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The discovery of Cancer/Testis antigens by autologous typing with T cell clones and the evolution of cancer vaccines

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Introduction

In the second half of the 1970s, Lloyd J. Old and his group at Memorial Sloan-Kettering Cancer Center (MSKCC) discovered the first human cancer antigens by establishing an approach called "autologous typing." The key preconditions of these discoveries were improvements in establishing human cancer cell lines from fresh tissue specimens and cultures from autologous healthy tissues, including fibroblasts and epithelial cells from different organs, as well as Epstein-Barr virus (EBV)-transformed B lymphocytes.

Based on the assumption that cancer cells are recognized by the immune system and thus elicit a cellular and humoral immune response, we postulated that serum and T cells from cancer patients may be used as tools to identify immunogenic tumor-associated antigens. Because methods to generate T cell lines and clones *in vitro* were not established yet, the first experiments to discover tumor-associated antigens were performed with patients' sera that were pre-absorbed extensively with autologous, allogeneic, and xenogeneic healthy cells. Subsequently, such sera were incubated with autologous cancer cells, which resulted in the discovery of a larger series of new human cancer antigens in different types of cancer like melanoma, leukemia, renal cell, brain, and others (1). As antibodies against these newly discovered cell surface antigens of human cancer generally were present in a rather low concentration, attempts to boost humoral immune responses *in vivo* were undertaken by vaccination with *in vitro*-expanded and lethally irradiated autologous cancer cells.

In the midst of these studies, a new era was ushered in when Georges Köhler and César Milstein developed a method to immortalize antibody-producing B cells (hybridomas) for which they were awarded the Nobel Prize in 1984 together with Niels Jerne (2). This new technique enabled the production of an unlimited amount of antibodies with a known and single specificity and opened a Pandora's Box for the discovery of multiple cell surface antigens in cancer and other tissues. The hope for antibodies as therapeutic reagents dominated the decade to follow.

In the late 1970s, methods to expand cytotoxic T lymphocytes (CTLs) after antigenic stimulation *in vitro* were developed (3, 4), which enabled the search for tumor-associated antigens that are recognized by T cells. One major advantage of T cells over antibodies as a discovery tool is that T cells also recognize antigens that are not expressed on the cell surface.

We initially focused on melanoma because we had established autologous sets of tumor cells and T cells mainly from this group of patients. At MSKCC, we started with a patient named A.V. who had stage IV disease (SK-MEL-29) and had been subjected to multiple surgical interventions without ever achieving tumor-free surgical margins. From the blood of this patient, we established T cell cultures that were reactive to autologous SK-MEL-29 melanoma cells *in vitro*. With the tools available at the time, an extensive specificity analysis was done showing tumor-restricted reactivity at the clonal level (5). Because A.V. had the strongest autologous T cell reactivity of a total of 13 melanoma patients, whom we extensively studied over the following years, we interpreted the fact that he was alive and free of disease for more than 30 years after his initial diagnosis by cancer immune surveillance.

Antigen discovery - the *MAGE* gene family

After returning to the University of Mainz in Germany in 1981, we identified another patient with strong T cell reactivity against autologous tumor cells in culture. This patient, MZ-2, had a stage IV amelanotic melanoma of an unknown primary tumor with metastatic disease to one kidney, the ovaries, lymph nodes, and spleen. Multiple surgical interventions followed by chemotherapy never achieved a complete remission.

Thierry Boon and his group at the Brussels Branch of the Ludwig Institute for Cancer Research (LICR) had shown in syngeneic murine cancer models that spontaneous non-immunogenic tumors could be rendered immunogenic by mutagenesis-induced expression of tumor rejection antigens that were recognized by cytotoxic T cell clones. When challenged with the original syngeneic tumor, immune protection was observed (6). As most human tumors at the time were considered 'non-immunogenic' or 'poorly immunogenic,' Thierry Boon's approach appeared as a reasonable option to be applied to human tumors.

Because we had already identified T cell reactivity in two cancer patients, A.V. (SK-MEL-29) and MZ-2, Lloyd Old contacted us and suggested a collaboration between Mainz and Brussels. A.K. paid a first visit to the Brussels Branch in 1984 to present his data of autologous typing with T cell clones in human melanoma. This was the start of an intensive and fruitful collaboration over many years.

