Cancer/Testis Antigens in Non Small Cell Lung Cancer: expression and immunogenicity

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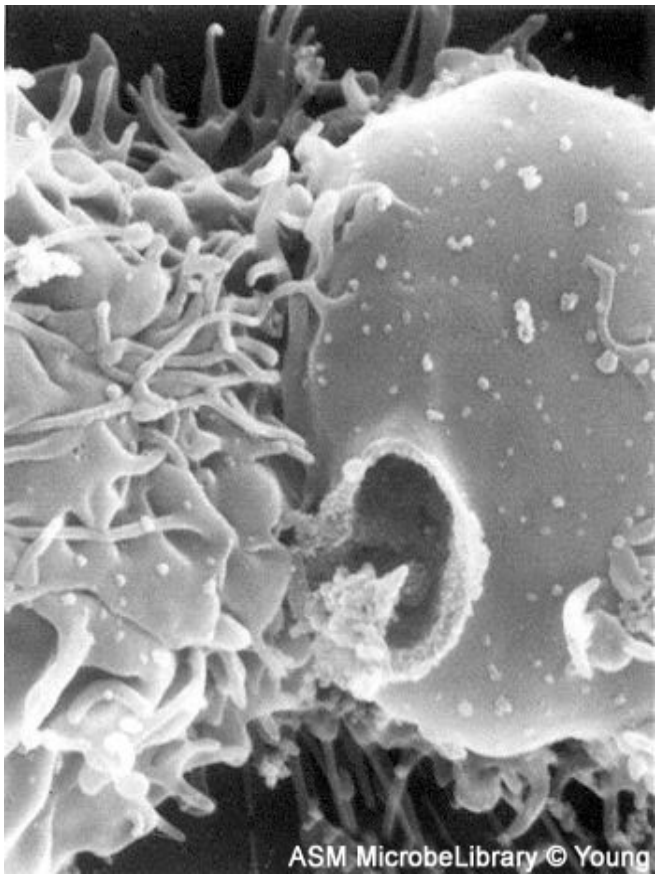

Cytotoxic Lymphocyte destroying a Tumor Cell.

The outer membrane of the tumor cell (right) has been shot full of holes by perforin, secreted from the cytotoxic T lymphocyte (left).

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Abbreviations:

APC	antigen presenting cell
CD	cluster of differentiation
CPE	cytopathic effect
CTA	cancer/testis antigen
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
EBV-BL	Epstein Barr virus-B lymphocytes
EDTA	ethylene diamine tetraacetic acid
ER	endoplasmic reticulum
ET ratio	effector to target ratio
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanite
HBSS	Hanks' balanced salts solution
IDO	indoleamine 2,3-dioxygenase
m.o.i	multiplicity of infection
MAGE	Melanoma antigen E
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHD	MAGE homology domain
MPA	mycophenolic acid
NSCLC	Non Small Cell Lung Cancer
PBL	pheripheral blood lymphocytes

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
qPCR	quantitative PCR
RNA	ribonucleic acid
RT	reverse transcriptase
SCLC	Small Cell Lung Cancer
TAA	tumor associated antigen
TGF	transforming growth factor
TIL	tumor infiltrating lymphocytes
TNF	tumor necrosis factor
TRIS	tris(hydroxymethyl)aminomethane
WT	wild type

1 Summary

Lung cancer is the leading cause of cancer-related mortality in the world, whereby Non Small Cell Lung Carcinomas (NSCLC) constitute 80% of all lung tumors. Whereas in Stage I and II NSCLC surgical resection with or without adjuvant chemotherapy currently represents the most frequently applied treatment, in late stage NSCLC, representing 70% of all cases, chemotherapy or radiotherapy are mainly palliative. Thus, the poor prognosis and the limited therapeutic options available urge the development of new approaches. Among these, active specific immunotherapy targeting Cancer/Testis Antigens (CTA) might represent a valuable additional treatment in NSCLC.

CTA have been shown to represent promising targets in different types of cancer as they are silent in healthy adult tissues except in testis and placenta. These tissues do not present antigenic epitopes as they are deficient in MHC expression. Moreover, CTA are expressed by various tumors of different histology, stage and grade, and in some tumors, expression has been found to be correlated with poor disease specific survival.

In this study first the prevalence and expression patterns of several CTA (MAGE-A1, -A2, -A3, -A4, -A10, -A12 and NY-ESO-1) in freshly excised NSCLC were investigated at gene and protein level. Tumor specimens (12 adeno-, 17 squamous cell and 4 large cell carcinomas) were obtained from HLA-A*0101 and/or HLA-A*0201 positive patients. CTA expression was detected in five adeno-, eight squamous cell and in two large cell carcinoma samples (45.5%). MAGE-A10 and -A12 were the most frequently (10/15 and 12/15 specimens, respectively) and MAGE-A1, -A4 and NY-ESO-1 the least frequently expressed genes (6/15, 6/15 and 4/15 specimens, respectively). In 10/15 positive cases at least four CTA genes were concomitantly expressed. These results at gene level were widely confirmed by protein detection, the few discrepancies being explained by focal CTA expression limited to defined tumor areas.

Immune responsiveness towards MAGE-A1 and -A3 (HLA-A*0101 restricted), MAGE-A4, -A10, multi-MAGE-A (an epitope shared by several MAGE-A antigens) and NY-ESO-1 (HLA-A*0201 restricted) epitopes was evaluated in cancer patients to assess whether a specific cellular response could be detected or generated upon *ex*

vivo stimulation. Induction of CTL was performed on expanded CD8+ T lymphocytes infiltrating the tumors (TIL), possibly enriched in activated specific T cells, eventually due to the presence of antigen. After successful expansion, CD8+ cells were repeatedly stimulated with autologous mature IL-4-DC pulsed with CTA peptides and/or infected with a recombinant vaccinia virus (rVV) encoding the corresponding epitopes together with the gene encoding human CD80. These vectors were constructed during the present study to provide highly effective immunogenic reagents with the perspective of possible clinical application.

CTA specific CTL response could be observed in 7/26 populations. In six cultures, cytotoxic activity was low and did not correlate with expression of specific CTA in the original tumor specimens. These CTL responses could possibly be attributed to a primary *in vitro* sensitization. However, in one case stimulation of TIL with rVV infected APC revealed a high level of MAGE-A10 specific CTL response detectable by cytotoxicity assays and multimer staining. The corresponding gene, encoding the target epitope, was highly expressed in the original tumor.

In NSCLC, CTA specific CTL sensitization in TIL, as detectable upon repeated stimulation with a panel of well defined peptides and highly effective APC, is rare. On the other hand, strong CTA specific CTL responses could frequently be generated from peripheral blood lymphocyes of healthy donors, upon stimulation of large numbers of effector cells with antigen pulsed DC obtained by GM-CSF/IFNα induction.

The concomitant expression of multiple CTA in NSCLC and the possibility of natural CTL responses in these cancers may support the development of specific vaccination protocols using multi antigen vaccine preparations of CTA.

2 Introduction

2.1 Immune system

The cellular immune system consists of two parts cooperating in the maintenance of healthy state. While the innate immune system, consisting of macrophages and natural killer cells, is the first alerted by potentially harmful stimuli, the adaptive, specific immune system (B and T cells) amplifies the protective mechanisms of non-specific immunity by focusing on specific antigens.

The specificity of lymphocyte responses resides in their surface receptors (B or T cell receptors, BCR and TCR) recognizing oligoaminoacidic structures whose triggering results in the production of antibodies (humoral immunity), or in the generation of cell-mediated immunity, respectively. Every BCR or TCR on an individual B or T cell has a unique specificity, resulting from somatic gene recombination, leading to an enormous diversity of B and T cells.

T cell selection processes take place in the thymus. As soon as the cell surface receptor is somatically rearranged, T cells pass positive selection by specific self-recognition of major histocompatibility complexes (MHC) and negative selection by demonstrating lack of specificity towards self-antigens. Mature T cells leave the thymus, enter the blood stream and migrate to the peripheral lymphatic organs. These selection processes are of particular importance for T cells, which cannot only directly kill virus infected cells or tumor cells, but also control the activation of other immune effector cells like, for instance, B cells.

For specific activation of each T cell subpopulation, potential target proteins must be fragmented and recognized in association with MHC products expressed on the surface of nucleated cells.

MHC class I molecules are expressed on the surface of all nucleated cell types. Cytotoxic T cells (CD8+ T cells), capable of causing target cell lysis once activated, recognize antigenic peptides only in association with MHC class I molecules. In contrast, helper T cells (CD4+ T cells) need to recognize peptides associated with class II MHC to secrete lymphokines, attract neutrophils, and enhance the ability of macrophages to engulf and destroy microbes. MHC class II molecules are

prevailingly expressed on the surface of antigen presenting cells (APC), the most important ones being macrophages, B cells, or dendritic cells (DC).

2.1.1 Antigen presenting cells

Main properties of APC are the ability to process endocytosed antigens and the expression of MHC molecules on their surfaces together with co-stimulatory receptors such as CD80 and CD86. The latter are among the molecules which distinguish professional antigen presenting cells such as DC from other MHC class II positive cells.

Upon stimulation by inflammatory challenges, APC mature and migrate to the spleen and lymph nodes to induce specific immune responses.

Triggering of CD40 receptor on APC by CD40 ligand (CD154), expressed on helper T cells, next to pathogenic stimuli may enhance the maturation process, as shown by increased expression of MHC, costimulatory factors, adhesion molecules, specific cytokines (IL-12, IL-15, TNF) and chemokines.

2.1.1.1 Antigen processing and presentation

The path leading to the association of protein fragments with MHC molecules differs for class I and class II restricted antigens. While MHC class II molecules present fragments derived from extracellular (exogenous) proteins, MHC class I molecules present peptides derived from cytosolic degradation of endogenously produced proteins.

Proteins are fragmented in the cytosol in proteasomes. Fragments are then transported across the membrane of the endoplasmic reticulum by transporter proteins (TAP). Synthesis and assembly of class I heavy chain, $beta_2$ microglobulin and peptides occur in the endoplasmic reticulum. Stable complexes are then transported to the cell surface.

2.1.2 Cytotoxic T cell activation

T cells reside in secondary lymphoid organs waiting for presentation of foreign antigens by APC migrating from periphery. Additional to the engagement of the TCR with MHC/Ag, co-stimulatory signals from the antigen-presenting cell are required, providing additional control mechanisms that prevent inappropriate and hazardous T cell activation. The signals may be provided by cytokines secreted by helper T cells or by costimulatory receptors expressed on professional APCs.

The CD28/CD80 or CD86 receptor/ligand system is one of the dominant co-stimulatory pathways. Interruption of this signaling pathway with CD28 antagonists not only results in the suppression of the immune response, but, in some cases, may induce antigen-specific tolerance.

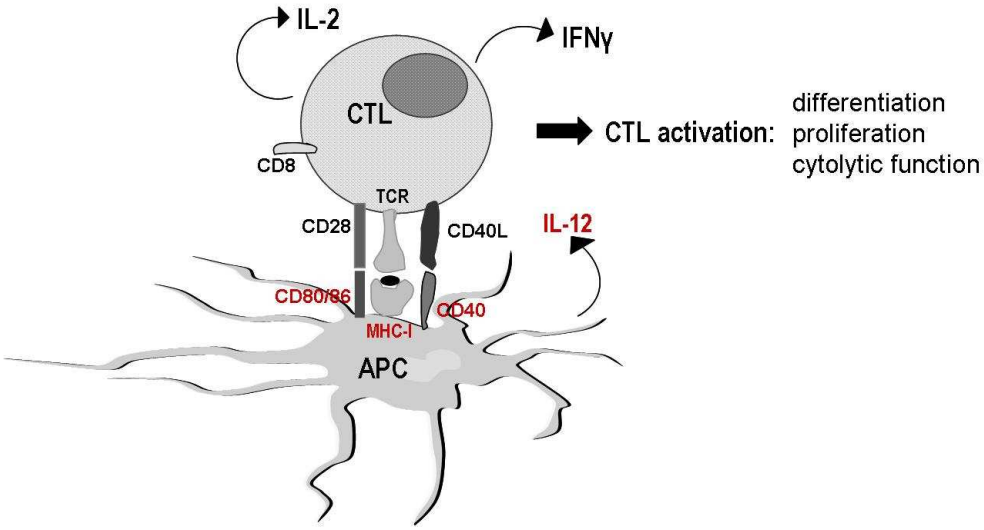

Figure 2.1: Stimulation of cytotoxic T cell for potent cytolytic immune response. Additional to TCR/MHC-Antigen binding, receptor/ligand formation of co-stimulatory factors are required for specific T cell activation. Upon stimulation, cytokines are released for further activation of the effector cells.

Upon activation, cytotoxic T cells migrate to sites of infection or, eventually, neoplastic growth, to kill target cells by a combination of apoptosis and osmotic lysis. Release of secretory granules, containing perforin and granzymes, induces membrane pore formation triggering osmotic swelling and lysis. In addition, interaction of T cell Fas ligand with the Fas receptor of target cell activates the caspase proteolytic cascade and other pathways involved in apoptosis.

2.2 Cancer

Despite a decrease in overall incidence, cancer remains a major public health problem. Each year 10.9 million people worldwide and 2 million in the European Union (EU) are diagnosed with cancer. There are 6.7 million (over 1 million in the EU) deaths from the disease, rates varying by gender and ethnicity [Jemal et al., 2005].

In the EU, cancer is a major cause of morbidity. 59% of people diagnosed with cancer are aged over 65 and around one out of three people will be diagnosed with cancer during their lifetime [Becker, 1998].

After heart diseases, cancer is the second leading cause of death in Switzerland (Figure 2.2). 28% of death in men and 21% in women were caused by cancer in the year 2002.

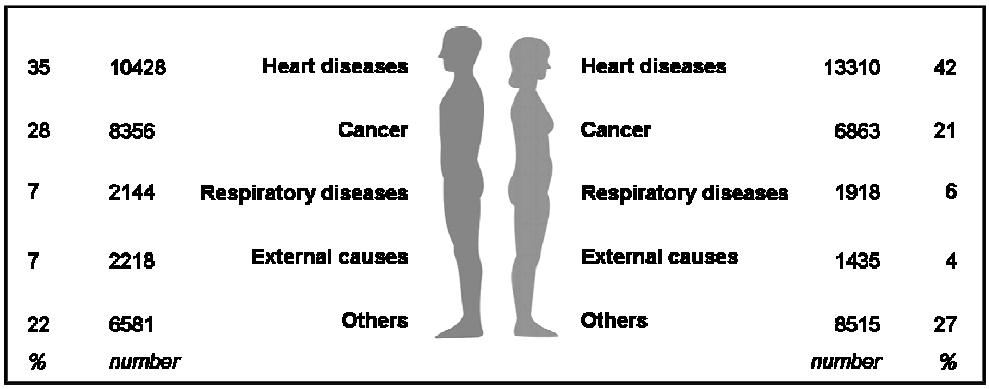

Figure 2.2: Death causes in Switzerland, 2002.
Source: 'Statistik der Schweiz', Bundesamt für Statistik (BFS), Neuchâtel 2005

The list of potentially causal and risk factors associated with cancer in general includes genetics (family history), behaviour (tobacco use, etc) and environment (radiation, etc). They all ultimately lead to dynamic changes in the genome driving the progressive transformation of normal human cells into malignant derivatives.

Tumorigenesis in humans is a multistep process. A multiplicity of changes, such as self-sufficiency in growth signals, limitless replicative potential, sustained

angiogenesis, capacity of tissue invasion and metastasis, insensitivity to anti-proliferative signals and evasion from apoptosis, collectively dictate malignant growth by breaching anti-cancer defence mechanisms [Hanahan and Weinberg, 2000].

These acquired capabilities are due to alterations in regulatory circuits that govern normal cell proliferation and homeostasis: mutations of the receptors or transcellular signal transducers or of the end target gene of the pathway itself. Common manifestations are overexpression of specific oncogenes (ras, c-myc, etc) and downregulation of tumor suppressor genes such as p53, [Robles et al., 2002].

Furthermore, beside autonomous mechanisms, mutant cancer cells can achieve heterotypic signaling between diverse cell types within a tumor by conscription and subversion of normal cells serving then as active collaborators in tumor cell deregulated proliferation.

These capabilities are probably shared by most types of human tumors. The paths, however, which cells take on their way to becoming malignant, are highly variable. Mutations in certain oncogenes and tumor suppressor genes can occur early in some tumor progression pathways and late in others. As a consequence, the acquisition of specific biological capabilities may appear at different times during progression. Finally, while in certain tumors a specific genetic event may *per se* contribute only partially to the acquisition of a single feature, in others, this event may aid in the simultaneous acquisition of several distinct characteristics.

2.3 Lung cancer

2.3.1 Epidemiology

Worldwide, lung cancer is the most common cause of cancer-related death. Since 1930, frequency of lung cancer as death cause increased constantly in men until it became, in the early 50ies, the leading cancer-related cause of death. Until the 90ies the rate of lung cancer death continued to increase dramatically. In comparison, prostate cancer became more important in the 80ies but the death rate remained still much lower (Figure 2.3A). In women, lung cancer death rate started to increase in the 60ies until it became a leading death cause before breast cancer in the 80ies (Figure 2.3B).

In the European Union lung cancer is the third most commonly diagnosed cancer (243 600 estimated cases in 2000). It affects men more than women, with the male: female ratio around 4:1.

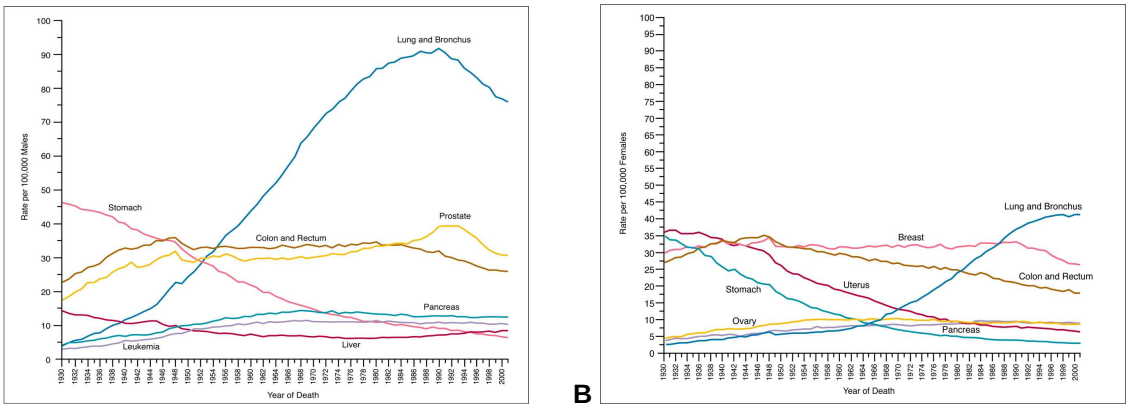

Figure 2.3: Cancer related death rates in the United States, from 1930 until 2000.
A: Death rates among males; **B:** Death rates among females;
Source: Cancer Statistics, 2005, CA A Cancer Journal for Clinicians 2005

Lung cancer is usually caused by a chronic exposure of the bronchial epithelium to multiple carcinogenic agents. The number of cases attributable to tobacco smoking varies between countries and regions depending on the historical levels of smoking for those regions. A recent estimate for Europe suggested that 90% of male and 60% of female lung cancers were caused by exposure to cigarette smoke. Besides

smoking, a small number of genetic polymorphisms have been associated with modest increases in lung cancer risk, thus excluding existence of highly-penetrant, strongly-predisposing genetic variants [Kiyohara et al., 2002].

In early stages of disease, lung cancers tend to be asymptomatic. As a consequence, most tumours are metastatic at the time of diagnosis (stages IIIB - IV, see below). Localised cancers (stages I - IIIA), resectable with available curative treatments, are only identified in approximately 20% of patients. Generally advanced stage at diagnosis implicates mostly non curative, palliative treatments and the relative resistance of the disease to currently available anti-cancer drugs leads to a high mortality rate, with 5-year survival typically between 10 and 15%.

2.3.2 Lung cancer forms

Lung cancers are generally heterogeneous, consisting frequently of cells of different histological subtypes. Still, pathological classifications emphasise the major cell type present in the tissue. Two broad categories of small cell lung cancers (SCLC), representing 20-25% of all bronchial carcinomas and non small cell lung cancers (NSCLC), representing 75-80% of cases, account for a large majority of lung cancers. Rarer tumor types such as carcinoids, carcinosarcomas, pulmonary blastomas and giant and spindle cell carcinomas are infrequently detected.

SCLC mostly arise centrally in a large bronchus. They grow extremely fast, are highly invasive and highly metastatic. Therefore, these types of tumors have a severe prognosis with a 3-year survival of less then 10%.

NSCLC are subdivided into three main histological subtypes, squamous cell lung carcinoma (35-40%), adenocarcinoma (25-30%) and large cell carcinoma (10%). Squamous cell lung carcinomas generally arise centrally within the lungs inside a large bronchus although they may sometimes be peripheral. Adenocarcinomas tend to occur in more peripheral locations arising from smaller airways but they can be found centrally in a main bronchus.

Large cell carcinomas are undifferentiated tumours which lack the diagnostic features of the other subtypes.

2.3.3 Staging

Lung cancers are classified according to UICC (International union against cancer) criteria. NSCLC patients are divided into different groups based on the standard TNM classification system, incorporating tumor size and location (T), lymph node involvement (N) and presence of distant metastases (M).

Tumors of stage I have a maximum primary tumor size of 5cm with the exclusion of local or distant metastasis (T1-2 / N0 / M0). For Stage II cases, the primary tumor has a minimum dimension of 5cm or extends to the breast wall or skin (T3-4 / N0 / M0). Stage III includes primary tumors of any size with local metastases affecting lymph nodes (T1-4 / N1-2/ M0). The highest stage tumors (Stage IV) present distant metastases in liver, skeleton, brain or adrenal glands (T1-4 / N0-3 / M1). The size of the primary tumor represents no crucial prognostic factor.

