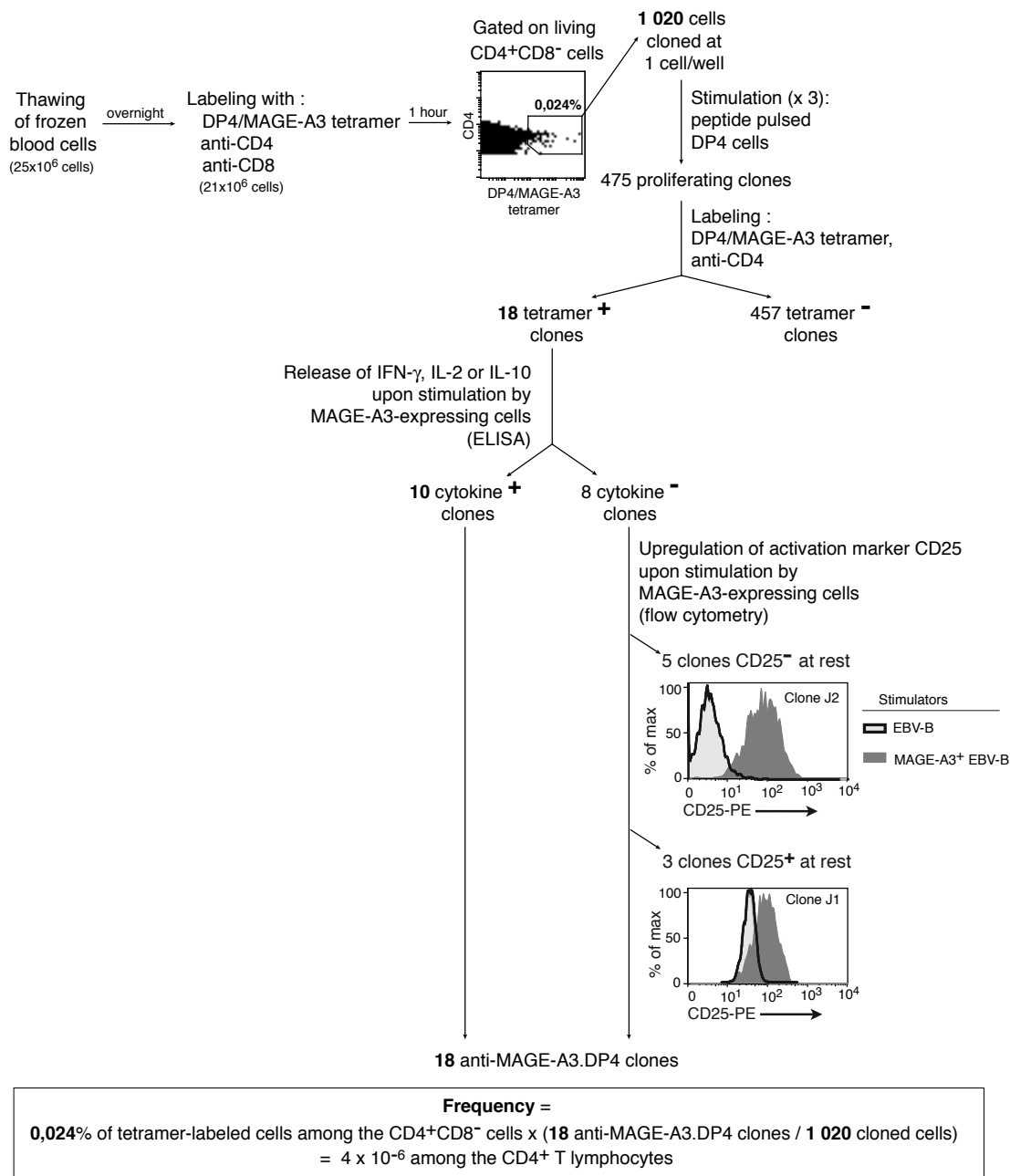


Figure 3. Example of the procedure used to obtain anti-MAGE-A3.DP4 T cell clones from blood cells of vaccinated patients. The numbers indicated correspond to an experiment performed with blood cells of patient EB97 collected after the sixth vaccination.

cell clones that we have isolated. In addition to these clones with an unmethylated *FOXP3* gene, several CD25 clones had a significant but lower suppressive activity, expressed *FOXP3* in the resting state but *FOXP3* demethylation was not observed.

This work is the first to describe the presence of anti-vaccine regulatory T cells not only on the basis of markers such as *FOXP3*, but also on the basis of their suppressive activity in vitro. *FOXP3* seems of doubtful value as unique marker for regulatory T cells, due to its transient expression in some activated non-

regulatory T cells and also in some of our resting T cells without suppressive activity. Exclusive to T cells with a suppressive activity and a stable expression of FOXP3 is the demethylation of an intronic sequence of FOXP3. Thus, a quantitative DNA methylation analysis of FOXP3 based on RT-PCR could become a routine technique to identify what, in our opinion, are the best regulatory T cell candidates.

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Pierre van der Bruggen

Ludwig Institute for Cancer Research

Av. Hippocrate 74-75

B - 1200 Brussels

[T] +32 02 764 74 31

[F] +32 02 762 94 05

[E] Pierre.vanderbruggen@bru.licr.org

[W] http://www.deduveinstitute.be/regulation_lymphocyte.php