We generated MZ-2 melanoma cell clones and subjected these to *in vitro* mutagenesis according to the work of Thierry Boon and his group. Subsequently, we injected those lethally irradiated cells intradermally into patient MZ-2. Delayed type hypersensitivity (DTH) and specific T cell reactivity were closely monitored over many months and showed increasing DTH responses. A local recurrence at the site where the metastatic spleen had been removed occurred early during this intradermal vaccination period. However, before the surgeons could remove it, this metastasis disappeared under continued vaccination with autologous melanoma cell clones. As the vaccinations were continued at regular intervals, the detectable T cell reactivity increased, allowing us to derive stable tumor reactive T cell clones. The patient survived more than 30 years without ever suffering recurrent disease.

Thierry Boon and his group had just cloned murine mutagenesis-induced tumor rejection antigens with a T cell-driven approach (7, 8) and then turned toward the identification of human tumor antigens in our MZ-2 melanoma model. In a first step, antigen loss variants of MZ-2 tumor cell clones were selected using MZ-2-derived CTLs. These loss variants were then transfected with MZ-2 DNA from a cosmid library generated from antigen-expressing MZ-2 melanoma clones. Transfected MZ-2 melanoma cells were tested for recognition by CTL clones, which led to the discovery of the *MAGE* (for melanoma antigen) gene family encoding the first human tumor antigen recognized by autologous T cells (9). Subsequently, multiple antigens were identified as targets of autologous T cells in the MZ-2 melanoma model. Among these are additional members of the *MAGE* gene family, as well as members of the *BAGE* and *GAGE* gene families (10). Analysis of the expression pattern of the *MAGE*, *BAGE*, and *GAGE* genes revealed that expression was only found in cancer cells and in germ cells of the testis, but not in any other normal tissues. After the discovery of more genes with a similar expression pattern, the term Cancer/Testis (CT) or cancer/germline family of antigens was later coined (11, 12).

Other T cell-defined human cancer antigens

Using similar approaches, Vincent Brichard, Aline van Pel, Pierre Coulie, and others of the LICR Brussels Branch identified and cloned the differentiation antigens tyrosinase and Melan-A from A.V. (SK-MEL-29) and another patient, LB39, which was a first indication that differentiation antigens can also be targets for tumor recognition and tumor rejection in human cancer (13, 14). At the same time and independently, the same melanocyte differentiation antigen Melan-A was discovered by Yutaka Kawakami in Steven Rosenberg's group at the National Cancer Institute (NCI) and was called MART-1 (15).

Thomas Wölfel and colleagues of our group in Mainz found a somatic mutation in the SK-MEL-29 (A.V.) melanoma that was efficiently targeted by A.V.'s CTLs. The antigen was a mutant cyclin-dependent kinase 4 (CDK4) that was insensitive to the tumor suppressor $p16^{INK4a}$ (16). Later, this mutation was found to occur also in the germline of melanoma families where it functions as a dominant oncogene due to the disrupted cell cycle regulation by tumor suppressor $p16^{INK4a}$ (17). Subsequent analysis of stored SK-MEL-29 (A.V.) lymphocytes uncovered that the majority of anti-melanoma T cells in the patient's blood were specific for this particular CDK4 mutation, an arginine-to-cysteine exchange at residue 24 (18). This in fact may have been a dominant tumor rejection antigen in patient A.V., who experienced a remarkable clinical course with long-term survival despite the fact that he never could be operated with tumor-free surgical margins. It is tempting to speculate that the strong and diverse T cell reactivity in both patients MZ-2 and A.V. kept them free of disease and saved their lives.

The SEREX technology

As technologies in molecular cloning further developed, Michael Pfreundschuh and his group discovered and established in the mid-1990s a molecular cloning technique for human cancer antigens by DNA expression cloning and autologous serum reactivity as a detection system. This serological strategy, termed SEREX, was a major breakthrough (19) and became a major tool for antigen discovery in the decade to follow, being employed by Pfreundschuh and his group and subsequently by many other researchers around the world.

The most striking experience was the fact that many of the antigens discovered by the tedious epitope cloning using T cells as a detection tool were rediscovered by the SEREX technology. Importantly, this indicated that antigens recognized by T cells may induce humoral immune responses at the same time.

