

## The Ludwig Institute for Cancer Research

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Cancer is a major concern in human health. The prospects for bringing cancer under control require linked innovative basic and clinical research. In this view, Daniel K. Ludwig created in 1971 the Ludwig Institute for Cancer Research, an international organization bringing together scientists and clinicians from around the world. Ludwig investigators are recognized leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.



**Thierry Boon**

Faithful to the organizing principles laid down by Mr Ludwig, the Institute conducts its research through ten Branches, located in seven countries. The Branch structure allows the Institute to interact with a number of different research and clinical environments. Each Branch is focused on a research program defined by the Branch Director in relation with the overall objectives of the Institute. The Branches are established in association with University Hospitals, to stimulate close collaborations between research laboratories and the clinic. By organizing and controlling its own clinical trials programs, the Institute has indeed created a continuum that integrates laboratory and clinical research.

Branch staffs vary in size from 30 to over 70, and internationally the Institute employs some 600 scientists, clinicians and support personnel. The quality of the research is monitored on an ongoing basis by the Institute's Scientific Committee and by an external peer review process.

The biological properties of any given cancer cell constantly change, allowing tumors to spread and become more aggressive. To overcome these obstacles, the Ludwig Institute has developed a broad-based discovery program that seeks to understand the full complexity of cancer. Research is organized according to the four major programmatic themes that define the Institute : genetics, cell biology, cell signalling and immunology.

# I. TUMOR IMMUNOLOGY AND IMMUNOTHERAPY

## OVERVIEW

T lymphocytes are capable of destroying cells that harbour viruses because they recognize viral antigens on their surface. They do so with great efficacy and specificity and, together with the anti-viral antibody response, they usually provide full recovery from viral diseases. For cancer the situation is very different : clearly the immune system does not eliminate most overt cancers. But cancer cells have long be known to differ from normal cells in many respects and it has been a long-standing hope of immunologists that cancer cells express abnormal antigens which could be recognized by the immune system. The existence of tumor-specific antigens was considered to have two major implications : the existence of cancer immunosurveillance and the feasibility of cancer immunotherapy. The hypothesis of cancer immunosurveillance implies that most cancers are eliminated at a very early stage by an immune rejection process. One could add that the immune system, while failing to eliminate some tumors, may nevertheless slow them down at all stages of their progression. The existence of immune surveillance has long been controversial but it is supported by recent evidence obtained with mouse tumors. Cancer immunotherapy is based on the notion that it is possible to artificially improve the response to tumor antigens to make it reach its full potential. Unlike responses directed against viral antigens, anti-tumoral responses may not have been perfected throughout evolution, because escaping cancer probably conferred little or no selective advantage.

Our interest in tumor immunology started with a fortuitous observation made with a mouse tumor which was strictly non-immunogenic. Mice from which this tumor was removed by surgery did not show any protection against a challenge with the same tumor cells. We observed that by treating the tumor cells in vitro with a mutagen we obtained tumor cell mutants that were rejected in the mice by a T lymphocyte mediated process (1). Remarkably the mice that had rejected these "tum-" mutants showed a degree of protection against a challenge with the original non-immunogenic tumor cells (2). The pattern of protection indicated that the tum- mutants owned their phenotype to the acquisition of new strong tumor rejection antigens. The response against these antigens evidently created conditions that facilitated a response against weaker antigens present on the original tumor, a phenomenon which has recently been named "epitope-spreading". These observations were found to apply to all mouse tumors, including spontaneous tumors (3). This led to two conclusions. First, all mouse tumors bear tumor-specific antigens recognized by T cells even though many of them are non-immunogenic.

Second, it is possible to create conditions that favor the T lymphocyte responses against the tumor antigens.