2.4 Cancer therapy

The primary objectives of cancer treatment are cure, prolongation of life, and improvement of the quality of life. Treatments usually include surgery, radiation therapy, chemotherapy, hormonal therapy, or combinations of them. The most advanced forms of treatment may produce 5-year survival rates of 75% or more for certain types of cancer e.g. cancer of the uterine corpus, breast, testis and melanoma. By contrast, 5-year survival rates in cancers of the pancreas, liver, stomach and lung are generally less than 15%.

2.4.1 Immunotherapy

Immunotherapy has matured as an additional treatment modality in the management of cancer. Two major approaches can be distinguished, passive immunotherapy, providing directly specific effector cells or antibodies, and active immunotherapy, requiring the participation of patients' own cells to elicit anti-cancer effects. The latter can be further divided in non-specific and specific active immunotherapy.

In active, non antigen specific immunotherapy multiple clinical trials have evaluated immunomodulators such as IFN-a (Phase I/II), BCG (Phase I/II) and *Mycobacterium vaccae* (Phase II) for the treatment of lung cancer [Agarwala et al., 2000; Millar et al., 1982; O'Brien et al., 2000]. These agents are thought to induce both cellular and humoral anti-tumor immunity by inducing strong inflammatory responses. However, the success of these immunomodulators in cancer immunotherapy has been very limited (no significant clinical responses by systemic application). Still, there has been a resurgence in these approaches by targeting co-stimulatory molecules such as B7 family members, CD40L and others [Raez et al., 2003].

In contrast, specific cellular immunotherapy focuses on therapeutic vaccination using autologous or allogeneic tumor cells, tumor cell lysates or defined antigens. Cancer vaccines induce anti-tumor immune responses mediated by effector cells including CD8+ and CD4+ T lymphocytes. This type of therapy is tumor specific and has a relatively low systemic impact.

Immune responses induced by vaccines that use whole tumor cells or tumor cell lysates instead of defined antigens are sometimes difficult to evaluate, as target-specific read-outs are not available.

On the other hand, a major limitation of peptide-based vaccination strategies is usually represented by the selective induction of CD8+ T cells against individual antigens. Antigen-specific CD4+ T cell responses and antigen-specific antibodies are less frequently involved. Using different immunostimulators such as cytokines (IL-2, IL-12, GM-CSF) or others adjuvants (CpG, incomplete Freund adjuvant) [Wilson et al., 2006] immune responses may be enhanced.

Tumor antigen-specific strategies imply the use of immunodominant peptides alone, protein- or peptide-pulsed dendritic cells, or antigens and co-stimulatory proteins simultaneously expressed by viral vectors. Preclinical and clinical studies are addressing immune responses, clinical outcome, feasibility and safety of different treatment approaches. Although the induction of antigen specific responses is frequently reported, clinical effectiveness is mostly limited.

2.4.2 Therapy for NSCLC

The prognosis of NSCLC patients is mainly dependant on the stage of the tumor, with 5-year survival ranging from 60-70% for early disease (stage I) to <1% for stage IV disease.

Treatment modalities currently employed are limited. Next to surgery, chemotherapy and/or radiotherapy may be used in patients who are not eligible for surgery. In metastatic NSCLC, primary chemotherapy is usually given with palliative *intention*. For radiotherapy, a radiation dose of 40 Gy or more in many fractions is commonly used.

There is an urgent need for new adjuvant therapies possibly effective in lung cancer with lower associated toxicity than chemotherapy.

2.4.3 Immunotherapy in NSCLC

In NSCLC immunotherapy could be considered as an additional treatment or even as a replacement of current therapeutic approaches. Clinical studies are evaluating a variety of antigen formulations, such as autologous or allogenic tumor cell vaccines, modified virus, pulsed autologous DC, or dexosomes, DC released vesicles, for their capacity to induce cancer-specific anti-tumor immunity.

A number of authors have attempt the generation of cellular immune responses by targeting Her-2/neu, MUC1 or CEA652 by peptide-based vaccination [Disis et al., 2002; Itoh et al., 2002; Kontani et al., 2003]. On the other hand, different studies aim at generating humoral responses targeting, for example, epidermal growth factor receptor bearing cells (EGF vaccine therapy; [Gonzalez et al., 2003]).

To increase the efficacy of vaccines, immunomodulators or adjuvants are frequently used to enhance specific immune responses. Vaccines including irradiated viable autologous tumor cells admixed with non-specific immunostimulant bacillus Calmette-Guerin (BCG modified tumor cell vaccine) showed no clear benefit [Schulof et al., 1988]. In contrast, allogeneic vaccination with CD80 and HLA gene-modified adenocarcinoma cell lines (phase I) [Raez et al., 2004] or autologous tumor cell vaccines expressing granulocyte macrophage colony-stimulating factor (GM-CSF) (phase I/II, [Nemunaitis et al., 2004]) have shown more promising results.

However, although a number of different immunotherapeutical procedures demonstrated some ability to induce anti-tumor immune responses in NSCLC, as illustrated sometimes by metastases shrinkage, responses were usually short-lived.

2.5 Tumor associated antigens

In the past 20 years, there has been a continuing search for tumor associated antigens (TAA), which could be used to direct the cytolytic potential of the human immune system against cancer. Human TAA can be classified, based on their expression pattern, into four major groups: while *unique antigens* can be distinguished from shared antigens, the latter group can be further divided into tumor-specific antigens, differentiation antigens and overexpressed antigens.

Unique antigens result from point mutations in defined genes potentially implicated in tumoral transformation. As these mutations are usually not shared by tumors from different patients, such tumor-specific antigens may only play an important role in the natural anti-tumor immune response of individual patients.

On the other hand, shared antigens are present on tumors from different patients and histologies. While *cancer/testis antigens* (CTA) are silent in healthy adult tissues except testis and placenta, and can therefore be considered as operationally tumor-specific, *differentiation antigens* are also expressed in the normal cells of the same tissue from which a tumor developed. Antigens of this last group represent specific markers for a cell lineage (tyrosinase, carcinoembryonic antigen (CEA), prostate specific antigen (PSA), etc) and are not tumor-specific. Their use as targets for cancer immunotherapy may result in auto-immunity towards the corresponding normal tissue (e.g. vitiligo in the case of melanocytes). The third group of shared antigens, such as MUC1 and Her-2/neu, comprise antigens expressed in a wide variety of normal tissues and *overexpressed* in tumors. The low level of expression in normal tissues should not implicate autoimmune damage, although a threshold is frequently difficult to define.

2.5.1 Cancer/testis antigens

2.5.1.1 Classification

About 90 cancer/testis antigens (CTA) grouped in over 40 families have been identified so far (see appendix I). The first CTA, the melanoma-associated antigen (MAGE) gene MAGE-A1, was characterized in 1991 through a newly developed

methodology for identifying tumor antigens based on tumor-specific CTL recognition [van der Bruggen et al., 1991].

Further analysis of the MAGE-A family revealed twelve closely related genes clustered at Xp28 [Chomez et al., 2001]. Other clusters of MAGE genes were rapidly identified, such as MAGE-B and MAGE-C, as well as B antigen (BAGE) and G antigen I (GAGE1). Tumor-antigen genes structurally different from MAGE were also discovered using similar techniques.

A new methodology, based on the screening of cDNA expression libraries with antibodies from patients' sera (SEREX), led to the identification of several categories of CTA such as SSX [Sahin et al., 1995], synaptonemal complex protein 1 (SCP1) [Tureci et al., 1998] and the highly immunogenic cancer antigen New York oesophageal squamous cell carcinoma 1 (NY-ESO-1) [Chen et al., 1997].

22 CTA families are encoded on the X chromosome (CT-X antigens). Many of them encode proteins with very high sequence similarities.

2.5.1.2 Expression

Proteins of the CTA families are expressed in a variety of malignant neoplasms, but silent in normal tissues except testis and placenta. Within the testis, CTA expression is restricted to germ cells [Jungbluth et al., 2000], while within the placenta they are expressed in the trophoblasts [Rimoldi et al., 1999; Simpson et al., 2005] (Figure 2.4). As these cells do not express MHC class I molecules, specific gene expression will not result in the presentation of antigenic peptides.

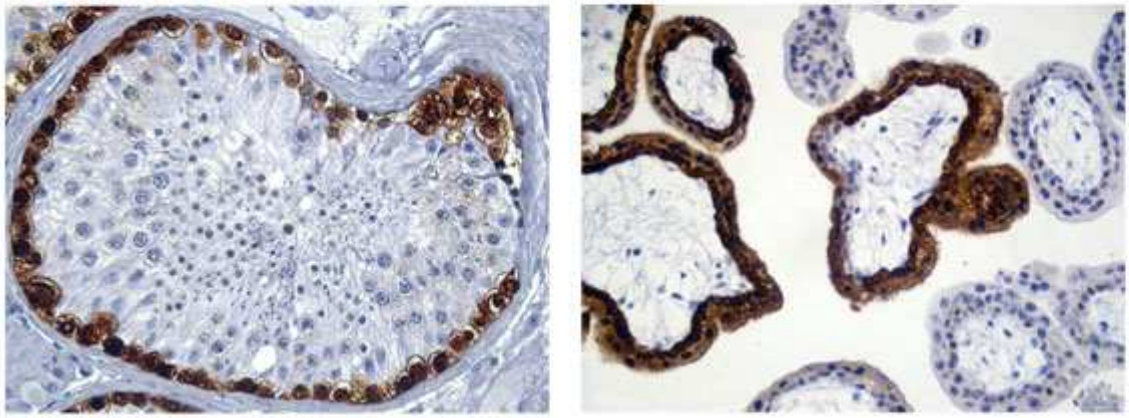

Figure 2.4: Cancer/testis antigen expression in normal tissues. Immunohistochemical staining of MAGE-A. **left:** staining of spermatogonia (testicular germ cells). **right:** staining of trophoblastic epithelia of placental villi.
Source: Cancer/testis antigens, gametogenesis and cancer. Nature Reviews, Cancer 2005

The expression of CT antigens varies between tumor types. While bladder cancer, lung cancer, ovarian cancer, hepatocellular carcinoma and melanoma frequently express CTA, these antigens were rarely observed in renal cancer, colon cancer, gastric cancer and leukaemia. Different studies demonstrate that CTA can highly be expressed within tumor tissues [Sugita et al., 2002] but, interestingly, rarely in homogeneous form by all tumor cells [Jungbluth et al., 2000].

The induction of CTA expression appears to be related to promoter demethylation [De Smet et al., 1996; Weber et al., 1994]. All CTA genes studied have methylated CpG islands within their promoters in normal somatic tissues, which may be responsible for the gene silencing [De Smet et al., 1999]. In contrast, in embryonic cells, CTA gene promoters have much less CpG methylation [Reik et al., 2001].

During tumorigenesis, global DNA hypomethylation and gene-specific hypomethylation is a known occurrence. A recent study indicated that hypomethylation in tumors is frequently associated with hypermethylation of tumor-suppressor genes [Kaneda et al., 2004]. However, hypomethylation alone is not sufficient for the induction of CTA expression, as DNA in colon cancer cells, for example, is frequently hypomethylated [Goelz et al., 1985], even though CTA gene expression is rare in this tumor type.

2.5.1.3 MAGE family

Based on sequence homologies many MAGE genes have been identified and classified into two subgroups: I, including MAGE-A, -B and -C, and II, including MAGE-D, -E, -F, -G and -H. In contrast to the genes of subgroup I, belonging to CTA, members of subgroup II are expressed in various normal adult tissues.

All proteins of the extended MAGE family share certain homologous regions, including the large central region MAGE homology domain (MHD) [Chomez et al., 2001]. The MHD does not contain any regions of significant homology with other known proteins, but it appears to be an important site of protein-protein interaction.

Various MAGE family members are suspected to play important physiological roles during embryogenesis, germ cell genesis and apoptosis. However, the biological function and their role in cell activities in both, germ line cells and tumors remain poorly understood.

Despite the common belief that CTA are found in tumor cells mostly as an unspecific result of the demethylation process, some studies suggest that the expression of CTA such as MAGE could play a role in human tumorigenesis.

Indeed, recent data indicate that expression of MAGE genes in cancer cells contributes directly to the malignant phenotype and response to therapy. In some human cells lines, for example, overexpression of at least one of the MAGE-A genes correlates with an increased resistance to TNF-mediated cytotoxicity [Park et al., 2002] or with resistance to the widely used chemotherapeutic drugs Paclitaxel and Doxorubicin, which are typical phenomena of aggressive cancer [Glynn et al., 2004].

2.6 Aim of the study

The purpose of this work is to evaluate the potential relevance of Cancer Testis Antigen specific immunization to envisage novel types of treatment for Non Small Cell Lung Cancer.

CTA represent interesting tumor associated antigens since next to their limited expression to non immunogenic testis cells in normal adult tissues, spontaneous specific responses to CTA have been demonstrated in cancer patients.

To first address the relevance of these TAA in lung cancer, prevalence and expression pattern of several CTA were evaluated in surgically excised lung tumor specimens of different stages and histology.

Moreover, the capacity of inducing cellular CD8+ T cell response towards CTA in cells from cancer patients was explored. Therefore *ex vivo* stimulations with HLA-A*0101 and HLA-A*0201 restricted epitopes were performed on CD8+ T cells infiltrating tumors (TIL). Based on prior data obtained from melanoma TIL, these populations were supposed to be enriched in activated specific T cells possibly related to specific antigen expression in cancer cells.

3 Materials & Methods

3.1 Reagents and Solutions

3.1.1 Media & Buffers

Complete medium	**RPMI 1640** + L-Glutamine[1] MEM Non-essential amino acids[1] Hepes buffer 10mM[1] Sodium pyruvate MEM 1mM[1] Glutamax 1mM[1] Kanamycin 100µg/ml[1]
HS medium	**Complete medium** 5% pooled Human serum[2]
DMEM-10% FCS	**DMEM** 10% FCS[1] Kanamycin 100µg/ml Glutamax 1mM
IFNα-DC medium	**Complete medium** 10% FCS IFNα_{2b} (10 000U/ml)[3] 50ng/ml GM-CSF[4]
IL-4-DC medium	**Complete medium** 10% FCS IL-4 (1000U/ml)[5] 50ng/ml GM-CSF
MACS Buffer	**PBS*** 0.5% FCS EDTA pH 8, 0.5mM[6]

[1] GIBCO, Paisley, UK; [2] Blood bank, University Hospital Basel, CH; [3] Intron A, Essex Chemie, Luzern, CH; [4] Novartis, Basel, CH; [5] courtesy of Dr. Lanzavecchia, Bellinzona, CH; [6] Fluka Chemie, Buchs, CH;

3.1.2 Peptides for cellular stimulation

Antigenic peptides were purchased from NeoMPS (Strasbourg, F).

Antigen	*Sequence*	*HLA-restrict.*	*Reference*
BMLF-1	GLCTLVAML	A*0201	[Steven et al., 1997]
LMP-2	CLGGLLTMV	A*0201	[Murray et al., 1998]
MAGE-A1$_{161-169}$	EADPTGHSY	A*0101	[Traversari et al., 1992]
MAGE-A3$_{168-176}$	EVDPIGHLY	A*0101	[Gaugler et al., 1994]
MAGE-A4$_{230-239}$	GVYDGREHTV	A*0201	[Duffour et al., 1999]
MAGE-A10$_{254-262}$	GLYDGMEHL	A*0201	[Huang et al., 1999]
Multi-MAGE-A[1]	YLEYRQVPV	A*0201	[Graff-Dubois et al., 2002]
NY-ESO-1$_{157-165}$	SLLMVVITQC	A*0201	[Jager et al., 1998]

[1] epitope in common between MAGE-A1, -A2, -A3, -A4, -A6, -A10, -A12.

3.1.3 Primer sets and probes for RealTime PCR

Primers and probes were obtained from Microsynth (Balgach, CH).

Gene	*Sequence*	*Reference*
β-actin	Pre-developed assay Applied Biosystem, Foster City, CA	
CD4	Assay-On-Demand Hs0018127_m1 Applied Biosystem, Foster City, CA	
CD8α	Fwd: CTCGGCCCTGAGCAACTC Rev: GGCTTCGCTGGCAGGA Probe: ATGTACTTCAGCCACTTCGTGCCGGTC	this work
FoxP3	Fwd: GGCACTCCTCCAGGACAG Rev: GCTGATCATGGCTGGGCTCT Probe: ATTTCATGCACCAGCTCTCAACGG	[Miura et al., 2004]

Gene	*Sequence*	*Reference*
IDO	Fwd: GGTCATGGAGATGTCCGTAA Rev: ACCAATAGAGAGACCAGGAAGAA Pb: CTGTTCCTTACTGCCAACTCTCCAAGAAACTG	[Uyttenhove et al., 2003]
IFN-γ	Fwd: AGCTCTGCATCGTTTTGGGTT Rev: GTTCCATTATCCGCTACATCTGAA Probe: TCTTGGCTGTTACTGCCAGGACCCA	[Kammula et al., 2000]
IL-10	Fwd: GTGATGCCCCAAGCTGAGA Rev: CACGGCCTTGCTCTTGTTTT Probe: CCAAGACCCAGACATCAAGGCGCA	[Giulietti et al., 2001]
MAGE-A1	Fwd: TACCTGGAGTACCGGCAGGT Rev: TTGGACCCCACAGGAACTCA Probe: CGGACAGTGATCCCGCACGCT	[Riker et al., 2000]
MAGE-A10	Fwd: CAGGGAGAGCAAGAGGTCAAGA Rev: AAGTCCTGCCCACACTCCC Probe: CAGCACTGAAGGAGAAGACCTGCCTGTG	this work
MAGE-A12	Fwd: TGGCATCGAGGTGGTGG Rev: CCCAGGCAGGTGACAAGG Probe: TGGTCCGCATCGGCCACTTGTAC	[Panelli et al., 2000]
MAGE-A2	Fwd: GACAAGTAGGACCCGAGGCA Rev: TGTGGGTCTTCATTGCCCA Probe: TGGAGGAGCATTGAAGGAGAAGATCTGC	this work
MAGE-A3	Assay-On-Demand Hs00366532 Applied Biosystem, Foster City, CA	
MAGE-A4	Fwd: CCACAGAGGAGCACCAAGGA Rev: GCTTTTGCCTGCACTCTTGC Probe: AAGATCTGCCTGTGGGTCCCCATTGC	this work
NY-ESO-1	Fwd: GCTGAATGGATGCTGCAGA Rev: CTGGAGACAGGAGCTGATGGA Probe: TGTGTCCGGCAACATACTGACTATCCGA	[Nakada et al., 2003]
TNFα	Fwd: CCCAGGGACCTCTCTCTAATCA Rev: GCTACAGGCTTGTCACTCGG Probe: CTGGCCCAGGCAGTCAGATCATCTT	[Razeghi et al., 2001]

Usually, a 200nM final concentration of each oligo was used. However, in some cases concentrations of primers and probes had to be optimized. For MAGE-A12 mRNA detection final concentrations of reverse primer and probe was adjusted to 400nM, to 250nM for NY-ESO-1 and 300nM for IFNγ detection.

3.1.4 Cell lines

CV1	African green monkey, kidney fibroblast	ATCC CCL-70
G401	Human, Wilms' tumor kidney epithelial cells	ATCC CRL-1441
SK-Mel37	Human, melanoma	
T2	Human, lymphoblast	ATCC CRL-1992

3.1.5 Plasmids

For the construction of recombinant virus, three different plasmids were used. These plasmids contain two homologous regions from loci I4L, A44L and A56R of the viral genome flanking the cloning site [Tsung et al., 1996], in which the different CTA in form of endoplasmic reticulum (ER)-targeted minigenes were inserted. Each minigene sequence is inserted under the control of a vaccinia specific early promoter and transcription termination signals.

The A44L plasmid contains the full gene encoding human CD80 [Zajac et al., 1998], while the full gene encoding CD40 ligand [Feder-Mengus et al., 2005] was cloned into plasmid A56R.

3.1.6 Virus

Vaccinia virus (Copenhagen strain) was chosen as expression vector. This virus belongs to the Orthopox virus gender characterized by an encapsulated double strand DNA. Poxviruses replicate in the cytoplasm of a large variety of cells. This vector features a strong capacity to express transgenes, a large insertion capacity (up to 30kb) and an intrinsic immunogenicity leading to powerful humoral and cellular responses [Moss, 1996; Paoletti, 1996]. Only limited virulence and side effects, such as cutaneous complications and encephalitis, were reported, following its administration.

3.2 Cellular immunology techniques

3.2.1 Cell cultures

3.2.1.1 Lymphocytes and Monocytes

PBMC were isolated from heparinized peripheral blood of each patient by Ficoll-Hypaque density gradient centrifugation. Monocytes or CD8+ T lymphocytes were purified from PBMC or respectively TIL by magnetic bead separation (Miltenyi Biotec, Gladbach, D).

3.2.1.2 Dendritic Cells

DC were derived from monocytes upon six days culture in DC medium containing either GM-CSF and IL-4 or GM-CSF and IFNα. For maturation (→ mDC), 1µg/ml LPS (abortus equi, Sigma, Buchs, CH) was added to DC cultures for eight hours before pulsing with specific peptides.

3.2.1.3 EBV-BL

As restimulation requires autologous antigen presenting cells, EBV-BL lines were generated by infecting CD14- cell fraction from patients' PBMC with Epstein-Barr virus containing cell (B95.8) supernatants. 5µg/ml Cyclosporin A (Novartis, Basel, CH) were added and infected cells were cultured in complete medium-10% FCS in the presence of IL-6 (supernatants from a transfected cell line; courtesy of Dr. Lanzavecchia, Bellinzona, CH).

3.2.2 Tumor Infiltrating Lymphocyte expansion

Tumor samples were surgically excised and transferred into PBS. In order to obtain a cell suspension, tumor samples were mechanically disrupted and incubated over night at 37℃ in complete medium containing 560µg/ml Collagenase II (Sigma, Buchs, CH). After washing the cell suspension, T lymphocyte proliferation was stimulated by solid phase bound anti-CD3 (500ng/ml, clone TR66; gift from Dr. Lanzavecchia, Bellinzona, CH) and anti-CD28 (100ng/ml; Pharmingen, San Diego, CA) on pre-coated plates in HS medium supplemented with 100U/ml IL-2

(Hoffmann-La Roche, Basel, CH) for two weeks. Medium was changed twice per week.