Developing a cancer vaccines program

Hans-Georg Rammensee and his group in Tübingen, Germany, were pioneers in the identification of minimal peptide epitopes for T cell recognition and their anchor residues in presenting MHC molecules (20). This work helped to identify peptide epitopes and HLA class I binding motifs from tumor antigens for early clinical trials that aimed to stimulate anti-cancer immunity.

Lloyd Old had the early vision to develop a cancer immunotherapy program including well-controlled clinical trials and extensive immune monitoring. It was his mission to provide clinical grade reagents, and to develop and share standardized means of immune monitoring and clinical trials management with highest standards independent from commercial interests of industry. As CEO of the LICR, he put major emphasis and funds into the establishment of a coordinated program with selected sites all over the world for the development of this cancer immunotherapy program.

As affiliates, we became part of the early clinical trials program of the LICR with the establishment of local clinical trial centers at selected academic sites. We developed early intradermal vaccination approaches with GM-CSF as an adjuvant and DTH as a readout for vaccine-specific immune responses. In addition, the development of assays to determine cytokine expression patterns and T cell profiles in DTH sites were important early contributions to the field. It was only because of the stringent immune monitoring, an integral part of all clinical trials, that we could recognize that the solubility of antigen, recall effects with repeated vaccinations at different sites, and specific T cell induction for the immunizing antigens were important variables in the assessment of early cancer vaccines.

Yao-Tseng Chen discovered the Cancer/Testis antigen NY-ESO-1 in 1997 (21). At the time, we observed a patient, NW38, with strong humoral and cellular reactivity against the autologous tumor. As none of the tumor antigens known at the time appeared to be the target in this elderly patient, we checked for the newly discovered NY-ESO-1 as the potential target. We found that patient NW38 had a high level of NY-ESO-1-specific IgG in the blood (titers up to 1:1,000,000), as well as a strong NY-ESO-1-specific T cell reactivity. Subsequently, we identified the relevant minimal T cell epitopes and their HLA class I

restriction elements. In the meantime, spontaneous immune responses to NY-ESO-1 have been detected in many cancer patients with different types of cancer and, based on cumulative evidence, NY-ESO-1 is currently considered as one of the most immunogenic human cancer antigens (22).

This knowledge prompted us to consider NY-ESO-1 as our first choice for cancer vaccines. The LICR and Cancer Research Institute (CRI) joined forces and established the Cancer Vaccine Collaborative in 2001 with a coordinated program of multiple parallel clinical trials with NY-ESO-1 as the focus. NY-ESO-1 peptides, recombinant protein, and recombinant NY-ESO-1 viral constructs were used alone or combined with different adjuvants to explore the most immunogenic method of therapeutic vaccination. Clinical outcomes were correlated with immunological findings, and several groups showed that favorable clinical courses of disease were associated with measurable immune responses, detectable intratumoral lymphocyte accumulation, and prolonged exposure to the vaccine.

Outlook

More recently, NY-ESO-1 came to fame as a target for an antibody-facilitated vaccination approach against this intracellular cytoplasmic antigen. Hiroyoshi Nishikawa and colleagues discovered that a NY-ESO-1-specific antibody enhanced the cellular and secondary humoral immune responses against NY-ESO-1-expressing tumors when given together with chemotherapy. Importantly, the enhanced immune response impacted on tumor size and survival in a preclinical mouse model using NY-ESO-1 transfected syngeneic cancer cells. This discovery was recently published in *Cancer Research* and covered as a "Highlight" in the cancer literature (23).

As cancer patients often have high levels of antibodies against NY-ESO-1, and sometimes against other CT antigens as well, we postulated that the efficacy of conventional cancer therapies that release intracellular antigens, for example chemotherapy or radiotherapy, may be enhanced by systemic application of NY-ESO-1-specific antibodies. A first human NY-ESO-1 antibody has recently been cloned from a patient with exceptionally high anti-NY-ESO-1 titers and a remarkably favorable clinical course. This human monoclonal antibody, 12D7, is currently in clinical development. It was Lloyd Old's input and vision to try this new approach, which he liked to call Antibody-Facilitated T Cell Induction in Cancer (AFTIC) as a new and promising option to integrate cancer immunotherapy into existing treatment modalities in cancer.

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