On the basis of these findings we launched an effort to identify the antigens recognized on mouse tumors by T cells. We focused our effort on mouse tumor P815. A first step was to obtain in vitro cytolytic T lymphocytes (CTL) that specifically lysed the P815 cells (4, 5). Then, with considerable help from Jean-Charles Cerrotini and other members of the Lausanne branch, we obtained stable CTL clones directed against tumor antigens of P815. Antigen-loss mutants of P815 were obtained in vitro by selection for resistance to lysis by a CTL clone (6). In addition, we observed that antigen-loss is a mechanism which is used by P815 tumors to escape T cell responses in vivo. The next step was the production of genomic libraries from antigen-bearing cells and the transfection of the DNA into antigen-loss variants. Antigenic transfectants could be detected on the basis of their ability to stimulate the proliferation of the relevant CTL clone and the genes coding for the antigens could be retrieved from these transfectants by using appropriate cosmid technology (7).

About at that time, Alan Townsend showed that influenza virus antigens derived from proteins that remained inside the cells could be detected by T cells. These antigens are recognized by T cells as small peptides presented by class I major histocompatibility complex (MHC) molecules. Our contribution was to show that this also applied to the proteins encoded by the genome of the cell (7, 8). Thus there is a permanent T cell based immune surveillance of the cellular genome and genetic abnormalities can result in the presentation of new antigens leading to elimination by T cells. Our results also demonstrated that there are two major genetic processes that produce tumor-specific antigens. The first is the acquisition of mutations by the cancer cell, which generate peptides which, because of an amino-acid change, either become capable of binding to MHC molecules or contain a new epitope (8). The second is the expression by the tumor of a gene which is not expressed in the normal cells of the adult (9)(Fig. 1).

Around 1985 we began to examine whether the results obtained in mice could be extended to man. We focused our efforts on melanoma because, contrary to most tumors, samples of metastatic melanoma can be converted into stable cell lines fairly frequently. Stimulation of T lymphocytes with autologous melanoma cells produced cytolytic T cells that appeared to lyse the tumor cells specifically. Stable CTL clones were obtained and immunoselection of antigen-loss variants indicated that, like mouse tumors, human tumors express not one but several antigens that are recognized by autologous CTL (10, 11). A cosmid genomic library derived from a tumor cell was then transfected into an antigen-loss variant and this led to the identification of the first gene coding for a human tumor-specific antigen recognized by T cells (12). This previously unknown gene was named *Mage* and it was soon found to be expressed in many melanomas and not in normal cells.