3.2.3 CTL induction

Autologous mDC were pulsed for two hours with 50µg/ml MAGE-A1, -A3, -A4, -A10, Multi-MAGE-A, NY-ESO-1 and EBV BMLF-1/ LMP-2 antigenic peptides or infected with UV-irradiated recombinant vaccinia virus (m.o.i. 1-3) for 16 hours at 37℃ and irradiated (3000 rad). Loaded / infected APC were cultured with tumor infiltrating CD8+ T lymphocytes (1x10e6 CD8+ and 5x10e5 APC per well) in presence of HS medium containing IL-2 at 37℃ for ten days by chan ging the medium on day four, six and eight. For restimulation, autologous mDC or EBV-BL pulsed with different peptides or infected with recombinant virus were used in alternating cycles (rVV - soluble peptide - soluble peptide).

3.2.4 Cytotoxicity test

CTL function was tested in standard ^{51}chromium release assays using T2 as target cell line. After labelling (100µCi/sample) for one hour, T2 cells were pulsed with the different specific peptides (10µg/ml) for two hours and added to CTL cells at effector to target ratios (ET ratio) of 100:1, 50:1, 25:1, 12.5:1 and 6.25:1. To minimize unspecific killing, K562 cells were added to each condition (K562:T2 ratio = 100:1). After four hours incubation, supernatants containing released ^{51}Cr were transferred to plates precoated with scintillation liquid and read by a luminescence counter. The percentage of specific lysis was calculated by the subtraction of spontaneous release from sample value divided by the subtraction of spontaneous from maximal release value x100. Each assay was performed in duplicate.

3.2.5 Phenotypic characterization

For characterization of immunocompetent cells in fresh tumor samples, after unspecific proliferation or antigen specific stimulation, cells were stained with fluorescent monoclonal antibodies and analysed by flow-cytometry (FACScalibur; Becton Dickinson, San Josè, CA) using Cell Quest software (Becton Dickinson). FITC or PE labelled anti-CD3, -CD4, -CD8, -CD14, -CD16, -CD45 (Becton Dickinson)

were used for tumor samples analysis, while specific CTL were characterized by using PE fluorochrome labelled pMHC complexes (pentamers specific for MAGE-A1, -A3, -A4, -A10, Multi-MAGE-A and NY-ESO-1, HLA class I restricted epitopes; Proimmune, Oxford, UK).

3.2.6 Immunohistochemistry

Formalin-fixed paraffin-embedded tumor specimens were processed in the Institute of Pathology according to standard methods [Bolli et al., 2002]. Sections were stained with the monoclonal antibody 57B for the identification of multiple MAGE-A gene products and D8.38 for NY-ESO-1 detection. Both antibodies were previously generated and characterized in our lab [Bolli et al., 2002; Kocher et al., 1995; Schultz-Thater et al., 2000].

The Histoscore was calculated by considering the intensity of the staining (0-3) and percentage of the stained tumor areas. Two evaluations of every section were done and analysed together. From a minimal Histoscore of 30, meaning a low-intensity staining (1) of 30% of cells or high-intensity staining (3) of no more than 10% of cells, the staining was considered as positive. Maximal staining reached a Histoscore of 300 (intensity 3 x area percentage 100).

3.3 Molecular biology techniques

The following methods were applied, if not specifically mentioned, according to the general procedures currently adopted in molecular biology [Sambrook et al., 1989].

3.3.1 Gene expression evaluation

For quantification of gene expression, surgical specimens were cut into small pieces and frozen in RNAlater at -70℃. After thawing, tum or tissues were mechanically disaggregated using the Medimachine System (Becton Dickinson, San Josè, CA). RNA was extracted following RNeasy® Mini Kit protocol (Qiagen, Basel, CH) and treated with DNase I (Invitrogen, Paisley, UK) before reverse transcription using M-MLV RT enzyme (Invitrogen, Paisley, UK). Specific amplification of reverse transcribed RNA was performed by quantitative real time PCR using primers and probes specific for MAGE-A1, -A2, -A3, -A4, -A10, -A12, NY-ESO-1**,** CD4, CD8α chain, FoxP3, IFNγ, IDO, IL-10, TNFα and β-actin, used as internal reference (see above). All assays were run in duplicates and results with a standard deviation >2% were excluded. SK-Mel37 melanoma cell line, expressing all CTA genes under investigation, was used as positive control for CTA expression.

Statistical analysis was performed by Statistica software (StatSoft, Tulsa, OK) using the Spearman rank correlation or Mann-Whitney's nonparametric U Test.

3.3.2 Virus construction

Recombinant vaccinia viruses were constructed by a two-step procedure. First, plasmids containing foreign genes were generated. These genes are controlled by a vaccinia virus promoter and flanked by sequences derived from non essential sites on the viral genome (see above). Second, the foreign genetic material of the plasmid vector was inserted into the viral genome by homologous recombination *in vivo* [Falkner and Moss, 1988].

3.3.2.1 Cloning of CTA oligos

Each CTA oligo cloned into plasmids starts with a sequence encoding the ER-targeting signal from the E3-19kD protein of adenovirus. The resulting fusion

peptide directly enters the ER, thereby bypassing discrete steps of the class I antigen processing pathway.

Plasmids were digested with restriction enzymes (Promega, Madison, WI) Nar I – Apa I (for MAGE-A4, -A3 and NY-ESO-1), Xho I – Nhe I (for MAGE-A2 and Multi-MAGE-A), Xho I – Sac I (for MAGE-A1) and BamH I – EcoR I (for MAGE-A10) in one or two steps depending on enzyme specific buffer required. After annealing of respective CTA oligos the inserts were ligated into the plasmids.

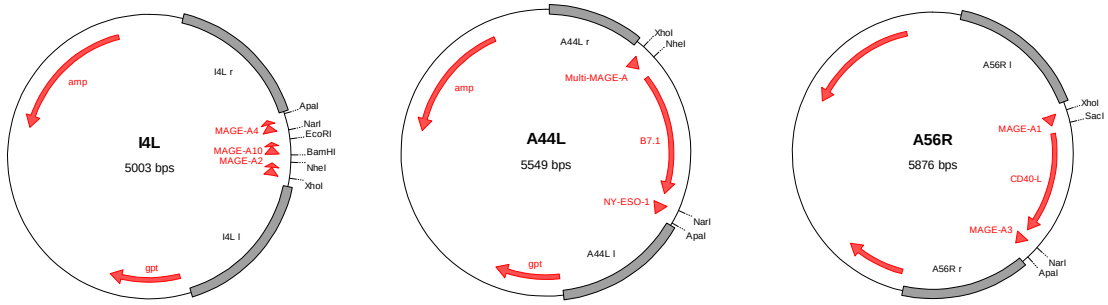

Figure 3.1: Maps of plasmids generated in this study. Each plasmid contained two regions, homologous of a viral genome locus (I4L, A44L and A56R; illustrated by grey boxes), flanking the cloning site. Minigenes MAGE-A2, -A4, -A10 were inserted in plasmid I4L. Multi-MAGE-A and NY-ESO-1 were cloned together with full gene CD80 (B7.1) in plasmid A44L. MAGE-A1 and MAGE-A3 and gene encoding for CD40 ligand (CD40-L) were inserted in plasmid A56R (all inserts illustrated by red arrows). amp = ampicillin resistance (β-Lactamase); gpt = guanine phosphoribosyl transferase, MPA resistance.

Competent E. coli (Top 10; Invitrogen, Paisley, UK) were transformed by electroporation (25µF and 2.5kV; Gene Pulser apparatus; Bio-Rad Laboratories, Hercules, CA) following manufacturers' protocols and plated on LB agar (GIBCO, Paisley, UK) containing 100µg/ml carbenicillin (Fluka Chemie, Buchs, CH). After colony selection and amplification of bacteria in LB medium (GIBCO), plasmid DNA was isolated using the NucleoSpin® Plasmid Kit (Macherey-Nagel, Oensingen, CH). The insert presence was verified on 0.8% - 1% agarose gel (GIBCO) after restriction with Bgl I.

Minigenes encoding MAGE-A2, -A4, -A10 epitopes were inserted into the plasmid I4L. Vector A44L was genetically modified with the minigenes encoding multi-MAGE-A and NY-ESO-1 epitopes. MAGE-A1 and -A3 epitope coding minigenes were inserted into plasmid containing the viral region A56R.

3.3.2.2 Transfection into viral vector

Insertion of the modified VV loci I4L, A44L and A56R into the viral genome was obtained after transfection of the plasmid onto infected cells and genetic recombination of homologous sequences (crossing-over with I4L, A44L or A56R sequence respectively).

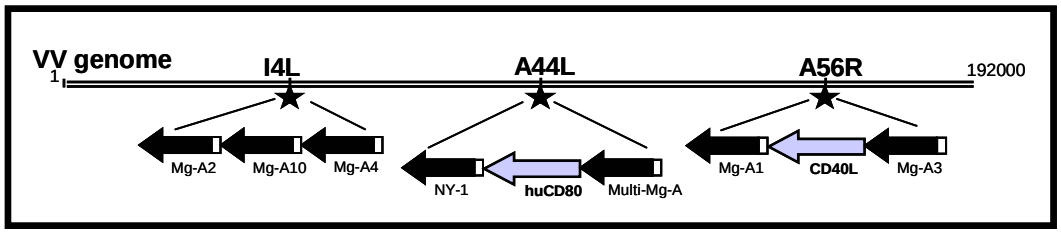

Figure 3.2: Recombinant vaccinia virus genomic map. Modified VV loci I4L, A44L and A56R in viral genome after genetic recombination. Loci I4L and A44L contain minigenes encoding for HLA-A*0201 restricted CTA epitopes and full gene huCD80. Minigenes encoding for HLA-A*0101 restricted CTA epitopes and full gene CD40L were inserted in locus A56R.

Briefly, subconfluent adherent CV1 cells were infected with sonicated WT vaccinia virus at m.o.i 0.1 for one hour at 37℃. Lipofectamine™ Reagent (160µg/ml; Invitrogen, Carlsbad, CA) and Plasmid DNA (2-5µg) were premixed for 20 minutes at room temperature and added to the infection in presence of serum free DMEM medium. After four hours of incubation at 37℃ DMEM-10% FCS was added to the reaction.

3.3.2.3 Recombinant viral selection

After complete infection of the cells (about two days), as monitored by cytopathic effect (CPE), viruses were harvested, sonicated and used for infection: 100µl of 10^{-3} and 10^{-4} of virus suspension were added to fresh subconfluent CV1 (non transfected WT virus served as control). For viral selection a combination of drugs, 25µg/ml MPA, 250µg/ml Xanthine and 25µg/ml Hypoxanthine (Sigma, St.Louis, MO), was added to the reaction and incubated at 37℃. Only recombinant virus expressing the enzyme ‘gpt’ can replicate in selective medium. Plaques were picked and resuspended in PBS. The selection of recombinant virus required two to four rounds with selective pressure and two or three more rounds of plaques selection without

pressure which enable the removal of the plasmid sequences containing the resistance genes.

Presence of inserted sequences in the viral genome was verified by conventional PCR using oligos specific for the viral flanking regions (I4L-5': GGAATAACTCGGATCTGC, I4L-3': CTATAATAACCAGGAACA; A44L-5': GTTGAAATCTAGTTCTGC, A44L-3': GAAAGTAAACTATTATGG; A56R-5': ACTCCACAGAGTTGATTGTA, A56R-3': GTATGTGACGGTGTCTGTAT). Expression of inserts was evaluated by antibody staining (CD80 and CD40L) or cytotoxicity tests with specific CTL for MAGE-A10 and multi MAGE-A.

3.3.2.4 Virus amplification and titration

Virus was amplified by infecting CV1 cells at m.o.i. 0.01 until complete CPE. Cultures were then harvested and centrifuged to collect infected cells. While supernatant was preserved, cells were resuspended in 10mM Tris pH 8.5, sonicated for one minute and centrifuged. Supernatants were pooled and poured onto a cushion of 36% sucrose, 10mM Tris followed by ultracentrifugation at 13 000 rpm for one hour. The pellet was resuspended in 1mM Tris pH 8.5.

For titration, dilutions from 1:10e6 to 1:10e9 were performed and CV1 cells were infected with 100µl for 24 to 48 hours. Plaques were revealed by crystal violet (Sigma, Buchs, CH)/ethanol solution staining of the cell monolayer.

3.3.2.5 Virus inactivation

To reduce CPE and prevent replication, viruses were inactivated prior to CTL stimulations. Virus solutions were diluted to a final concentration of 5x10e8pfu/ml in HBSS (Invitrogen, Carlsbad, CA). 10µg/ml Psoralen (Trioxsalen; Calbiochem, Cambridge, MA) was added for ten minutes at room temperature and suspensions were exposed to UV light (365nm) for twelve minutes. This photoreaction can eliminate a wide range of infectious agents while leaving protein antigens and other surface components relatively unmodified [Hanson, 1992]. Expression of genes encoded under early viral promoters is not affected.

3.4 The procedure at a glance

From each patient, blood and tumor samples were collected. While evaluation of CTA expression was assessed on small pieces of tumor, the bulk of the tissue was enzymatically disrupted for TIL isolation. Monocytes and B lymphocytes were isolated from patients' blood for the generation of potent antigen presenting cells. Expanded tumor infiltrating CD8+ T lymphocytes were repeatedly stimulated with either mature DC or autologous EBV-BL, together with CTA epitopes in form of soluble peptides or encoded by a recombinant vaccinia virus developed during this study. Specific CTL generation was analysed by phenotypic tests as well as by cytotoxic assays.

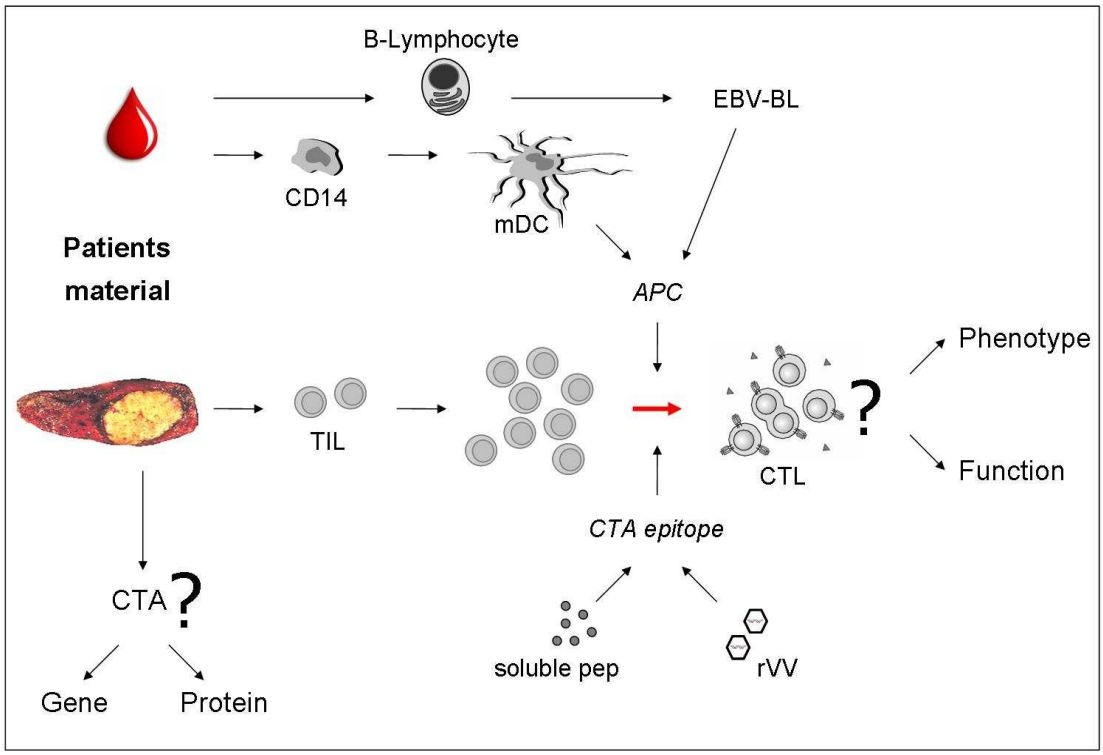

Figure 3.3: Illustration of the procedure.
mDC = mature dendritic cells; EBV-BL = Epstein-Barr virus-B lymphocytes; APC = antigen presenting cells; TIL = tumor infiltrating lymphocytes; CTL = cytotoxic T lymphocytes; CTA = Cancer/Testis antigens; pep = peptides; rVV = recombinant vaccinia virus.

4 Patients

From over 70 patients operated during the period April 2003 - May 2006 in the Department of Thoracic Surgery of Basel University Hospital, 33 patients with NSCLC (mean age 64.3 ± 8.18) were included in this study (Table 4.1). Main inclusion criterion was represented by expression of HLA determinants HLA-A*0101 and HLA-A*0201, restricting CTL responses to the epitopes under investigation.

Patient	Gender/Age	Histology	Stage	Grade	NeoAdj Tx	HLA-type
LT 1	M/77	AC	IV	3	-	A*0201
LT 2	M/68	SCC	I	2	n.a.	A*0201
LT 3	M/65	SCC	III	3	n.a.	A*0101, A*0201
LT 4	F/80	SCC	II	2	-	A*0201
LT 5	M/65	AC	I	3	-	A*0101
LT 6	M/67	AC	II	1	-	A*0101
LT 7	F/56	SCC	II	3	+	A*0101, A*0201
LT 8	M/68	AC	III	3	-	A*0201
LT 9	F/44	AC	I	2	-	A*0201
LT 10	M/54	SCC	I	2	-	A*0201
LT 11	F/61	SCC	I	2	-	A*0201
LT 12	M/63	LCC	IV	3	-	A*0201
LT 13	M/69	AC	III	2	-	A*0201
LT 14	M/59	LCC	II	3	-	A*0101, A*0201
LT 15	M/72	SCC	III	3	-	A*0101
LT 16	F/60	SCC	I	3	-	A*0101
LT 17	M/61	SCC	IV	n.a.	-	A*0201
LT 18	M/56	AC	II	2	-	A*0201
LT 19	M/48	SCC	II	3	-	A*0101, A*0201
LT 20	F/63	LCC	II	3	-	A*0101, A*0201
LT 21	M/73	AC	I	2	-	A*0101, A*0201
LT 22	M/73	SCC	I	2	-	A*0201
LT 23	F/74	AC	I	1	-	A*0101
LT 24	M/60	SCC	III	2	+	A*0201
LT 25	F/66	AC	I	3	+	A*0201
LT 26	M/60	LCC	I	3	-	A*0101
LT 27	M/63	SCC	II	3	-	A*0201
LT 28	M/65	AC	III	3	-	A*0201
LT 29	M/62	SCC	II	2	-	A*0101, A*0201
LT 30	M/62	SCC	III	2	+	A*0101
LT 31	M/57	SCC	III	2	-	A*0201
LT 32	M/75	SCC	I	3	+	A*0101
LT 33	M/77	AC	III	3	-	A*0201

Table 4.1: Clinico-pathological characteristics of patients included in the study. LT = lung tumor; AC = adenocarcinoma; LCC = large cell carcinoma; SCC = squamous cell carcinoma; n.a. = not available.

The majority of tumors were squamous cell carcinomas (17), followed by adenocarcinomas (12) and four large cell carcinomas of the lung. Twelve tumor were in stage I, nine each in stage II and stage III, and three in stage IV. In 13 patients neither lymph node nor distant metastases were evident. 17 patients had lymph node but no distant metastases. In the three patients with tumors of stage IV, metastases were investigated, since primary tumors at this stage are not routinely resected, further explaining the low number of stage IV specimens included in this study.

Histological grade varied among the tumor specimens with a majority of grade 3 (17), 13 of grade 2 and only two of grade 1.

Prior to surgical treatment, five patients underwent neo-adjuvant therapy.

5 Results

5.1 Construction of viral vectors

Vaccinia viruses are considered as highly attractive vectors for cancer gene therapy. Their large genome can be engineered by recombination of multiple genes, which will be simultaneously expressed.

$rVV_{combi\text{-}MAGE}$ contains minigenes encoding HLA-A*0201 restricted epitopes from MAGE-A2, -A10 and -A4 in the region I4L. To obtain rVV_{CGA}, supplementary inserts encoding for NY-ESO-1 and multi-MAGE-A HLA-A*0201 restricted epitopes together with CD80 (region A44L), and for MAGE-A1 and -A3 HLA-A*0101 restricted epitopes with CD40L (region A56R), were added to $rVV_{combi\text{-}MAGE}$. $rVV_{CTA\text{-}A1}$ is a virus recombinant in the A56R locus for MAGE-A1 and -A3 epitopes coding minigenes and full gene CD40L (Table 5.1).

Recombinant viruses	***co-stimulatory molecules***	***CTA minigenes***	***HLA-restrict.***
$rVV_{combi\text{-}MAGE}$	huCD80	MAGE-A2,-A4,-A10,multi-MAGE-A, NY-ESO-1	A*0201
rVV_{CGA}	huCD80; huCD40L	MAGE-A1,-A2,-A3,-A4,-A10, multi-MAGE-A, NY-ESO-1	A*0101; A*0201
$rVV_{CTA\text{-}A1}$	huCD40L	MAGE-A1, -A3	A*0101

Table 5.1: Characteristics of viral constructs.