GENETIC EVENTS PRODUCING TUMOR-SPECIFIC ANTIGENS RECOGNIZED BY T LYMPHOCYTES

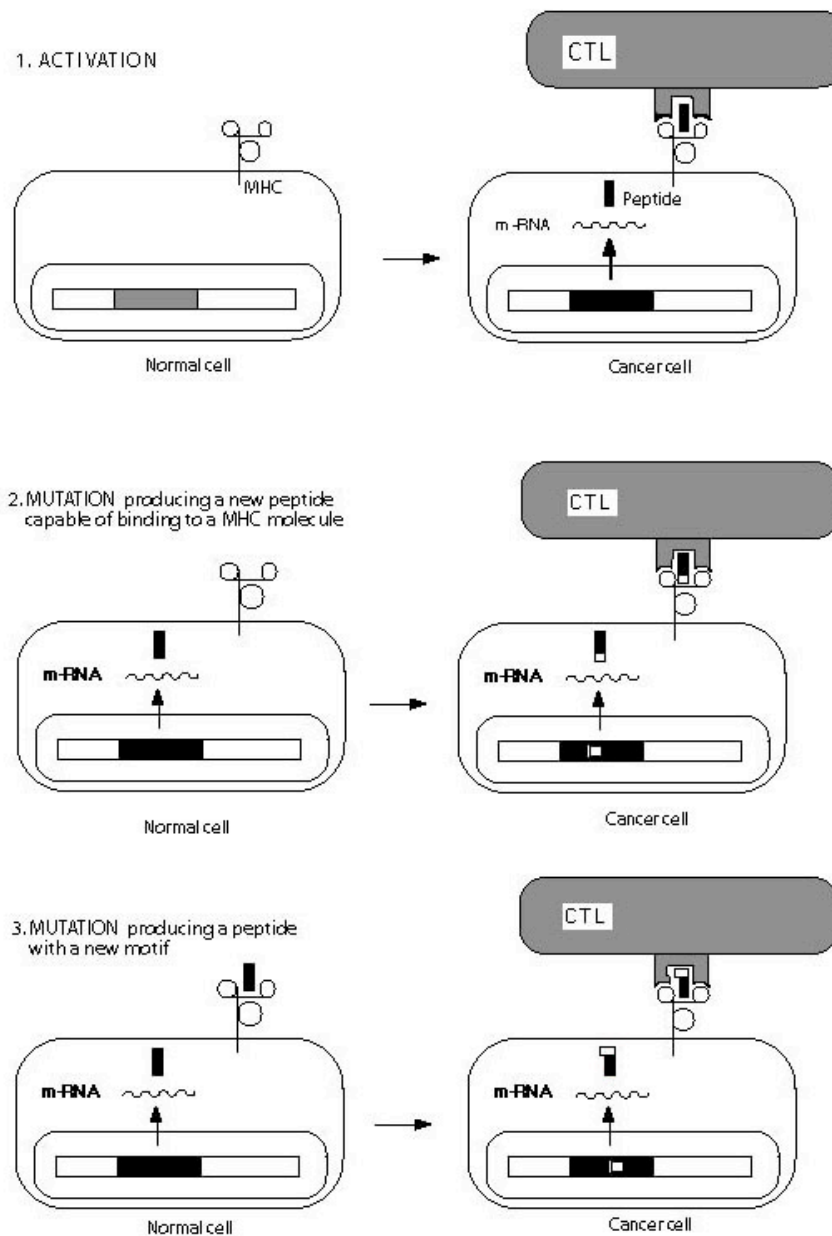


Fig.1

Gene *Mage* belongs to the *Mage-A* gene cluster, which comprises 12 genes (13). It is located on the X chromosome. The homologous families *Mage-B* and *C* are located on the same chromosome. All these genes have the same expression pattern. They are expressed in many tumors of various histological types and not in normal cells with one exception, male germline cells (13). It is therefore

appropriate to refer to these genes by the term cancer-germline genes. It is important to note that the germline cells do not express HLA molecules and therefore cannot present antigens to T cells. The antigens encoded by *Mage* genes appear therefore to be strictly tumor-specific as far as T cell responses are concerned, contrary to what is suggested by the term cancer-testis antigen which is often used to refer to these antigens. The activation of *Mage* genes in cancer and germline cells is not due to the presence of specific transcription factors. It is caused by the demethylation of the promoter in these cells (14). In cancer cells this appears to be a consequence of a global demethylation, which is observed in many cancer cells.

Using similar approaches we identified other families of cancer-germline genes, such as the *Bage* and *Gage* families (15, 16). cDNA enrichment approaches capitalizing on the cancer-specific pattern of expression was used to find additional cancer-germline gene families such as *Lage*, *Sage* and *Hage*. Another approach, Serex, based on antibody responses of cancer patients was initiated by Michael Pfreundschuh. It enabled him to identify the SSX and SCP-1 families. Using this approach, members of the New York Branch identified a second member of the Lage family, named NY-ESO-1, shortly after our discovery of *Lage-1* (17). It is likely that by now most of the cancer-germline genes have been identified.

Antigens encoded by cancer-germline genes ought to be very suitable as therapeutic vaccines for cancer-patients as they are strictly tumor-specific and present on a large proportion of tumors. But genes that are strongly overexpressed in cancer cells relative to normal cells could also be a source of acceptable antigens for cancer immunotherapy. An example is gene *Prame*, which is expressed at a high level in a very large proportion of melanoma and other tumors (18).