5.1.1 Genetic characterization of rVV

DNA insertions were verified by PCR using primers specific for the flanking regions of each insert (Figure 5.1). As control, genomic DNA of VV_{WT}, $rVV_{B7.1}$ and rVV_{154} was used. Amplicons lengths directly depended on each specific construct:

I4L-MAGE-A2/-A10/-A4 = 529bp
I4L-WT = 2224bp

A44L-NY-ESO-1/huCD80/multi-MAGE-A = 1361bp
A44L-huCD80 = 1208bp
A44L-WT = 699bp

A56R-MAGE-A1/huCD40L/MAGE-A3 = 2154bp
A56R-huCD40L = 1972bp
A56R-WT = 974bp

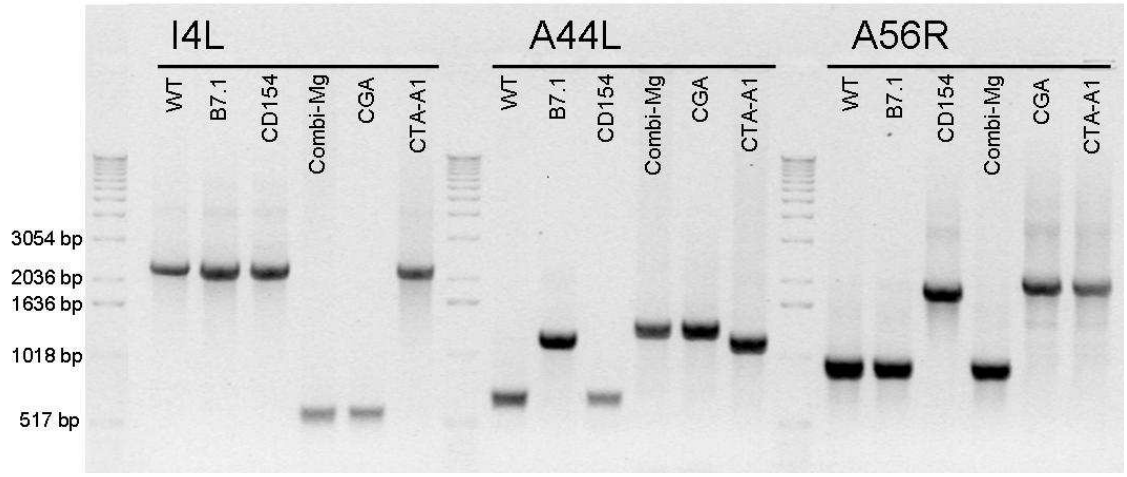

Figure 5.1: Characterization of recombinant viruses: PCR amplification of loci I4L, A44L and A56R. Genomic DNA of VV_{WT} (WT), $rVV_{B7.1}$ (B7.1), rVV_{CD154} (CD154), $rVV_{combi\text{-}MAGE}$ (Combi-Mg), rVV_{CGA} (CGA) and $rVV_{CTA\text{-}A1}$ (CTA-A1) was amplified using primers for flanking regions of each insert and analysed in a 1% agarose gel. 1Kb ladder.

All three viral vectors displayed the expected genetic profile confirming the presence of the recombinant sequences in their genome. $rVV_{combi\text{-}MAGE}$ contains three minigenes encoding MAGE-A2, MAGE-A10 and MAGE-A4 epitopes within the I4L region, and sequences for NY-ESO-$1_{157\text{-}165}$ and multi-MAGE-A epitopes together with human CD80 within the A44L region. In this construct, the A56R region was not modified and remains wild type-like, whereas in $rVV_{CGA,}$ which derives from $rVV_{combi\text{-}MAGE}$, minigenes encoding MAGE-A1 and MAGE-A3 epitopes together with the gene encoding CD40L were also inserted in this locus. $rVV_{CTA\text{-}A1}$ contains the full gene CD80 in the A44L region and sequences for MAGE-A1, MAGE-A3 epitopes and CD40L in the A56R region.

5.1.2 Evaluation of recombinant genes expression

The capacity of the three recombinant vaccinia virus generated ($rVV_{combi\text{-}MAGE}$, rVV_{CGA} and $rVV_{CTA\text{-}A1}$) to express human CD80 and CD40L proteins was verified by antibody staining of infected cell surfaces followed by flow cytometry analysis. CV-1 cells were infected with replicative virus at m.o.i. 1. As control, similar infections were performed with VV_{WT}, $rVV_{B7.1}$ and rVV_{154} (Figure 5.2).

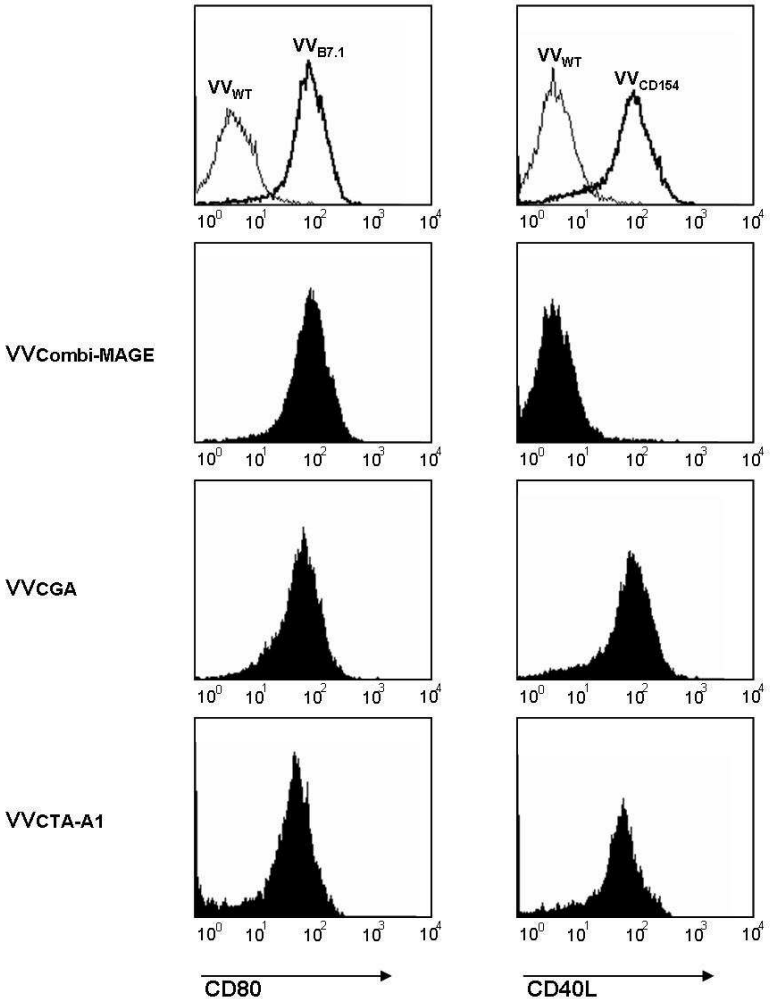

Figure 5.2: Cell surface expression of co-stimulatory molecules CD80 and CD40L following infection with rVV. CV1 cells were infected with replicative VV_{WT}, $rVV_{B7.1}$, rVV_{CD154}, $rVV_{combi\text{-}MAGE}$, rVV_{CGA} and $rVV_{CTA\text{-}A1}$ at m.o.i. 1. Phenotypic analyses were performed using antibodies specific for human CD80 and CD40L.

$rVV_{combi\text{-}MAGE}$, rVV_{CGA} and $rVV_{CTA\text{-}A1}$ infected cells showed surface expression of human CD80 protein comparable to that of the positive control $rVV_{B7.1}$. Similarly

expression of human CD40L was detectable following infection with rVV_{CGA} and rVV_{CTA-A1} and not with $rVV_{combi-MAGE}$. CV1 infected with VV_{WT} remained negative for either protein.

The immuno-modulatory activities of the vaccinia-expressed human CD80 and CD40L molecules, e.g. the activation of immune cells through CD28 and CD40 binding, respectively, were previously demonstrated [Feder-Mengus et al., 2005; Zajac et al., 1998].

Viral expression of recombinant CTA minigenes and HLA-A*0201 presentation of their products was indirectly tested by measuring the expression of MHC molecule on the surface of infected T2 cells. Indeed, due to the lack of peptide transporters (TAP), T2 cells are not able to correctly process protein and therefore do not stably express MHC-class I on the cell surface. However, since the different CTA epitopes in the viral constructs are encoded in form of ER-targeted peptides, these products should migrate to the ER irrespective of TAP proteins. After cleavage of the signal moiety, peptides might bind the resident empty MHC possibly leading to upregulation of HLA-A*0201 expression on T2 cell surface.

T2 cells were infected over night at m.o.i. 20 with rVV_{CGA} and rVV_{CTA-A1}, $rVV_{combi-MAGE}$ and as control with VV_{WT}. Surface HLA-A*0201 expression was detected by a specific antibody and measured by flow cytometry (Figure 5.3).

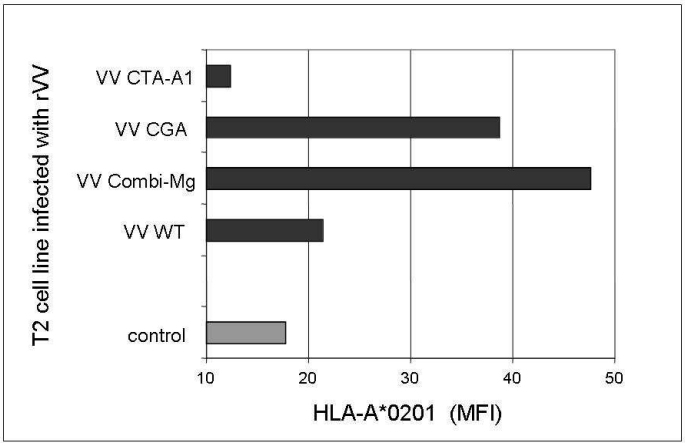

Figure 5.3: Upregulation of HLA-A*0201 at the surface of T2 cells following CTA epitope expression. Phenotypic analyses were performed following over night infection of T2 cells with VV_{WT}, $rVV_{combi-MAGE}$, rVV_{CGA} and rVV_{CTA-A1} at m.o.i. 20. As control, non infected T2 cells were stained with HLA-A*0201 antibody.

As compared to the non infected control, T2 cells HLA-A*0201 upregulation was detected only upon infection with rVV_{CGA} and $rVV_{combi\text{-}MAGE}$ but not with VV_{WT} nor with $rVV_{CTA\text{-}A1}$. Indeed, minigenes of the latter viral construct do only encode HLA-A*0101 restricted epitopes, whereas T2 cell line is HLA-A*0101 negative.

Further analysis of virus induced expression of each recombinant MAGE-A epitope was tested by sensitising target cells upon infection to killing by specific CTL recognizing MAGE-A10 or multi-MAGE-A (Figure 5.4).

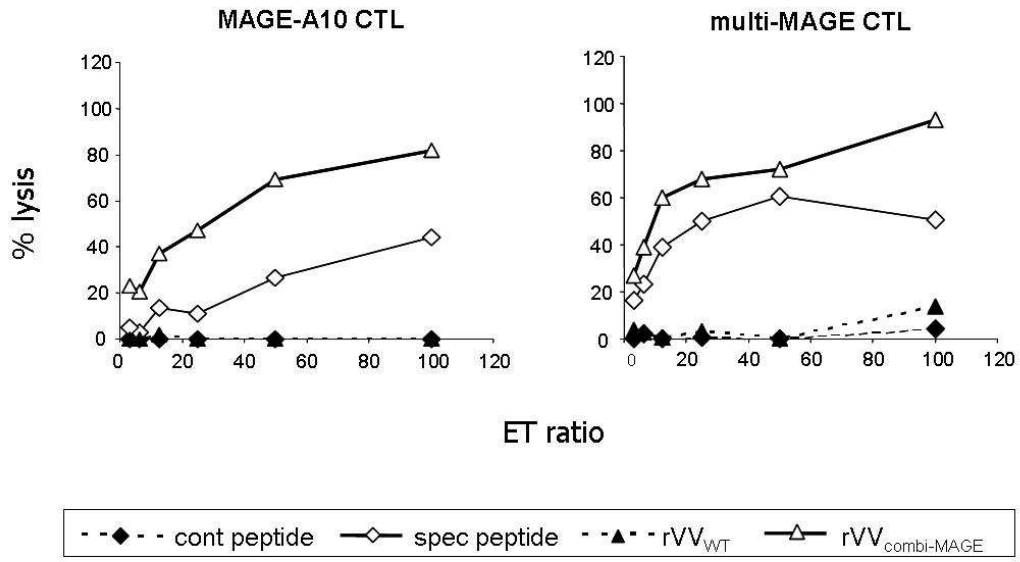

Figure 5.4: Cytotoxicity of CD8+ T lymphocyte cell line specific for MAGE-A10 and multi-MAGE-A. As target, T2 cells were infected either with rVVcombi-MAGE or VV_{WT} at m.o.i. 10. As positive control, cells were pulsed with 20µg/ml of the corresponding soluble peptide.

The cytotoxicity activity of CTL specific for MAGE-A10 and multi-MAGE-A against $rVV_{combi\text{-}MAGE}$ infected targets confirmed expression of the corresponding epitopes. Moreover, levels of epitope presentation were at least comparable to those elicited by 20µg/ml soluble peptide pulsing. As expected, VV_{WT} infected T2 cells were unaffected by MAGE specific CTL.

5.2 Lung Tumor samples characterization

5.2.1 Cancer Testis Antigens expression

5.2.1.1 Gene expression

5.2.1.1.1 Evaluation of the technique

Analysis of CTA gene expression by real-time qRT-PCR in tumor samples required validation of qualitative and quantitative features of the methods employed.

Following the definition of optimal concentrations for each oligonucleotide set, efficacy of qPCR was first evaluated. For this purpose, serial dilutions of cDNA from CTA positive cell line SK-Mel37 were tested (Figure 5.5A).

First, for most CTA, ratio to reference gene β-actin (delta Ct) remained stable up to a thousand fold dilution reaching then the limit of detection. A linear correlation between β-actin and MAGE expression was observed.

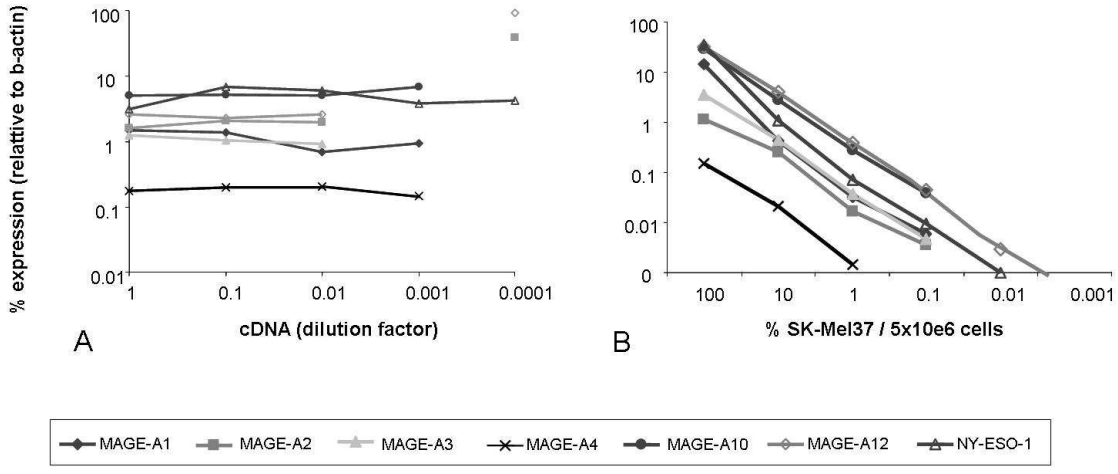

Figure 5.5: Evaluation of CTA mRNA detection by quantitative real time PCR. A: Efficacy of CTA detection. Stability of amplification was tested on diluted SK-Mel37 cDNA; **B:** Sensitivity of CTA detection. Gene expression was assessed from RNA mixture obtained from SK-Mel37 admixed with G401 cells to different dilutions.

Second, the sensitivity of the whole procedure from RNA extraction, reverse transcription to quantitative amplification of gene of interest was tested by spiking

experiments. CTA positive SK-Mel37 cells were diluted with CTA negative G401 cells to a total of 5x10e6 cells. Gene expression of CTA MAGE-A1, -A2, -A3, -A4, -A10, -A12 and NY-ESO-1 was measured in every condition (Figure 5.5B).

Depending on expression level of each gene in SK-Mel37 cells, the procedure of RNA purification, cDNA synthesis and PCR amplification enabled the detection of CTA positive cells ranging from 50000 cells (1% of total) to 50 cells (0.001% of total) for MAGE-A4 and MAGE-A12, respectively.

5.2.1.1.2 Analysis of NSCLC samples

Expression of MAGE-A1, -A2, -A3, -A4, -A10, -A12 and NY-ESO-1 genes was measured in lung tumor samples. Figure 5.6 illustrates representative CTA expression patterns in individual clinical specimens. The expression level of each CTA gene varied from negative for all genes (LT1) to high expression levels of few (LT20) or several CTA genes tested (LT24). Remarkably, CTA expression level in the latter tumor sample was comparable to that detected in the positive control tumor cell line SK-Mel37.

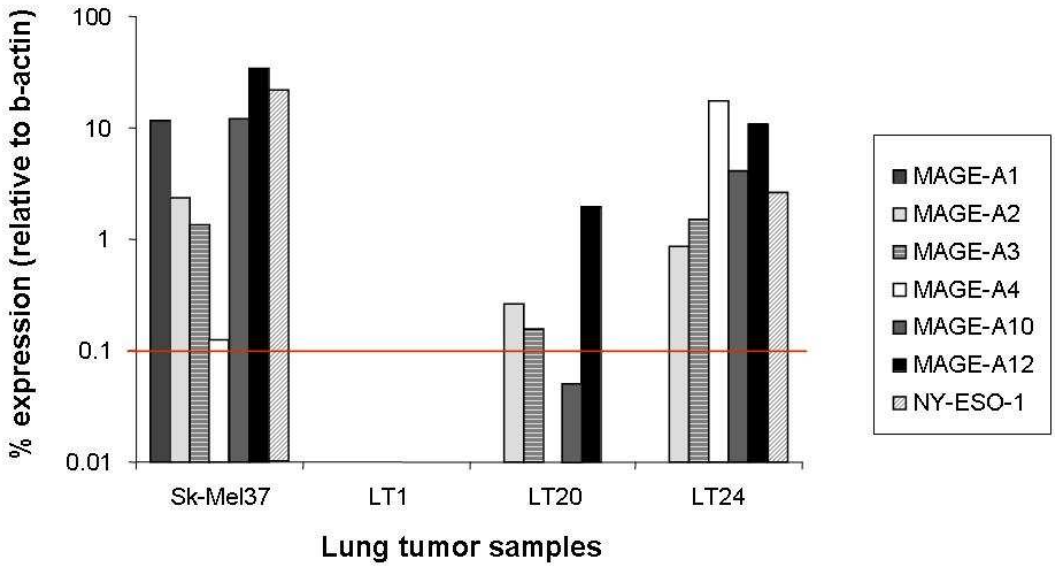

Figure 5.6: CTA expression patterns from individual NSCLC specimens. CTA expression observed in lung tumor samples (LT) was compared to expression in positive control cell line SK-Mel37. Expression of individual CTA genes representing less than 0.1% of β-actin expression was considered as negative.

For a better characterization of each tumor sample, CTA gene expression was independently evaluated from two pieces of each tumor. The results from both measurements were averaged and are represented in Figure 5.7.

Following the processing and analysis, tumor samples were classified as “CTA positive” if gene expression of at least one CTA was >0.1% of β-actin level. The quantitative detection of each CTA mRNA resulted in grading the expression from “weak” (between 0.1% and 1% of β-actin signal), “moderate” (between 1% and 10% of β-actin signal) to “strong” (above 10% of β-actin signal).

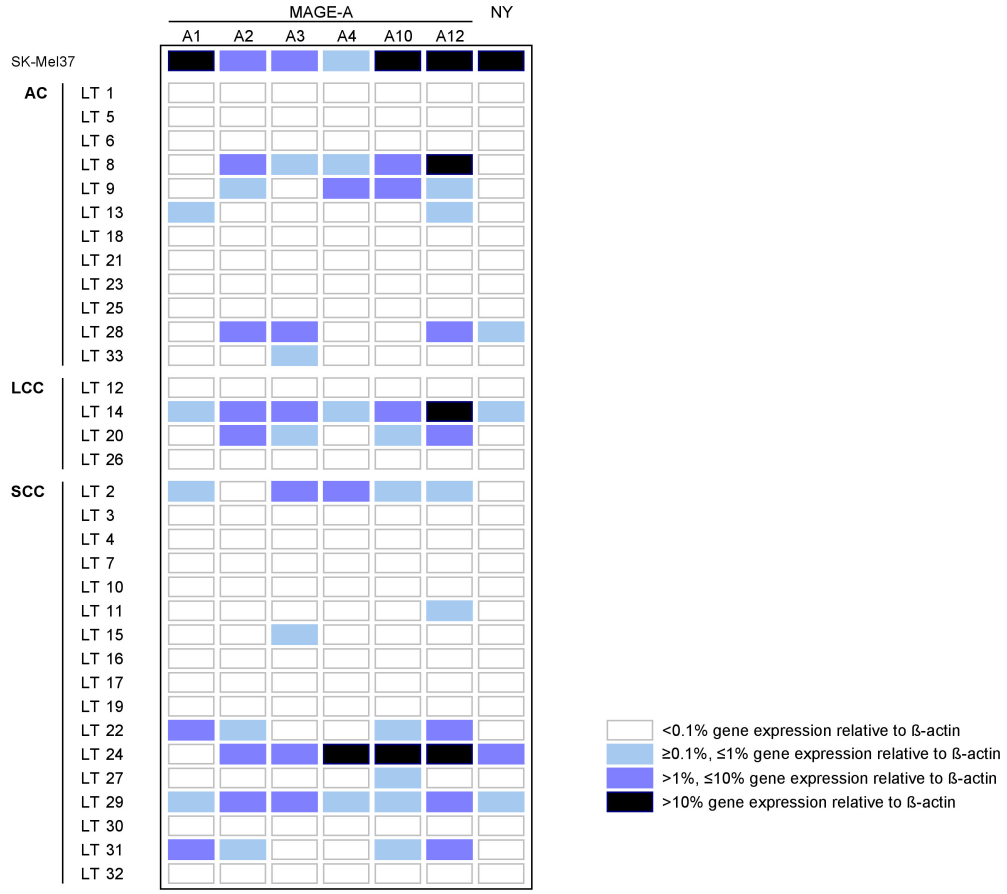

Figure 5.7: CTA gene expression of NSCLC specimens. Upon reverse transcribtion, cDNA of MAGE-A1, -A2, -A3, -A4, -A10, -A12 and NY-ESO-1 (NY) was amplified by quantitative real time PCR. Two individual samples were investigated for each specimen; data illustrate the mean of both measures. AC = adenocarcinoma; LCC = large cell carcinoma; SCC = squamous cell carcinoma; LT = lung tumor.

In total, 15/33 samples were CTA positive (45%). At least one CTA gene was expressed in 5/12 adenocarcinomas (42%), 2/4 large (50%) and 8/17 squamous cell

carcinomas (47%). In particular, MAGE-A12 and MAGE-A10 were the most frequently expressed CTA genes with 12/15 and 10/15 cases, respectively. NY-ESO-1 was only expressed in 4/15, MAGE-A1 and MAGE-A4 genes in 6/15 positive samples. In most cases, multiple CTA were concomitantly expressed. In 10/15 positive tumor samples including 3/5 adenocarcinomas, 2/2 large and 5/8 squamous cell carcinomas, expression of at least four CTA genes was detected.

5.2.1.2 Protein expression

Immunohistochemical studies were performed in 19/33 NSCLC samples in order to detect CTA protein expression. Our group previously developed two antibodies, 57B and D8.38, recognizing several MAGE-A proteins (MAGE-A1, -A2, -A3, -A4, -A6 and -A12; [Rimoldi et al., 1999]) and NY-ESO-1, respectively.

The results obtained by staining tumor sections with these antibodies were graded, taking into account the intensity and the area percentage of the staining, according to a histoscore (see materials & methods, 3.2.6).

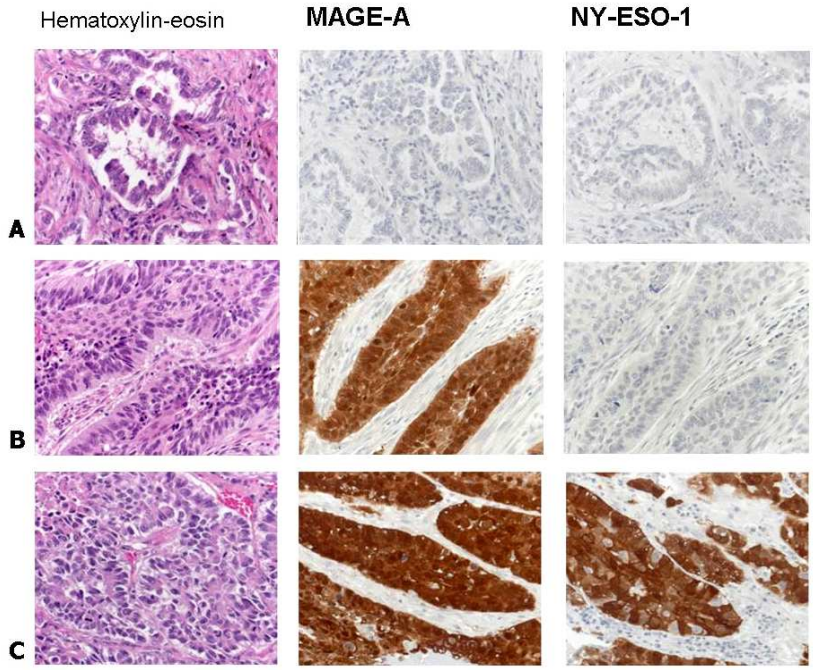

Figure 5.8: Immunohistochemical detection of CTA protein in NSCLC. Tumor sections were stained with antibodies 57B, specific for MAGE-A1, -A2, -A3, -A4, -A6 and -A12 proteins, and D8.38, specific for NY-ESO-1 protein. **A** = no CT protein expression; **B** = strong MAGE-A protein staining (histoscore 300), but negative for NY-ESO-1 protein; **C** = strong expression of MAGE-A and NY-ESO-1 proteins (histoscore 300 for both stainings).

14 out of 19 NSCLC sections tested were positive for MAGE-A staining whereas for five specimens, the histoscore was below 30 and were therefore classified as negative. NY-ESO-1 protein was detected in six sections only. In 2 of 19 tumor samples (LT14 and LT24) the maximum histoscore of 300 was detected for MAGE-A as well as for NY-ESO-1 proteins.

A large heterogeneity of MAGE-A and NY-ESO-1 protein expression was detectable in the majority of specimens. Expression was frequently focal and limited to defined tumor areas. This heterogeneity was even visible within relatively small areas, where strongly stained cells were detected close to negative cells (Figure 5.9).

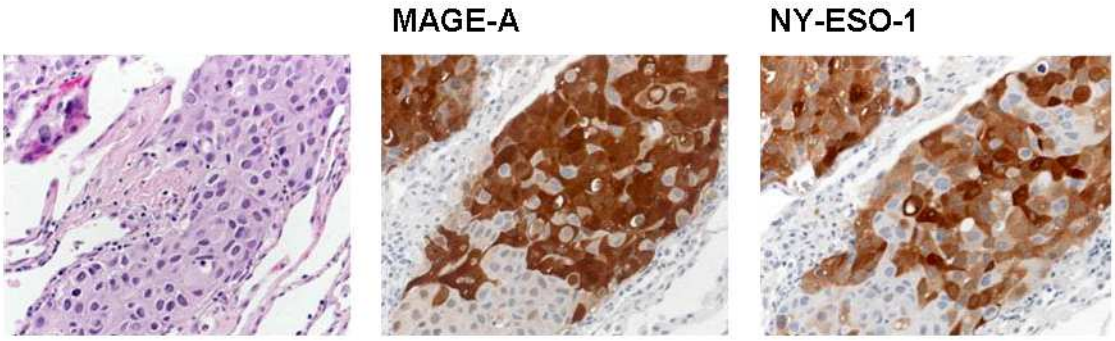

Figure 5.9: Heterogeneity of expression. Tumor section stained with antibodies 57B and D8.38. For both MAGE-A and NY-ESO-1, intensity of protein expression displays strong variation from cell to cell within observed tumor area.

5.2.1.3 Correlation gene/protein expression level

PCR results of CTA genes average expression levels were evaluated in comparison with those obtained at the protein level by immunohistochemistry, performed on tumor sections. Overall, these techniques did generate matching results except in a few cases (Figure 5.10).

MAGE-A expression was detected in 14 of 19 NSCLC specimens tested, at gene as well as at protein level. In one case, immunohistochemistry was negative in the presence of a weak MAGE-A12 gene expression (LT11). In LT32, although no CTA gene expression was observed, a weak MAGE-A specific staining was detectable. Similarly, positivities for NY-ESO-1 were detected by immunohistochemistry in two

samples where PCR were negative. However, in both cases, staining was focal with a low histoscore (≤60) (LT8 and LT13).

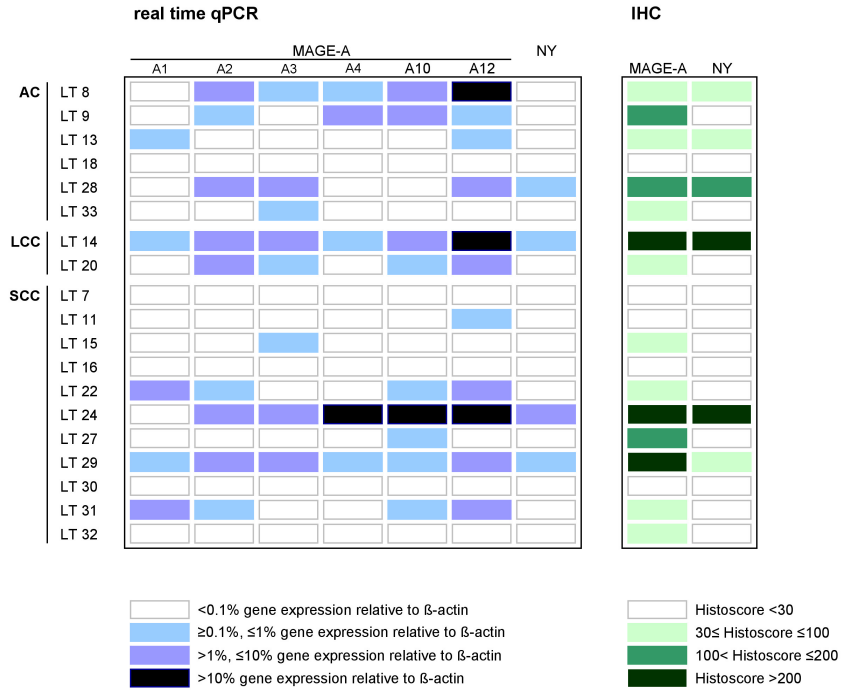

Figure 5.10: Comparison of CTA gene and protein expression in NSCLC specimens. CTA gene expression was evaluated by quantitative real time PCR, protein expression by immunohistochemistry (IHC). Only specimens investigated by both techniques are illustrated. AC = adenocarcinoma; LCC = large cell carcinoma; SCC = squamous cell carcinoma; LT = lung tumor; NY = NY-ESO-1.

5.2.1.4 CTA expression related to Tumor Stage and Grade

Expression of CTA genes in tumors is often thought to be related to their advanced stage or histological grade [Bolli et al., 2002; Gure et al., 2005]. Therefore, this correlation was evaluated in the NSCLC samples involved in this study.

Relative intensity of CTA expression, as detected by PCR, was graded based on the number of genes expressed and their relative expression level in each tumor specimen. Intensity of expression varied from 0 = absent or below threshold to 1 = weak, 2 = moderate, or 3 = strong. Maximum theoretical value of this index is 21, corresponding to a strong expression of all seven CTA under investigation. Thus, SK-Mel37 has a relative intensity of CTA expression of 17.

Considering all NSCLC specimens tested in this study (n=33), expression of CTA appeared to increase with tumor stage (Figure 5.11) although not reaching statistical significance due to high standard deviations. The majority of stage I tumors poorly expressed CTA (mean score = 1.67). The mean score value of CTA expression in stage II was twice as high (mean score = 3.11), and continued to increase in stage III tumors (mean score = 4.56). Remarkably, no CTA expression was detected in any of the three stage IV tumors. Of note, these samples refer to metastases only, as opposed to primary lung tumors sampled in the other stages.

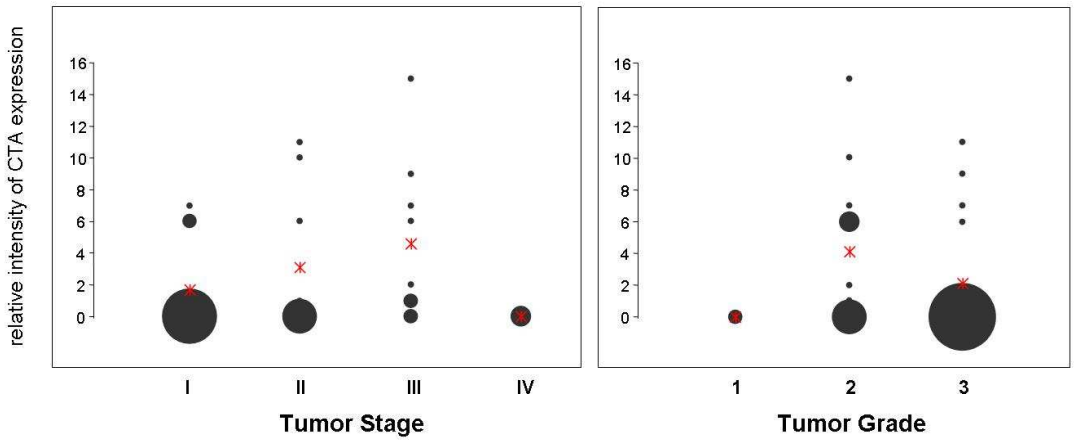

Figure 5.11: CTA expression related to stage and grade of NSCLC specimens. Relative intensity of CTA expression was graded based on the number of genes expressed and their relative expression level in each specimen. **left:** Tumor stage. Mean score values (x) of stage I = 1.67±2.8, stage II = 3.11±4.6, stage III = 4.56±5.1, stage IV = 0; **right:** Tumor grade. Mean score values (x) of grade 1 = 0, grade 2 = 4.08±4.75, grade 3 = 2.12± 3.66.

Regarding tumor grade, no correlation was observed with expression of CTA from the NSCLC specimens under investigation. Relative intensity of CTA expression was highest in tumors of grade 2 (mean score = 4.08), while 13/17 grade 3 tumors were only poorly expressing CTA (mean score = 2.12). The two specimens of grade 1 were negative for CTA expression.

5.2.2 Lymphocyte infiltration

Characterization of lymphocytes infiltrating the tumors was performed by flow cytometry. After disruption of tissues by mechanical and enzymatic treatments, cells

were labelled with antibodies specific for CD3, CD4, CD8, CD14, CD45 and CD16/56. In general, numbers of lymphocytes detected in NSCLC samples were small. Only a minority of cells expressed the T cell marker CD3 (on average <2.5% of total cells). Accordingly, CD8+ and CD4+ cells were detectable in small percentages (0.7±0.8% and 0.17±0.09%, respectively). Monocytes/macrophages (CD14+) and NK cells (CD16/56+) represented on average 0.2% of total cells.

Whereas phenotypic characterization could not be systematically performed, presence of T cells was also determined by quantitatively measuring the expression of CD4 and CD8 genes in total RNA from tumor samples of different stages and grades (Figure 5.12).

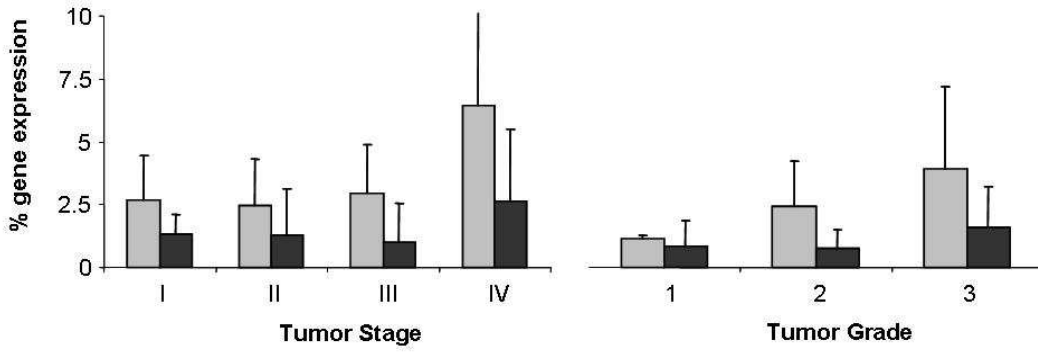

Figure 5.12: CD4+ and CD8+ T cell infiltration related to stage and grade of tumor.
□ CD4 gene expression; ■ CD8 gene expression.

Expression of both CD4 and CD8 genes was detected in tumors of all stages with similar intensity. Comparatively, higher expression of these markers was found in stage IV tumor samples, although these data resulted from three metastatic specimens only. No significant correlation with tumor grade was observed.

To preliminarily assess the activation status of TIL from NSCLC, expression of the gene encoding IFNγ was tested by qPCR. IFNγ transcripts could indeed be amplified from the majority of specimens, however, the level of expression only exceeded 0.1% of β-actin gene expression in five out of 32 cases.

5.3 Cytotoxic T lymphocytes

One of the aims of this study was the detection / generation of CTA specific CTL from TIL. Thus, tumor infiltrating CD8+ cells needed to be purified, characterized and specifically stimulated. However, due to their limited amount in fresh samples, a direct measurement of CTA specific immune response could not be performed. Thus, TIL were unspecifically expanded prior to antigenic stimulation.

5.3.1 Expansion

TIL expansion was performed by antibody mediated CD3/CD28 triggering in the presence of IL-2. After ten days, the T cell fraction represented up to 95% of the cultured cells (Figure 5.13, panel A). Average data from the samples under investigation are reported in figure 5.13, panel B. In seven cases, TIL could not be amplified due to contamination.

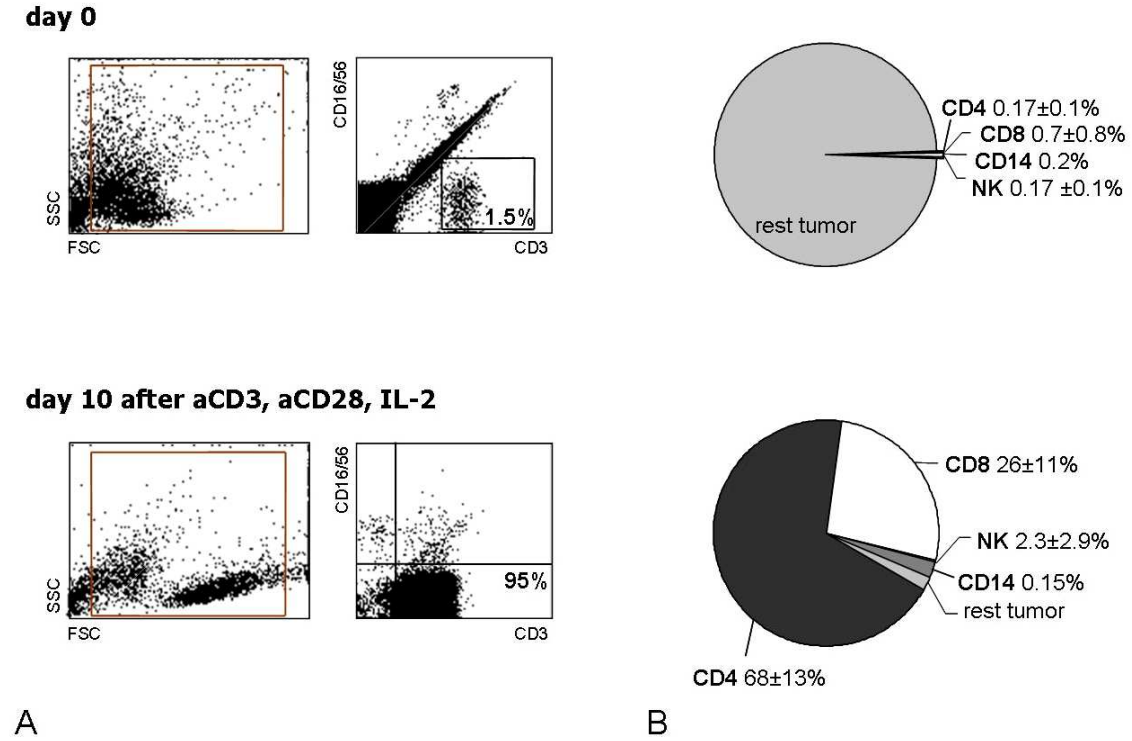

Figure 5.13: TIL population before and ten days after non specific expansion.
A: Example of phenotypic characterization with specific antibody for CD3+ cells.
B: Phenotypic average from 33 NSCLC specimens.

Expanded cell populations included a majority of CD4+ (68±13%) and a substantial percentage of CD8+ cells (26±11%). NK cells and monocytes percentages remained modest (<3% of total expanded population) (Figure 5.14).

Importantly, expanded cells represent a selected population out of original tumor infiltrating lymphocytes.

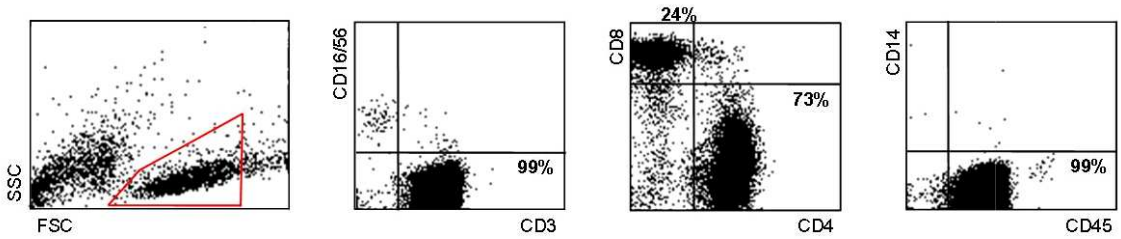

Figure 5.14: Characterization of TIL after non specific expansion. Cells were stained using specific antibodies for CD3, CD4, CD8, CD14, CD45 and CD16/56. Analysis was performed on gated population (figure left).

Non specifically expanded CD8+ T lymphocytes were phenotypically characterized for CTA specificity. Staining with multimers specific for CTA peptides was barely detectable. Even when the tumor expressed the antigens under investigation, TIL did not seem to be particularly enriched in specific CTL.

5.3.2 Specific CTL induction

Testing the CTA specificity and the cytolytic potential of CD8+ effector cells purified from expanded TIL populations required repeated specific *in vitro* stimulations with autologous mature dendritic cells (mDC). These stimulations were performed using either two (MAGE-A1 and -A3, HLA-A*0101 restricted), four (MAGE-A4, -A10, multi-MAGE-A and NY-ESO-1, HLA-A*0201 restricted) or six epitopes (MAGE-A1, -A3, -A4, -A10, multi-MAGE-A and NY-ESO-1), depending on patients' HLA type.

Out of 26 expanded TIL populations, 15 were stimulated with autologous APC pulsed with CTA derived soluble peptides. The other eleven CD8+ lymphocyte populations were stimulated with APC alternatively infected with $rVV_{combi\text{-}MAGE}$ or pulsed with peptides. In six of them both protocols were comparatively evaluated.