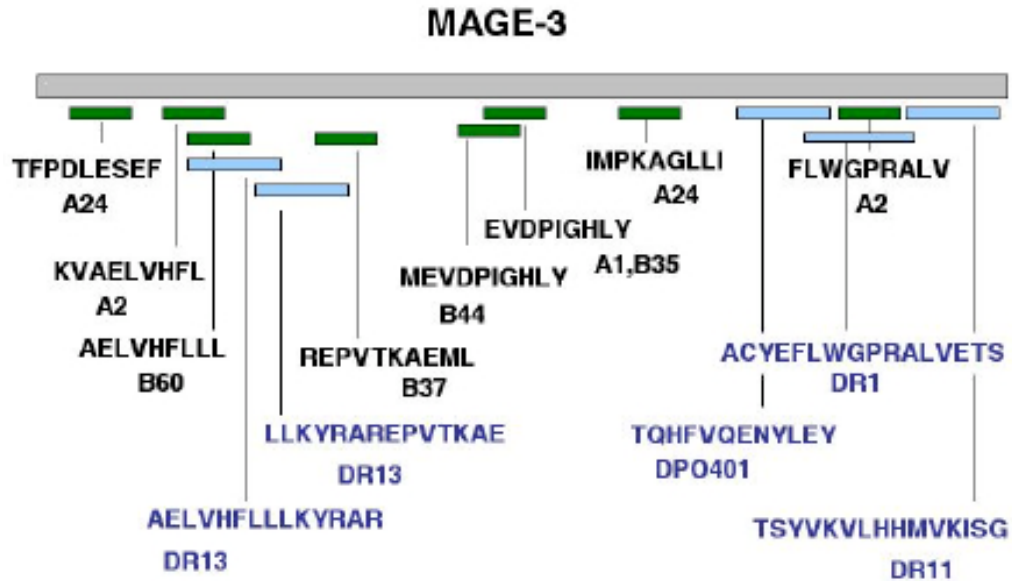
Gene mutation was also found to be a major source of human tumor-specific antigens. Interestingly, several mutations that were found to be antigenic also appear to play a role in oncogenesis. One interesting example is a mutation in cyclin-dependent kinase-4, which prevents the binding of this protein to P16 (19). As a result CDK4 permanently phosphorylates Rb causing excessive cell cycling. Another example is a mutation in caspase 8, which reduces the sensitivity of cells to pro-apoptotic factors (20). Unfortunately, when a tumor-specific antigen results from a mutation it is expressed on an extremely small proportion of tumors. This precludes the use of this class of tumor-specific antigens as cancer vaccines.

Finally, we observed that CTL of melanoma patients can respond to antigens encoded by melanocytic differentiation genes, such as tyrosinase and Melan-A (also referred to as Mart-1)(21, 22). This is surprising as one would have expected natural tolerance to put a tight block on responses against such self antigens. Other groups proceeded to vaccinate melanoma patients with these antigens and observed tumor regressions in some patients.

The peptides that are presented by MHC molecules at the cell surface result from the degradation of intracellular proteins by the proteasome in the cytosol. The peptides are then transported into the endoplasmic reticulum where they combine with newly synthesized MHC molecules on their way to

the cell surface. The proteasome plays a central role in this pathway, known as the class I antigen processing pathway. Some cells, such as dendritic cells and cells exposed to interferon-gamma, express a different type of proteasome named immunoproteasome, whose catalytic activity is slightly different from that of the standard proteasome. We have shown that a number of human tumor antigens are not produced with the same efficiency by the two proteasome types, some of them are produced only by the standard proteasome whereas others are produced exclusively by the immunoproteasome (23, 24). This means that the peptide repertoire displayed at the cell surface depends not only on the proteins expressed by the cells but also on the type of proteasome they harbor. This parameter should be considered in the context of immunotherapy.

For the vaccination of cancer-patients with a tumor antigen recognized by T lymphocytes, several forms of the antigen can be used. These include antigenic peptides, whole protein, recombinant defective viruses carrying a sequence coding for the antigen, or naked DNA. In addition, dendritic cells derived from blood cells of the patient can be reinfused into the patient after being pulsed with antigenic peptides or protein or after being transfected with encoding cDNA or RNA. The simplest vaccines are the antigenic nona- or decapeptides. However each peptide binds and can be presented only by the protein encoded by one or a few HLA alleles. It is therefore necessary to identify many antigenic peptides to be able to provide an adequate vaccine to the majority of cancer patients. The identification of these antigenic peptides on the basis of the gene sequence involves the obtention of a T cell clone that recognizes this antigen. We have devised approaches to obtain such CD8 T cell clones by stimulating T cells with dendritic cells infected with a recombinant virus carrying the encoding gene (25). CD4 T cell clones have been obtained by stimulation with dendritic cells pulsed with the relevant protein. As a result we have now identified a large number of antigenic peptides encoded by genes *Mage-1* and *Mage-3* (Fig.2).