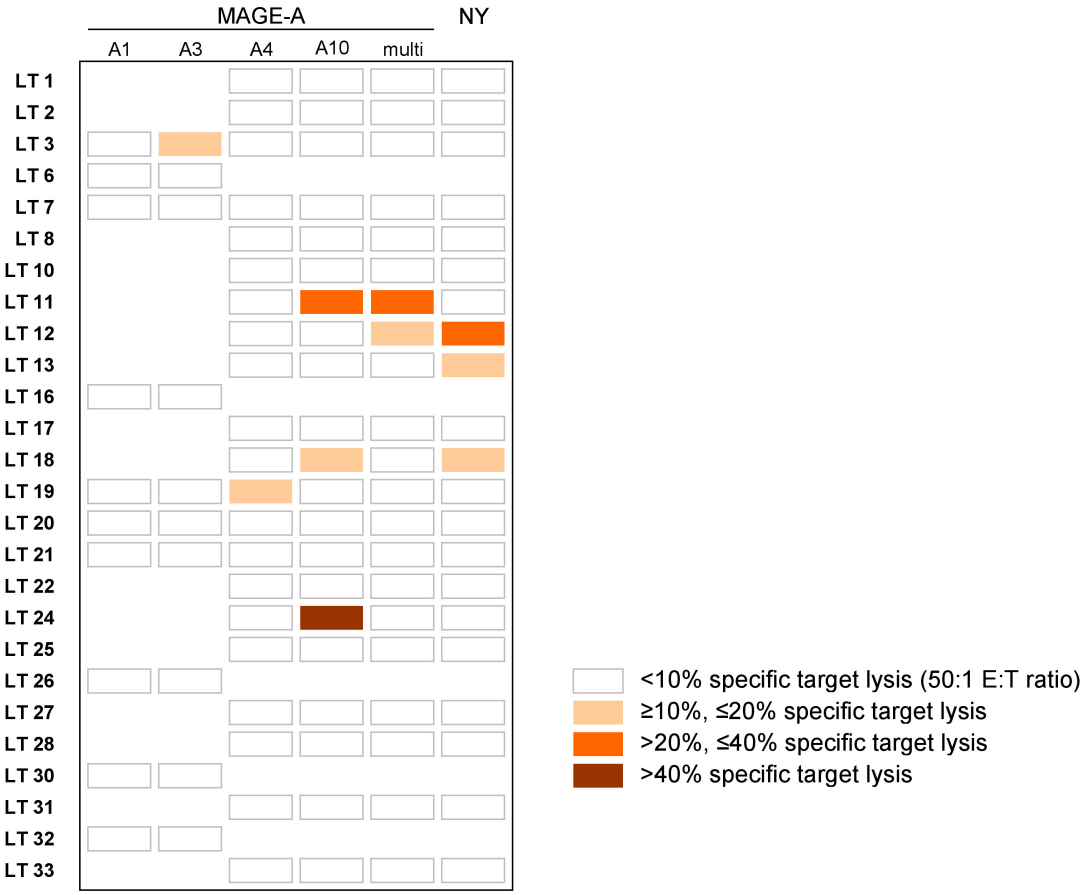

Figure 5.15: CTL response detection / generation in NSCLC upon specific *in vitro* stimulation. CTA specific CTL from expanded CD8+ T lymphocytes infiltrating the tumor specimens (LT) stimulated with mature IL-4-DC and specific CTA epitopes in form of soluble peptides or encoded by minigenes in rVV. NY = NY-ESO-1.

Out of a total of 104 CD8+ cultures restimulated up to five times with CTA, in ten from seven different patients a significant specific lysis (>10% at 50:1 ET ratio) was detected (Figure 5.15).

In six of ten cultures, only a minor cytotoxic activity not exceeding 20% at 50:1 ET ratio was observed. In three antigen stimulated cultures, specific CTL response ranged between 20% and 40% specific lysis (at 50:1 ET ratio). Representative examples of minor and negative epitope specific cytotoxicity are reported in figure 5.16.

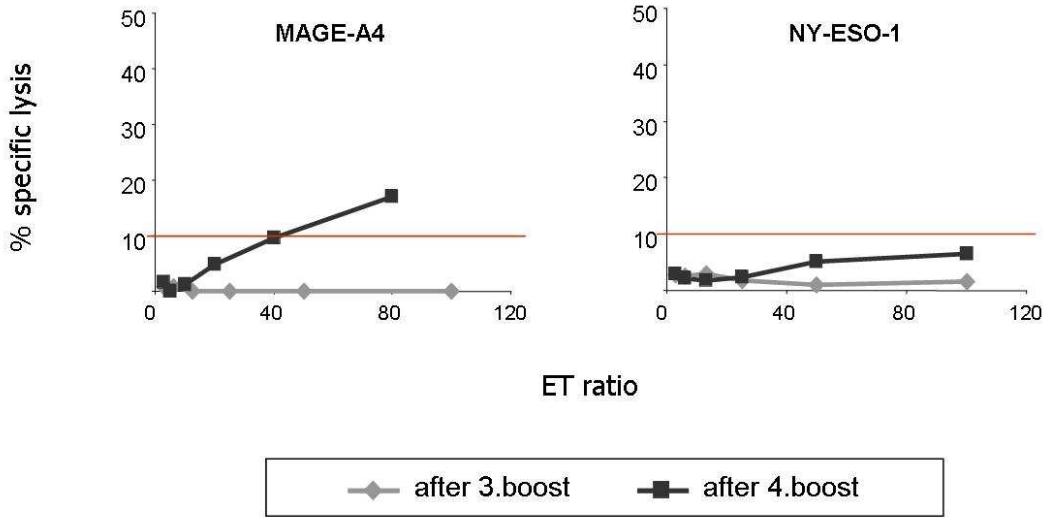

Figure 5.16: CTA specific cytotoxic activity of CD8+ T lymphocytes infiltrating NSCLC. Following three and four boosts with HLA-A*0201 restricted CTA epitopes (example with MAGE-A4 and NY-ESO-1, LT19) specific target lysis was assessed by ^{51}chromium release assays. Cytotoxic activity of less than 10% specific lysis at 50:1 ET ratio was considered as negative.

However, in these nine cultures, despite several specific stimulations, CTA specific multimer staining of effector cells remained below 0.5%, as illustrated in figure 5.17.

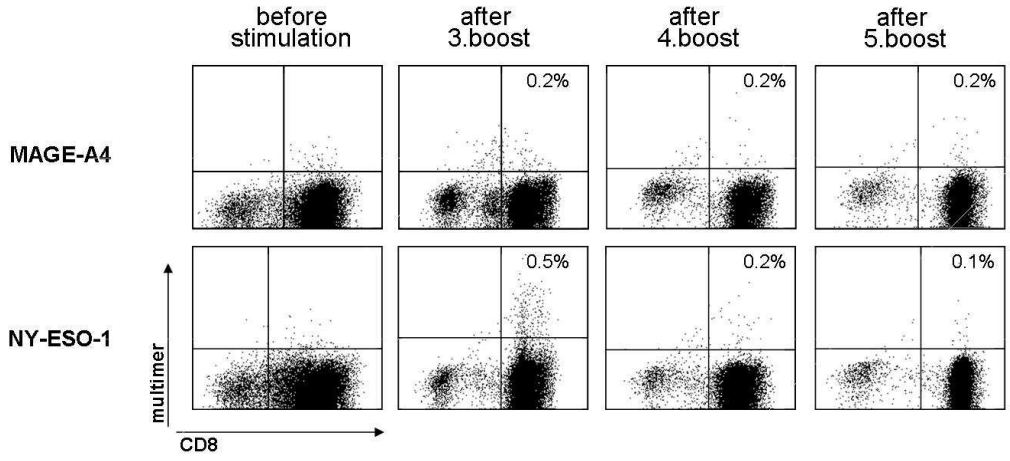

Figure 5.17: Phenotypic analysis of tumor infiltrating CD8+ T cells after specific stimulation with CTA epitopes. After each specific stimulation with CTA epitopes (example with MAGE-A4 and NY-ESO-1, LT19) CD8+ T lymphocytes were stained with corresponding MHC-multimers and CD8 specific antibodies.

However, following stimulation of CD8+ TIL from NSCLC patient LT24, a strong specificity for one of the CTA under investigation could be observed. While upon anti CD3/CD28 non antigen specific stimulation only 0.4% of CD8+ cells displayed the MAGE-A10 HLA-A*0201 phenotype as detected with specific multimers, this small population became a much larger fraction of CD8+ T cells (>35%) following antigen stimulation. A corresponding cytotoxic activity exceeding 60% specific lysis at 50:1 ET ratio was observed following only three stimulations of TIL (Figure 5.18).

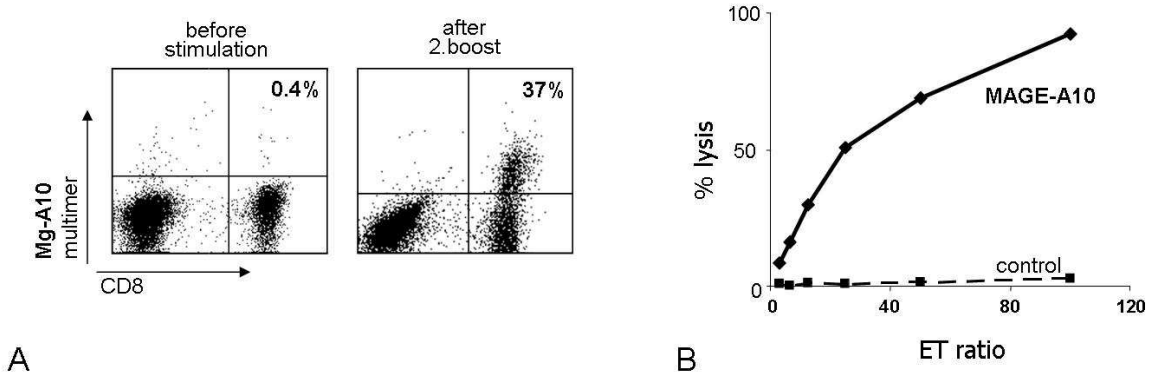

Figure 5.18: MAGE-A10 specific CTL characterization of specimen LT24. **A:** MAGE-A10 effector T cells phenotype before and after specific stimulations. CD8+ cells were stained with MAGE-A10 multimer. **B:** Cytotoxic activity of CTL following three stimulations with MAGE-A10 antigenic epitopes. Specific lysis was assessed by ^{51}chromium release assay.

5.3.2.1 Use of rVV for specific CTL stimulation

Comparative studies on the efficacy of antigenic stimulation with the viral vector versus soluble peptides were performed in six NSCLC patients. In 21/24 cultures CTA specific CTL response was undetectable. In one case cytotoxicity was only generated upon soluble peptide stimulation, whereas in the remaining two, MAGE-A10 and NY-ESO-1 specific responses were only observed in the virus stimulated cultures (Figure 5.19).

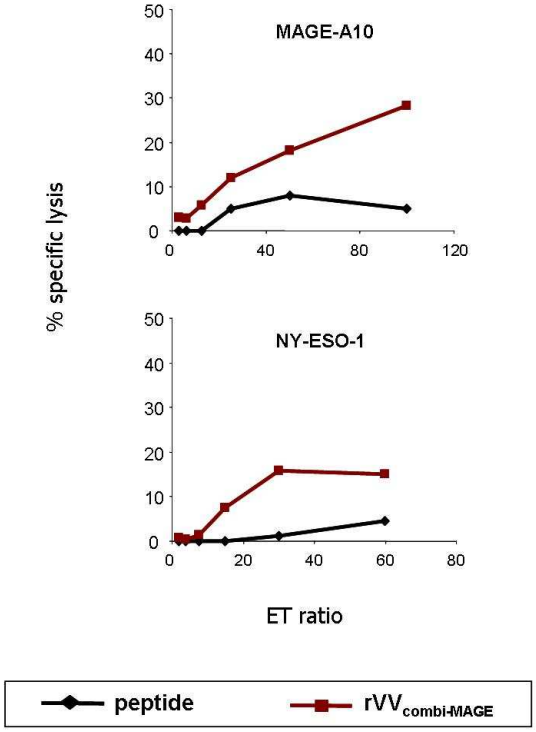

Figure 5.19: Comparative stimulations of CD8+ T lymphocytes using either soluble peptides or $rVV_{combi\text{-}MAGE}$ as immunogenic reagents. CD8+ T cells stimulated with mDC either only pulsed with soluble peptides (example with MAGE-A10 and NY-ESO-1, LT18), or in alternating cycles pulsed and infected with $rVV_{combi\text{-}MAGE}$ encoding the respective epitopes. Cytotoxicity was tested after five stimulations by ^{51}chromium release assay.

Interestingly, the high CTL specificity against MAGE-A10 observed in CD8+ T cell population of LT24 (Figure 5.18) had been primed by rVV infected APC.

5.4 Parameters influencing CTL detection/generation

All in all, despite several stimulations using strong APC and immunogenic reagents, detection / generation of CTA specific CTL from expanded TIL populations of NSCLC was extremely rare. To obtain an insight into this lack of cellular immune responsiveness, several parameters of the tumor micro-environment possibly influencing CTL presence or activation, were investigated.

5.4.1 Expression of CTA

The use of tumor infiltrating lymphocytes for the detection of CTA specific CTL is based on the assumption that these CTL are enriched in the environment where the antigens are expressed. However, comparison of specific CTL with the expression of corresponding CTA in the original tumor specimens demonstrated no obvious correlation (Figure 5.20).

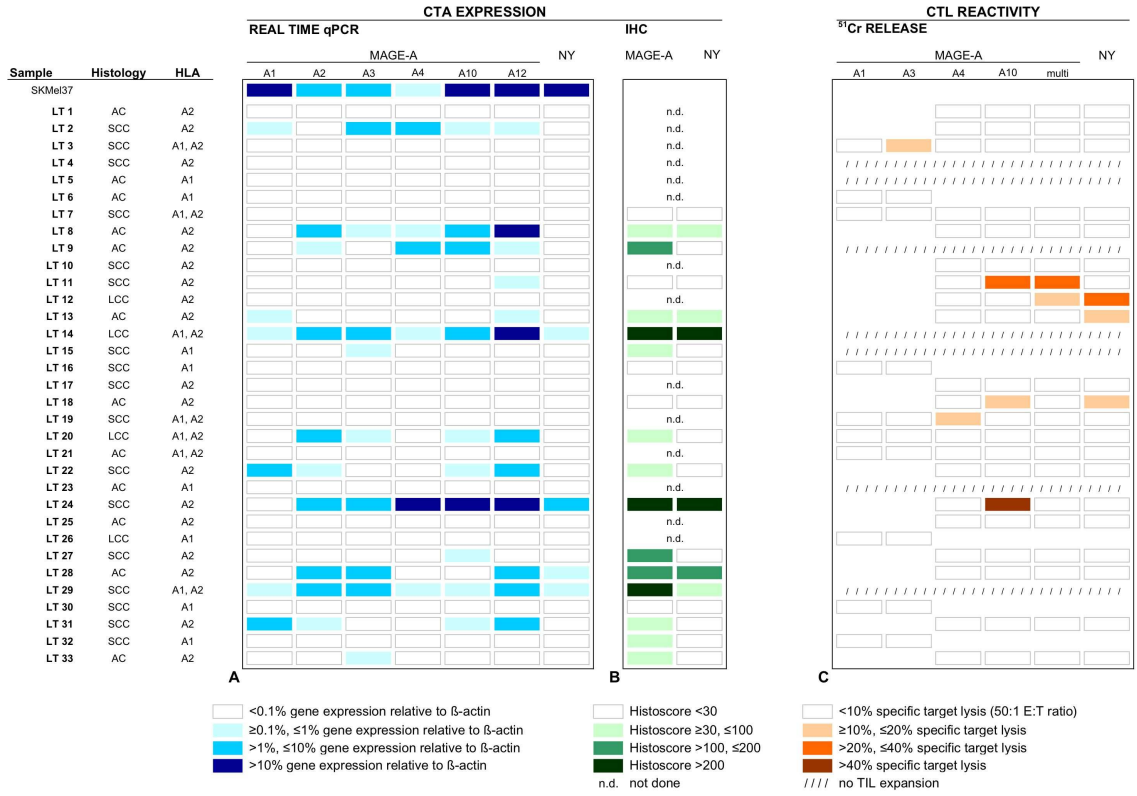

Figure 5.20: Overview of CTA expression and immune responses obtained from 33 NSCLC specimens. LT = lung tumor; AC = adenocarcinoma; LCC = large cell carcinoma; SCC = squamous cell carcinoma; IHC = immunohistochemistry; NY = NY-ESO-1.

In four of 15 tumor specimens expressing CTA, TIL could not be expanded and therefore evaluation of immune response was not possible.

Among the seven patients showing evidence of CTL response, LT24 emerged not only in terms of CTA expression level, but also in terms of expression pattern. Indeed, MAGE-A10 was highly expressed in the original tumor, while the tumors of the other six patients either did not express any CTA or not those targeted by the CTL generated *in vitro*.

Thus, results obtained from these 15 CTA positive NSCLC specimens did not obviously confirm the hypothesis of a CTA specific CTL enrichment in TIL.

5.4.2 Evaluation of Immunocompetence of CD8+ TIL

In order to attempt an assessment of the overall immunocompetence of TIL from NSCLC, infiltrating CD8+ cells were stimulated with two HLA-A*0201 restricted epitopes from the respiratory Epstein-Barr virus (EBV; epitopes derived from BMLF-1 and LMP-2 proteins). Following non antigenic CD3/CD28 expansion *in vitro*, specific CTL could easily be induced, as detected by both phenotypic and cytotoxic assays (Figure 5.21).

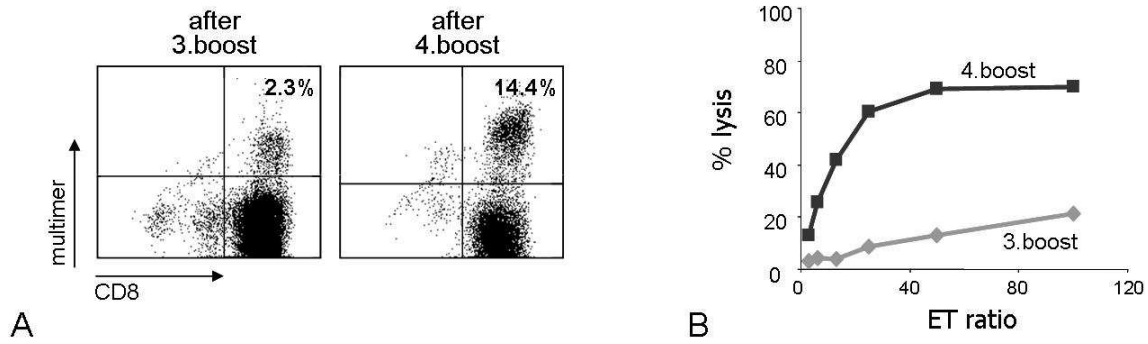

Figure 5.21: Antiviral CTL response from expanded TIL. CD8+ T cells (LT19) were stimulated with mDC pulsed with EBV derived epitopes. **A:** Phenotypic characterization after three and four boosts. Cells stained with CD8 specific antibodies and BMLF-1 and LMP-2 multimers; **B:** CTL sensitisation measured by cytotoxicity assay after three and four boosts.

However, these results *per se* do not exclude the presence of immunomodulatory factors in the tumor micro-environment possibly impairing local responses.

5.4.3 Immunomodulatory factors in freshly excised tumors

The expression of genes encoding factors playing a critical role on the activation status of effector cells was evaluated by qRT-PCR in a number of freshly excised tumor tissues (n=21). Among factors which might inhibit CTL generation, expression of FoxP3, IDO and IL-10 genes was measured and correlated with expression of IFNγ and TNFα genes, suggestive of an activated status of resident TIL. For each NSCLC specimen, levels of potential modulatory factors were put in relation with CTL generation or CTA expression.

5.4.3.1 Markers of activation

In situ activation of CD8+ T cells might represent a marker suggesting a capacity to generate specific CTL. Interestingly, expression of the key T cell activation marker IFNγ gene was found to correlate with CD8 gene expression level in the NSCLC samples tested in this study (Figure 5.22).

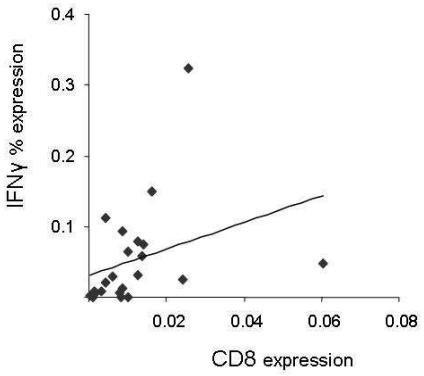

Figure 5.22: Correlation of CD8 gene expression with IFNγ gene expression. RNA expression profiles of tumor specimens (n=21) were analysed by nonparametric Spearman's correlation test; r = 0.62.

Thus, to evaluate if the activation status of CD8+ T cells might have affected their capacity to generate specific CTL, IFNγ/CD8 ratios were studied in TIL yielding or not specific CTL upon repeated in vitro stimulations.

The mean ratio of IFNγ/CD8 in TIL from which no CTA specific CTL could be raised was three times lower than the one from the lymphocyte populations, where CTA

specific immune responses could be generated (Figure 5.23). A significant correlation between IFNγ/CD8 ratio and CTL generation was thus detectable (p = 0.022).

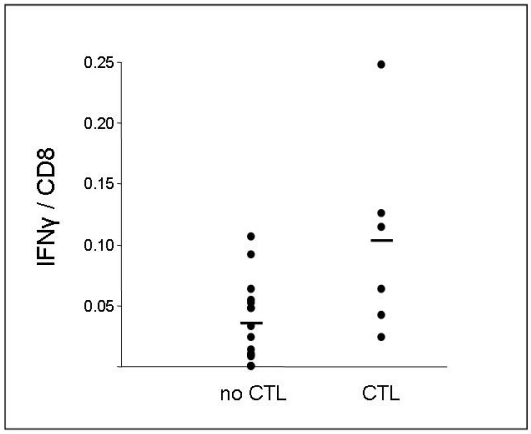

Figure 5.23: IFNγ/CD8 ratio from NSCLC specimens, grouped according to their capacity to generate specific CTL responses. Ratios were compared using Mann-Whitney's nonparametric test. Significant correlation, p = 0.022.

Importantly, the highest value observed in specimens with specific CTL responses (0.25) corresponds to sample LT24, which is the only one where CTL response is specific for a CTA expressed by the tumor.

TNFα is an important maker of local inflammation and immune activation. Interestingly, within the tumor samples tested in this study expression of TNFα gene is significantly lower than that detectable in healthy lung tissue (p = 0.025) (Figure 5.24). However, data obtained from different specimens did not support a correlation with CTL response nor with presence or absence of CTA.

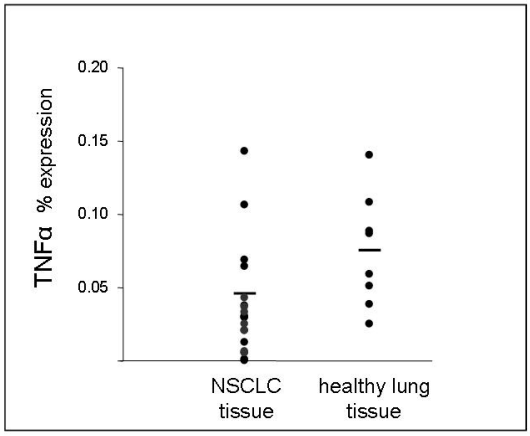

Figure 5.24: TNFα expression in NSCLC specimens and healthy lung tissue specimens. Mann-Whitney's nonparametric test; significant correlation, p = 0.025.