**Fig.2**

Our first clinical trial involved the vaccination of metastatic melanoma patients with an antigenic peptide which is encoded by *Mage-3* and presented by HLA-A1. Seven patients out of 26 showed evidence of tumor regression (26, 27)(Fig.3). No toxicity was observed. We then examined in a series of small trials whether changes in the vaccine modalities would significantly improve the outcome. We tried more frequent injections of the Mage-3.A1 peptide, combination with an adjuvant or combination with another Mage peptide binding to either a class I or class II HLA molecule. No improvement was observed. Similar results were observed after vaccination with the Mage-3 protein or with an ALVAC recombinant virus coding for *Mage* sequences. In these trials we failed to detect CTL responses against the vaccine. Even though our approach lacked sensitivity, this indicated that the CTL responses were weak at best. To sum up the observations made on about 200 patients, we can say that some evidence of tumor regression is observed on about 20% of the patients with half of them, i.e. 10%, showing complete or partial clinical responses.

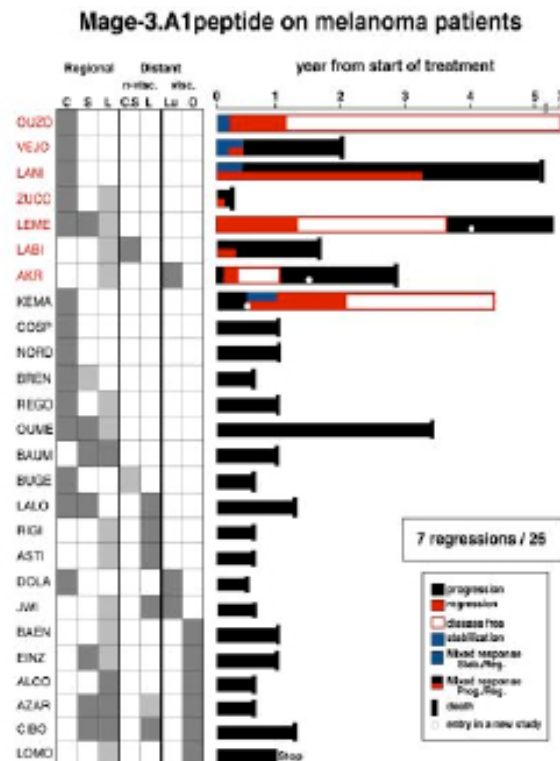


Fig.3

To explain why our vaccinations fail to exert any detectable effect on the tumors of 80% of the patients one can consider two, unfortunately non-exclusive, causes. First, the anti-vaccine CTL response might be inadequate. Second, the tumor might be resistant to immune attack. We set out to analyze the first possibility, considering that if the level of the CTL response to the vaccine was a limiting factor for clinical efficacy, we ought to observe a correlation between the observations of CTL responses and those of tumor regressions. We therefore developed highly sensitive approaches for the detection of CTL responses combined with an analysis of the T cell receptor diversity of the responding CTL (28). These approaches are beginning to show a correlation between CTL responses and tumor regressions. If this trend is confirmed, it will be crucially important to understand why some patients make CTL responses to the vaccines whereas others do not. When patients vaccinated with antigenic peptides or vaccinia-like recombinant viruses produce a T cell response, it is monoclonal (28). In contrast, patients vaccinated with peptide-pulsed dendritic cells produce either polyclonal responses or no response. We will engage in a systematic study of patients recruited in trials involving dendritic cell vaccination to try to identify factors in their pre-vaccination state that influence their propensity to make a T cell response to the vaccine.

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