5.4.3.2 Markers of local cellular immune response inhibition

As compared to healthy lung tissue (n=8), genes encoding inhibitory factors such as IDO and IL-10 were overexpressed in freshly excised NSCLC specimens (Figure 5.25).

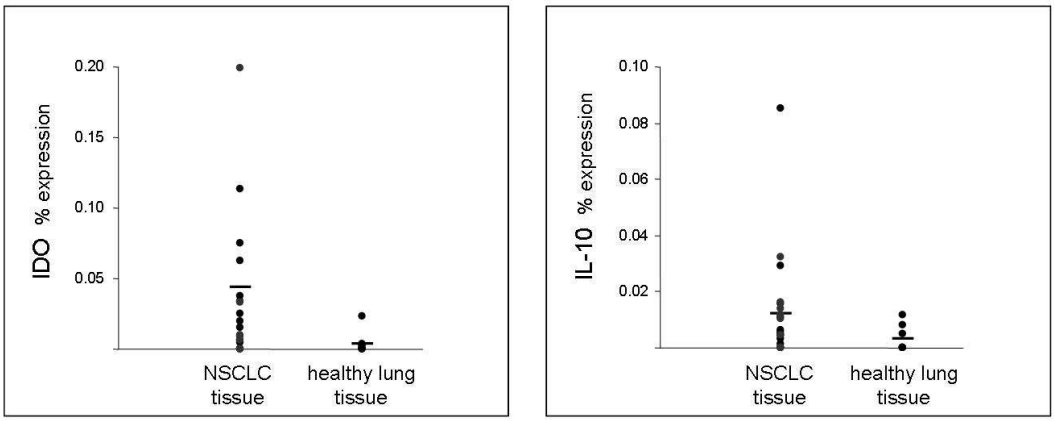

Figure 5.25: Overexpression of IDO and IL-10 in NSCLC specimens compared to healthy lung tissue specimens. Marker gene expression is relative to β-actin gene expression. Mann-Whitney; significant correlation for IDO, p = 0.022 (IL-10, p = 0.088).

These markers have been suggested to impair tumor surveillance by inhibiting the generation of CTL activity. Similarly, expression of FoxP3 gene, as a marker of regulatory T cells known to inhibit proliferation of effector cells, was analysed.

No specific negative correlation could be observed for any of the inhibitory markers (Figure 5.26). In addition, the level of IFNγ/CD8 ratio in the specimens was not found to be influenced by these factors.

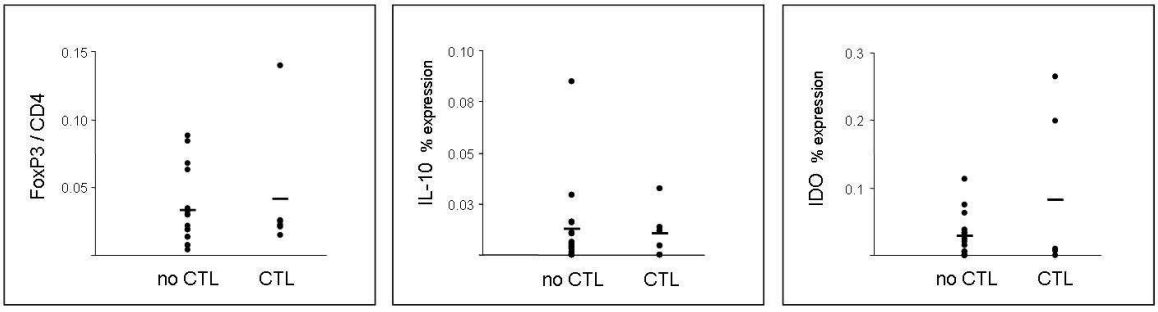

Figure 5.26: Expression of inhibitory factors in NSCLC specimens: comparison of tumors from which CTA specific CTL responses were generated or not. Each marker gene expression is relative to the expression of β-actin gene.

Despite the correlation between CTA expression and poor survival [Gure et al., 2005] and the trend linking stage and CTA also observed in this study, a relation between IDO, IL-10 or FoxP3/CD4 and CTA presence could not be observed for the NSCLC specimens under investigation.

5.4.4 Origin and number of CD8+ cells

One of the aims of this study was to demonstrate that the generation of specific CTL responses in NSCLC patients is possible, given an appropriate set of immunogenic reagents.

However, relatively small numbers of lymphocytes were initially obtained from the tumors, implying a limited diversity of antigen recognition. Moreover, despite demonstration of immunocompetence of TIL for viral antigens, limited CTA specific CTL generation was obtained, without correlation in 9/10 cases to CTA expression. On the other hand, the frequency of naive CTL specific for CTA is known to be low in PBL from healthy donors (in the range of 1 per 10e7 to 1 per 10e6 CD8+ T cells; [Chaux et al., 1998]). These findings may indicate that numbers of CD8+ cells purified from tumor specimens were not adequate to generate CTA specific CTL responses.

Thus, in order to attempt to increase the size of the CD8+ T cell pool, induction of CTA specific CTL was investigated on CD8+ peripheral blood lymphocytes obtained from patients bearing CTA expressing tumors as well as from healthy donors. CTA specific stimulations were performed in cultures including a minimum of 3x10e6 CD8+ cells and 1x10e6 APC per condition.

5.4.4.1 PBL from healthy donors

Peripheral blood isolated CD8+ cells from four different healthy donors (HD) were stimulated with CTA peptide pulsed IFNα-DC. These cells were recently shown to provide APC functions comparable to mDC [Lapenta et al., 2006].

In three out of four donors, CTA specific CTL were detected after two to four stimulations. Remarkably, in all of them specificity against MAGE-A10 epitope was induced. In one case, as shown in figure 5.27, MAGE-A10 specific multimer staining

was detected in nearly 27% and 50% of CD8+ population after only two and four stimulations respectively.

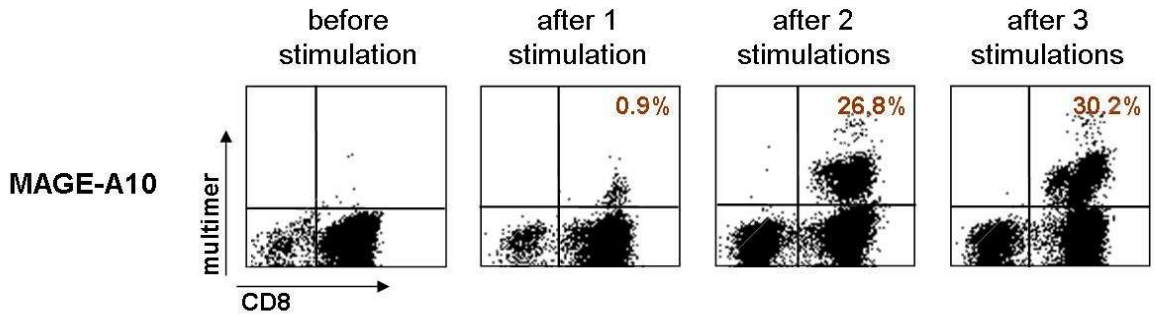

Figure 5.27: MAGE-A10 specific CTL generation following multiple specific stimulations. Peripheral blood CD8+ lymphocytes of healthy donors stimulated with MAGE-A10 peptides pulsed autologous IFNα-DC. Phenotypic characterization of CD8+ cells with MHC-multimer specific for MAGE-A10.

In addition to MAGE-A10, CTL specific for multi-MAGE-A and NY-ESO-1 epitopes were generated in two out of three cultures (Figure 5.28). While, after five stimulations, 30% of CD8+ T cell population was multi-MAGE-A specific, positivity for NY-ESO-1 multimers pertained to 7% only of CD8+ T cells after six stimulations.

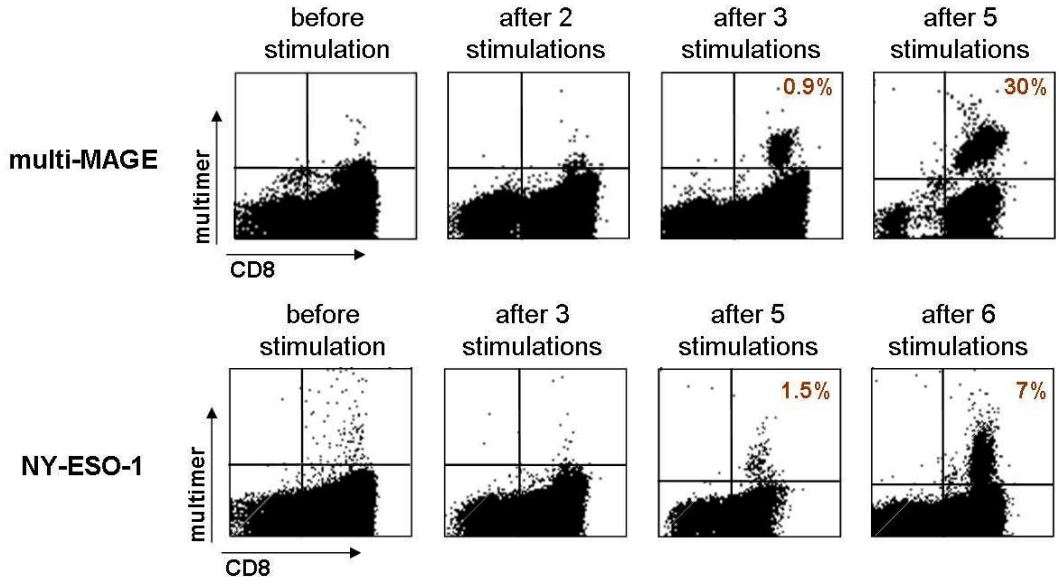

Figure 5.28: multi-MAGE-A and NY-ESO-1 specific CTL generation after multiple specific stimulations. Peripheral blood CD8+ lymphocytes of healthy donors stimulated with multi-MAGE-A or NY-ESO-1 peptides pulsed autologous IFNα-DC. Phenotypic characterization of CD8+ cells with corresponding MHC-multimers.

The effector function of these MHC-multimer positive populations was confirmed by standard chromium release assays. Strong cytotoxicity activity was detected in all these cases except in the NY-ESO-1 specific cell culture (Figure 5.29).

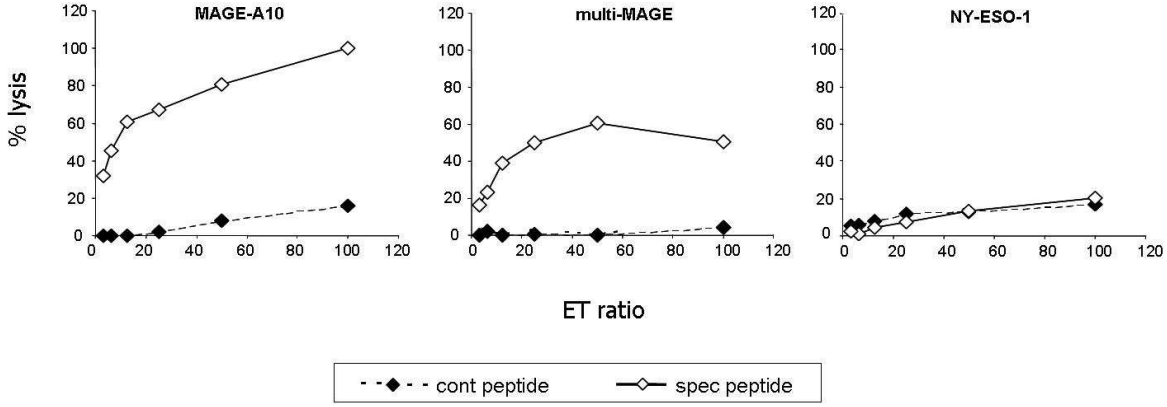

Figure 5.29: Functional analysis of CTL specific for MAGE-A10, multi-MAGE-A and NY-ESO-1. Peripheral blood CD8+ lymphocytes of healthy donors specifically stimulated for three (MAGE-A10), respectively five (multi-MAGE-A and NY-ESO-1) times. Cytotoxicity was measured by ^{51}chromium release assay.

Despite the successful CTL generation with MAGE-A10, multi-MAGE-A and NY-ESO1 epitopes, generation of specific CTL against MAGE-A4 failed in all four attempts with HD-PBL. As illustrated in figure 5.30, regardless of the number of stimulations with respective antigenic peptide, multimer detection of MAGE-A4 specific CD8+ cells remained negative. Likewise, no specific cytotoxicity against MAGE-A4 was detectable.

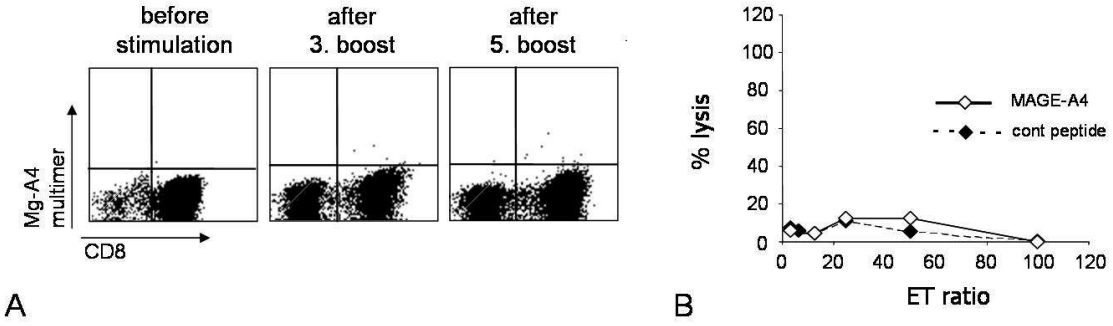

Figure 5.30: Phenotype and functional analysis of CD8+ cells after multiple MAGE-A4 specific stimulations. Peripheral blood CD8+ lymphocytes of healthy donors stimulated with MAGE-A4 peptides pulsed IFNα-DC. **A:** Flow cytometric analysis of MAGE-A4 CD8+ cells stained by MAGE-A4 multimers. **B:** Cytotoxicity analysis by ^{51}chromium release assay.

5.4.4.2 PBL from patients

Accordingly, in two NSCLC patients, peripheral blood CD8+ lymphocytes were isolated and stimulated with epitopes from CTA expressed in the tumor (multi-MAGE-A and MAGE-A10). As APC, either peripheral blood CD14+ cells directly, CD14+ derived IFNα-DC or tumor derived fibroblasts were used.

No responses against multi-Mage-A or MAGE-A10 could be observed in these patients.

6 Discussion

In contrast to other tumors such as melanoma, in lung cancer relevant immunologically dominant antigens remain unknown.

The ideal target antigen for cancer vaccines should be highly immunogenic, be expressed at a high level in tumor tissue, optimally by all malignant cells, and not or only at low levels in normal tissues.

Cancer/testis antigens represent interesting targets because of their highly restricted expression patterns in normal tissues [Chen and Old, 1999; Simpson et al., 2005] and their expression in a wide range of different human tumor types [Juretic et al., 2003]. Apparently, the expression levels vary between malignant tissues. Importantly, they have been found to be frequently expressed in non small cell lung cancers [Fischer et al., 1997; Weynants et al., 1994]. In addition, CT antigens such as NY-ESO-1 may be spontaneously immunogenic, inducing an integrated response involving both cellular und humoral arms of the immune system [Korangy et al., 2004].

These data led us to comparatively investigate in this work CTA expression in NSCLC and the capacity to induce a specific immune response in non vaccinated patients. 33 tumors with different histologies, stages and grades were studied. Inclusion criteria was the expression of A*0201 or A*0101 HLA alleles.

CTA expression was measured at gene level and confirmed at protein level. We observed frequent expression of CTA in squamous cell, adeno- and large cell carcinoma of non small cell lung cancer specimens. Nearly half of all tumor samples tested expressed at least one of MAGE-A or NY-ESO-1 antigens. Moreover expression was equally distributed in the three histological subtypes. Other works, however, described a more frequent CTA expression in squamous cell carcinoma as compared to other subtypes [Grunwald et al., 2006; Peikert et al., 2006].

CTA gene expression was not observed to increase with tumor grade. However, a trend could be detected regarding the stage, which depends on size and metastatic characteristics but not on differentiation status of primary tumor. This may remind previous works where expression of these antigens was shown to correlate in

NSCLC with poor prognosis [Bolli et al., 2002; Gure et al., 2005]. The inherent variability of expression and the relatively small number of specimens involved in this study might explain the poor correlation found in the 33 NSCLC specimens under investigation.

Protein detection data revealed that in many tumor tissues, CT antigens are not found to be expressed by all tumor cells. This phenomenon of focal expression was already observed in other carcinomas [Bolli et al., 2002; Chitale et al., 2005; Sarcevic et al., 2003]. Clearly, in this context, complete destruction of the whole malignant tissue by CTL of a single specificity might appear unrealistic. However, the occurrence of local antigen spreading may compensate the limitations of the initial immune response. This phenomena may lead to the activation of different CTL specificities by antigens released by tumor cells killed by the vaccine specific T cells [Ma et al., 2004; Vanderlugt and Miller, 2002].

As previously described for CTA [Gure et al., 2005], concomitant expression of multiple MAGE-A and NY-ESO-1 genes was frequently detected within the same specimen, suggesting the use of polyvalent CT antigen specific vaccines in subgroups of NSCLC patients [Chianese-Bullock et al., 2005]. By targeting various CTA, the number of eligible patients can be increased. Furthermore, a vaccination against multiple CT antigens simultaneously expressed in tumors might decrease the risk of tumor escape from the immune system, through the loss or down-regulation of single target antigens.

Recently, MAGE-A4 was shown to be involved in the positive regulation of apoptosis, thus, potentially acting as a tumor suppressor protein [Peikert et al., 2006]. Overexpression of this CTA may sensitize malignant cells to apoptotic triggering by chemotherapeutical agents. Remarkably, MAGE-A4 was one of the least expressed antigens detectable among the seven CTA tested in this study.

Spontaneous regression of tumors without therapy and the heterogeneity of clinical progression of disease among patients with the same histologic malignancy suggest the existence of pre-sensitization of the immune system, possibly induced by tumor cells, playing a role in the clinical course of the disease [Shankaran et al., 2001]. Thereby, a strong incentive to the development of vaccination protocols, able to boost immune responses for therapeutic purposes in defined types of cancer, may be provided.

Clinical trials based on the administration of synthetic peptides or recombinant proteins suggest that specific T cells can indeed be induced by immunization [Atanackovic et al., 2004; Morse et al., 2005; Thurner et al., 1999]. Thus, for the first time, our study systematically investigated whether CTL recognizing specific CTA epitopes could be expanded from lymphocytes infiltrating NSCLC in non vaccinated patients.

Classical TIL expansion protocols were updated by using mitogenic anti-CD3 and anti-CD28 after collagenase digestion of tumor specimens. However, numbers of lymphocytes infiltrating the tumor were relatively low before non specific expansion, thus providing a limited repertoire for generation of CTA CTL. No correlation of T cell infiltration with tumor stage or grade could be observed. After non specific expansion, CTL responses were undetectable in tumor infiltrating lymphocytes. Several stimulations were performed using peripheral blood monocytes derived dendritic cells pulsed with soluble antigen or infected with a recombinant viral vector developed during this study.

CTA specific T cell stimulation was induced using mature DC as APC [Salio et al., 2001; Thurner et al., 1999]. Many clinical trials are addressing the feasibility and safety of tumor antigen loaded DC, as they are known to be highly effective APC. Upon stimulation with maturation factors such as inflammatory cytokines or stimulation via CD40 or toll-like receptors, DC upregulate MHC and adhesion molecules as well as costimulatory molecules thereby becoming terminally differentiated stimulators of T cell immunity [van Kooten and Banchereau, 1997].

CTA epitope specific CTL were only detected in a few expanded TIL populations, and cytotoxicity was usually weak and not complemented by specific multimer staining.

These observations suggest that the effector cells were possibly endowed with low functional avidity [Dutoit et al., 2002]. Furthermore, in the original tumor specimens, either no CTA was expressed (4/7 cases) or some CTA were indeed expressed, but not those encoding the specifically recognized epitopes (2/7 cases).

In one case, however, a high level of CTA specific CTL response was concomitantly detectable by cytotoxicity assays and multimer staining. MAGE-A10 gene encoding the target epitope was highly expressed in the original tumor, and MAGE-A specific staining was evident in a large majority of tumor cells.

Although among the few responses detected from TIL only one was against a CTA actually expressed in the same tumor, self antigen expression may influence the activation status of cytotoxic cells. Indeed in the CTL positive cases of this study, IFNγ level as related to CD8 gene expression was significantly higher when CTA were expressed. In the single case of high cytotoxic response, IFNγ/CD8 ratio was higher then that detectable in the other samples.

Still unclear is whether this constellation of data relates to immuno-editing of the antigenic phenotype of the tumors by specific CTL responses [Dunn et al., 2004] or if the detection of weak specific cytotoxicity could rather be attributed to primary *in vitro* sensitization. However, high CTA specificity detected in the one NSCLC specimen was most likely pre-existing and not *in vitro* induced.

In contrast to specific CTL induction frequently detected in melanoma TIL after stimulation with differentiation antigens [Spagnoli et al., 1995], in the present study, the majority of TIL populations of CTA positive NSCLC was unable to generate specific CTL responses despite repeated stimulation with a panel of well defined peptides and highly effective APC. These data suggest that specific CTL sensitization in NSCLC is not frequent. Importantly, efficient expansion of CTA specific CTL *in vivo*, has been shown to require long immunization courses [Coulie et al., 2001; Thurner et al., 1999].

In a number of previous reports frequency of CTL precursors specific for a CTA epitope has been shown to be very low in the blood of healthy donors or melanoma

patients prior to specific immunotherapy [Chaux et al., 1998; Godelaine et al., 2003]. This low frequency could explain the absence of CTL response, as numbers of lymphocytes infiltrating the tumor specimens were relatively small and therefore the potential TCR diversity reduced. Most interestingly, expression of a number of CTA has recently been shown to take place also in the thymus [Gotter et al., 2004], thereby facilitating the induction of specific tolerance.

Apparently, many tumors are able to avoid attacks of the immune system by tumor escape mechanisms, such as downregulation of MHC or antigens, or creation of a tumor microenvironment unfavourable to immunocompetent cells. Secretion of factors (e.g. Indoleamine 2,3-dioxygenase (IDO), transforming growth factor-β, IL-10) and induction of regulatory T cells are known to suppress anti-tumor immune responses [Kim et al., 2006; Uyttenhove et al., 2003]. Recently, T regulatory CD4+ cells expressing FoxP3 were found to be accumulated in NSCLC, especially in early stages [Ishibashi et al., 2006]. Furthermore, other studies suggested infiltration of IDO expressing cells as prognostic marker of poor survival in NSCLC [Astigiano et al., 2005].

Intervention of some of these tumor escape mechanisms, as a possible explanation for the low number of positive results obtained, was investigated. Expression of genes encoding inhibitory factors IL-10, IDO or expressed by regulatory T cells, such as FoxP3, was evaluated in the different NSCLC specimens. No major correlation between absence of CTL response or expression of CTA and levels of these genes could be observed.

Tumor infiltrating lymphocytes freshly derived from surgically excised tumor specimens have been suggested to be anergic, unable to proliferate and to produce factors, such as IFNγ, upon T cell receptor triggering [Zippelius et al., 2004]. However, it has been demonstrated that TIL derived CD8+ cells can be expanded after purification and restimulation [Ratto et al., 1996]. Furthermore, in melanoma studies using TIL, these cells were shown to be indeed at least as efficient as peripheral lymphocytes in the generation of effector cells [Spagnoli et al., 1995].

Peripheral blood T cells (PBL) may display a comparatively enhanced reactivity, possibly targeting a wider range of epitopes. By testing naive PBL from healthy donors, in absence of any CTA expression, CTL responses specific for MAGE-A10, multi-MAGE-A and NY-ESO-1 could indeed be induced. In this case, to compensate the likely absence of pre-sensitization, high numbers of CD8+ T cells were stimulated with CT antigens. These data indicate that CTA cellular immune responses can be generated from appropriate pools of healthy donors' T cells.

However, despite attempts to generate similar responses from PBL of NSCLC patients, we were unable to raise CTL specific for the two epitopes tested.

Optimal CTL activation depends on efficient antigen presentation by MHC molecules loaded through the class I endogenous pathway of antigen processing [Maffei et al., 1997; Townsend et al., 1986; van Endert, 1999]. In contrast to the CTL response evaluation in NSCLC specimens, in healthy donors DC were derived from GM-CSF stimulated monocytes by using IFNα instead of IL-4. As recently shown, these cells were more effective antigen presenting cells than IL-4-DC [Lapenta et al., 2006]. Still unclear is whether generation of CTA specific CTL from PBL decisively depends on the use of a different subtype of DC as APC.

This study also demonstrated the potential of DC pulsed-peptides as immunogenic reagents for stimulation of PBL *in vitro*. HLA-A*0201 restricted CTA epitopes were able to efficiently induce CTL responses mostly for MAGE-A10, but also for multi-MAGE-A and NY-ESO-1. Soluble peptides were used at high concentration, clearly saturating the antigen uptake of APC. However, the similar use of peptide MAGE-A4 for PBL stimulation of healthy donors did not induce any response even after numerous stimulations. Among the many possible reasons leading to these differences one may mention a lower immunogenicity of the epitope, peptide instability or a lower frequency of MAGE-A4 CTL precursors.

Notably, the clinical use of peptides alone is limited by their rapid proteolytic digestion, leading to low bio-availability and poor immunogenicity. In contrast, tumor specific cell mediated immune responses could be elicited by infection of APC with recombinant viruses encoding multiple TAA, mimicking the endogenous pathway of antigen presentation by MHC class I molecules.

Vaccinia virus vectors were shown to be highly immunogenic and induce humoral and cellular responses [Graham et al., 1991]. Among others, our group demonstrated safety and immunogenicity of recombinant vaccinia viruses (rVV) in a phase I/II clinical trial in metastatic melanoma patients [Zajac et al., 2003]. Genes encoding costimulatory molecules such as CD80 and CD86 were also shown to enhance immune responses [Marti et al., 1997; Oertli et al., 2002].

Based on previous experience, we constructed viral vectors encoding the different CTA epitopes assessed for immune response induction in NSCLC. Immunogenicity of this vector was demonstrated on a few TIL populations isolated from NSCLC specimens. Of note, in the one case where high CTL response was detected, response was induced by using $rVV_{combi\text{-}MAGE}$, containing minigenes encoding HLA-A*0201 restricted MAGE-A4, MAGE-A10, multi-MAGE-A and NY-ESO-1 epitopes together with human CD80. Direct comparison of the use of peptides with the viral vector suggests the usefulness of vaccinia virus encoding epitopes as a potent vector for therapeutic vaccination.

Our extensive work on 33 NSCLC specimens and on the reactivity of TIL leads us to the conclusion that CTA may be relevant targets for an immunotherapy approach in this disease. Indeed, their expression is frequent and often concomitantly detectable in individual samples. Spontaneous immune response toward CTA was observed only in one patient and generation of CTA specific CTL remains rare. However, we demonstrated that it is nevertheless possible to generate multiples cellular immune responses also from peripheral blood lymphocytes from healthy donors.

Active specific immunotherapy approaches in NSCLC are currently limited. Phase I or II clinical trials have been reported using MUC1, HER-2/neu or telomerase peptides as well as whole protein MAGE-3 or EGF based vaccines [Atanackovic et al., 2004; Brunsvig et al., 2006; Gonzalez et al., 2003; Palmer et al., 2001; Salazar et al., 2003]. Beneficial clinical and immune responses have been described. In a phase II clinical study with cancer/testis protein MAGE-3, patients developed strong antigen specific CD4+ helper T cells along with specific antibodies and CD8+ T cell responses [Atanackovic et al., 2004].

However, these active specific immunotherapy trials are limited by the use of a single antigen. The *in vitro* and *in vivo* efficacy of our approach, based on an immunogenic vaccinia vector encoding for multiple antigens, was previously demonstrated for melanoma antigens. In the present study we showed the immunogenicity of a vector encoding CTA epitopes. Hence, it is tempting to speculate that a multi-CTA recombinant virus similar to the one described in this work might prove useful in the implementation of clinically effective antigen specific immunotherapy in NSCLC.

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Annex I – CT Gene Database

Gene Family	CT Identifier	Family Member	CT Identifier
MAGEA	CT1	MAGEA1	CT1.1
		MAGEA2	CT1.2
		MAGEA3	CT1.3
		MAGEA4	CT1.4
		MAGEA5	CT1.5
		MAGEA6	CT1.6
		MAGEA8	CT1.8
		MAGEA9	CT1.9
		MAGEA10	CT1.10
		MAGEA11	CT1.11
		MAGEA12	CT1.12
BAGE	CT2	BAGE	CT2.1
		BAGE2	CT2.2
		BAGE3	CT2.3
		BAGE4	CT2.4
		BAGE5	CT2.5
MAGEB	CT3	MAGEB1	CT3.1
		MAGEB2	CT3.2
		MAGEB5	CT3.3
		MAGEB6	CT3.4
GAGE1	CT4	GAGE1	CT4.1
		GAGE2	CT4.2
		GAGE3	CT4.3
		GAGE4	CT4.4
		GAGE5	CT4.5
		GAGE6	CT4.6
		GAGE7	CT4.7
		GAGE8	CT4.8
SSX	CT5	SSX1	CT5.1
		SSX2a	CT5.2a
		SSX2b	CT5.2b
		SSX3	CT5.3
		SSX4	CT5.4

NY-ESO-1	CT6	NY-ESO-1 LAGE-1a LAGE-1b	CT6.1 CT6.2a CT6.2b
MAGEC1	CT7	MAGEC1 MAGEC3	CT7.1 CT7.2
SYCP1	CT8	SYCP1	CT8
BRDT	CT9	BRDT	CT9
MAGEC2	CT10	MAGEC2	CT10
SPANX	CT11	SPANXA1 SPANXB1 SPANXC SPANXD	CT11.1 CT11.2 CT11.3 CT11.4
XAGE	CT12	XAGE-1a XAGE-1b XAGE-1c XAGE-1d XAGE-2 XAGE-3a XAGE-3b XAGE-4	CT12.1a CT12.1b CT12.1c CT12.1d CT12.2 CT12.3a CT12.3b CT12.4
HAGE	CT13	HAGE	CT13
SAGE	CT14	SAGE	CT14
ADAM2	CT15	ADAM2	CT15
PAGE-5	CT16	PAGE-5 CT16.2	CT16.1 CT16.2
LIPI	CT17	LIPI	CT17
NA88A pseudogene	CT18	NA88A pseudogene	CT18
CTAGE-1	CT21	CTAGE-1 CTAGE-2	CT21.1 CT21.2
CSAGE	CT24	CSAGE TRAG3	CT24.1 CT24.2
MMA1	CT25	MMA1a MMA1b	CT25.1a CT25.1b

CAGE	CT26	CAGE	CT26
BORIS	CT27	BORIS	CT27
HOM-TES-85	CT28	HOM-TES-85	CT28
AF15q14	CT29	AF15q14	CT29
HCA661	CT30	HCA661	CT30
LDHC	CT32	LDHC	CT32
MORC	CT33	MORC	CT33
SGY-1	CT34	SGY-1	CT34
SPO11	CT35	SPO11	CT35
TPX1	CT36	TPX1	CT36
NY-SAR-35	CT37	NY-SAR-35	CT37
FTHL17	CT38	FTHL17	CT38
NXF2	CT39	NXF2	CT39
TDRD1	CT41	TDRD1 NY-CO-45	CT41.1 CT41.2
TEX15	CT42	TEX15	CT42
FATE	CT43	FATE	CT43
TPTE	CT44	TPTE	CT44

Updated: March 29, 2006

Source: http://www.cancerimmunity.org/CTdatabase

Annex II – Publication

Cancer/testis antigen expression and specific cytotoxic T lymphocyte responses in non small cell lung cancer.

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Cancer/testis antigen expression and specific cytotoxic T lymphocyte responses in non small cell lung cancer

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Non small cell lung cancers (NSCLC) express cancer/testis antigens (CTA) genes and MAGE-A expression correlates with poor prognosis in squamous cell carcinomas. We addressed cytotoxic T lymphocytes (CTL) responses to HLA class I restricted CTA epitopes in TIL from NSCLC in an unselected group of 33 patients consecutively undergoing surgery. Expression of MAGE-A1, -A2, -A3, -A4, -A10, -A12 and NY-ESO-1 CTA genes was tested by quantitative RT-PCR. Monoclonal antibodies (MAb) recognizing MAGE-A and NY-ESO-1 CTA were used to detect CTA by immunohistochemistry. $CD8^+$ TIL obtained from tumors upon culture with anti CD3 and anti CD28 mAb and IL-2 were stimulated with autologous mature DC (mDC) and HLA-A*0101 restricted MAGE-$A1_{161–169}$ or MAGE-$A3_{168–176}$ peptides or HLA-A*0201 restricted MAGE-$A4_{230–239}$, MAGE-$A10_{254–262}$, NY-ESO-$1_{157–165}$ or multi-MAGE-A (YLEYRQVPV) peptides or a recombinant vaccinia virus (rVV) encoding MAGE-A and NY-ESO-1 HLA-A*0201 restricted epitopes and CD80 co-stimulatory molecule. Specificity was assessed by ^{51}Cr release and multimer staining. At least one CTA gene was expressed in tumors from 15/33 patients. In 10 specimens, at least 4 CTA genes were concomitantly expressed. These data were largely confirmed by immunohistochemistry. TIL were expanded from 26/33 specimens and CTA-specific CTL activity was detectable in 7/26 TIL. In 6, however, specific cytotoxicity was weak, (<40% lysis at a 50:1 E:T ratio) and multimer staining was undetectable. In one case, high (>60% lysis at 50:1 E:T ratio) MAGE-$A10_{254–262}$ specific, HLA-A*0201 restricted response was observed. Supportive evidence was provided by corresponding multimer staining. Although CTA genes are frequently expressed in NSCLC, detection of CTL reactivity against CTA epitopes in TIL from nonimmunized NSCLC patients represents a rare event.

Key words: NSCLC; cancer/testis antigens; tumor infiltrating lymphocytes; cytotoxic T lymphocytes

Cancer/testis antigens (CTA) were the first human tumor associated antigens to be molecularly characterized.[1] This group of antigens comprises products of different gene families expressed, under physiological conditions, predominantly in spermatogonia and in placenta.[2,3]

The physiological role of CTA is unclear. Their expression has been shown to be enhanced by drugs inducing DNA demethylation[4,5] and its pattern suggests a role in germ cell development[6,7] although functional mechanisms have not been elucidated so far. Data suggesting that defined CTA might be involved in the control of cell cycle progression have also been reported.[8,9]

CTA have been shown to be expressed in a large variety of tumors of different histological origin.[10] Furthermore, since a number of them encompasses both HLA class I and class II restricted epitopes,[11] they represent attractive targets for active specific immunotherapy in different areas of clinical oncology.

Lung cancers are some of the most frequent tumors in males and their incidence is rising among females as well. They are often characterized by poor prognosis and the limitations of therapeutic options available beyond surgery,[12] urge the development of novel approaches.

Among lung cancers, a frequent expression of CTA has been repeatedly observed in non small cell lung cancer (NSCLC).[13–15] However, immune responses against CTA epitopes in patients bearing these tumors have never been investigated in detail.

In this article, we have systematically addressed the expression of a panel of CTA in NSCLC tumors from a group of patients consecutively operated in our clinic, and we have assessed the responsiveness of lymphocytes infiltrating these tumors against a panel of defined CTA derived, HLA class I restricted, epitopes.

Patients

Patients whose tumors were investigated in this study (n = 33, 25 males and 8 females, mean age 64.3 ± 8.18) were operated for the excision of NSCLC during the period May 2003 to May 2005 in the Department of Thoracic Surgery of the University of Basel (Table I). Inclusion in the study was limited to patients expressing HLA-A*0101 and HLA-A*0201 determinants that restrict the response of cytotoxic T lymphocytes (CTL) to well defined CTA epitopes.[11] Five patients underwent neo-adjuvant therapy[16] prior to surgical treatment.

The study was approved by the regional ethical committee (EKBB, reg. nr 175/02) and all patients were required to sign an approved informed consent prior to inclusion.

Material and methods

Reagents

Peptides used in this study were. HLA-A*0101 restricted MAGE-$A1_{161–169}$,[17] and MAGE-$A3_{168–176}$,[18] and HLA-A*0201 restricted MAGE-$A4_{230–239}$,[19] MAGE-$A10_{254–262}$,[20] multi-MAGE-A (YLEYRQVPV)[21] and NY-ESO-$1_{157–165}$.[22] They were obtained from NeoMPS (Strasbourg, France). Monoclonal antibodies (mAbs) specific for CD3, CD4, CD8, CD16, CD56 and a monomorphic HLA-DR determinant were obtained from Becton Dickinson (San Josè, CA). Fluorochrome labelled multimers specific for HLA-A*0201 restricted epitopes (see earlier) were from Proimmune (Oxford, UK).

Quantitative real time PCR

RNA were extracted from 2 pieces of each tumor, according to the RNA easy Mini Kit protocol (QIAGEN AG, Basel, CH),

Abbreviations: APC, antigen presenting cells; CTA, cancer/testis antigens; CTL, cytotoxic T lymphocytes; EBV, Epstein-Barr virus; mAb, monoclonal antibody; mDC, mature dendritic cells; NSCLC, non small cell lung cancer; rVV, recombinant vaccinia virus; TIL, tumor infiltrating lymphocytes.

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TABLE I – CLINICO-PATHOLOGICAL CHARACTERISTICS OF THE PATIENTS INCLUDED IN THE STUDY

Patient	Gender/Age	Histology	Stage	Grade	NeoAdj Tx	HLA-type
LT1	M/77	AC	M1	3	−	A*0201
LT2	M/68	SCC	pT2, pN0	2	n.a.	A*0201
LT3	M/65	SCC	pT3, pN1	3	n.a.	A*0101, A*0201
LT4	F/80	SCC	pT3, pN0	2	−	A*0201
LT5	M/65	AC	pT2, pN0	3	−	A*0101
LT6	M/67	AC	pT2, pN1	1	−	A*0101
LT7	F/56	SCC	pT2, pN1	3	+	A*0101, A*0201
LT8	M/68	AC	pT2, pN3	3	−	A*0201
LT9	F/44	AC	pT2, pN0	2	−	A*0201
LT10	M/54	SCC	pT2, pN0	2	−	A*0201
LT11	F/61	SCC	pT1, pN0	2	−	A*0201
LT12	M/63	LCC	pT2, pN0, M1	3	−	A*0201
LT13	M/69	AC	pT1, pN3	2	−	A*0201
LT14	M/59	LCC	pT2, pN1	3	−	A*0101, A*0201
LT15	M/72	SCC	pT4, pN2	3	−	A*0101
LT16	F/60	SCC	pT1, pN0	3	−	A*0101
LT17	M/61	SCC	M1	n.a.	−	A*0201
LT18	M/56	AC	pT2, pN1	2	−	A*0201
LT19	M/48	SCC	pT2, pN1	3	−	A*0101, A*0201
LT20	F/63	LCC	pT1, pN1	3	−	A*0101, A*0201
LT21	M/73	AC	pT2, pN0	2	−	A*0101, A*0201
LT22	M/73	SCC	pT2, pN0	2	−	A*0201
LT23	F/74	AC	pT1, pN0	1	−	A*0101
LT24	M/60	SCC	pT1, pN2	2	+	A*0201
LT25	F/66	AC	pT1, pN0	3	+	A*0201
LT26	M/60	LCC	pT2, pN0	3	−	A*0101
LT27	M/63	SCC	pT2, pN1	3	−	A*0201
LT28	M/65	AC	pT1, pN3	3	−	A*0201
LT29	M/62	SCC	pT2, pN1	2	−	A*0101, A*0201
LT30	M/62	SCC	pT2, pN3	2	+	A*0101
LT31	M/57	SCC	pT3, pN2	2	−	A*0201
LT32	M/75	SCC	pT2, pN0	3	+	A*0101
LT33	M/77	AC	pT3, pN2	3	−	A*0201

NeoAdj Tx, neoadjuvant cytoreductive therapy; LT, lung tumor; AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; staging (TNM classification); pT, primary tumor; pN, regional lymph node metastases; M, distant metastases.
n.a.: not available.

treated with DNAse I (Invitrogen, Paisley, UK) and reverse transcribed (M-MLV RT, Invitrogen, Paisley, UK). Resulting cDNA were amplified by quantitative real time PCR,[23] in the presence of primers and probes specific for IFN-γ,[24] CD8α chain (forward 5′-CTC GGC CCT GAG CAA CTC-3′; reverse 5′-GGC TTC GCT GGC AGG A-3′; probe 5′-VIC-ATG TAC TTC AGC CAC TTC GTG CCG GTC-TAMRA-3′), MAGE-A1,[25] MAGE-A2 (forward 5′-GAC AAG TAG GAC CCG AGG CA-3′; reverse 5′-TGT GGG TCT TCA TTG CCC A-3′; probe 5′-FAM-TGG AGG AGC ATT GAA GGA GAA GAT CTG C-TAMRA-3′), MAGE-A3, MAGE-A4 (forward 5′-CCA CAG AGG AGC ACC AAG GA-3′; reverse 5′-GCT TTT GCC TGC ACT CTT GC-3′; probe 5′-FAM-AAG ATC TGC CTG TGG GTC CCC ATT GC-TAMRA-3′), MAGE-A10 (forward 5′-CAG GGA GAG CAA GAG GTC AAG A-3′; reverse 5′- AAG TCC TGC CCA CAC TCC C-3′; probe 5′-FAM-CAG CAC TGA AGG AGA AGA CCT GCC TGT G-TAMRA-3′), MAGE-A12,[26] NY-ESO-1[27] and β-actin house keeping gene, used as internal reference. Primers and probes for IFN-γ, CD8α, MAGE-A1, -A2, -A4, -A10, -A12 and NY-ESO-1 were commercially synthesized (Microsynth, Balgach, CH). MAGE-A3 and β-actin oligos were predeveloped assays from Applied Biosystem (Foster City, CA). SK-Mel37 melanoma cell line, expressing all CTA genes[28] under investigation was used as positive control. Specimens were considered positive if the average specific gene expression in each sample exceeded 0.1% of the relative expression of β-actin gene, simultaneously amplified from the same cDNA.

Immunohistochemistry

Generation, characterization and specificity of 57B and D8.38 mAb, used to detect MAGE-A and NY-ESO-1 CTA, respectively, by immunohistochemistry, were described in detail previously.[13,29,30] Sections from paraffin embedded tissues were stained as described.[13] Histoscores were calculated by considering the intensity of staining (0–3) and the overall percentage of the section stained (intensity X cell percentage). Tumors were considered positive from a minimal histoscore of 30, meaning a low intensity staining of 30% or a strong intensity staining of 10% of tumor cells.

Expansion of tumor infiltrating lymphocytes

Tumor samples were mechanically disrupted and incubated o/n at 37°C in the presence of collagenase type II (Sigma, Buchs, CH). To selectively expand TIL, cell suspensions were cultured at 37°C in RPMI 1640 supplemented with Kanamycin [100 μg/ml], Hepes [10 mM], sodium pyruvate [1 mM], Glutamax [1 mM] and non-essential amino acids (all from GIBCO, Paisley, UK; thereafter referred to as complete medium) and 5% human serum (Blood bank, Universitätsspital Basel, CH) supplemented with recombinant interleukin-2 (rIL-2, 100 U/ml, Hoffmann-La Roche, Basel, CH) in plates coated with anti CD3 (TR66, a gift from Dr. Lanzavecchia, Bellinzona, CH) and anti CD28 mAbs (Pharmingen, San Diego, CA) at 0.5 and 0.1 μg/ml concentration, resepctively. Fresh medium containing rIL-2 was added twice per week.

Construction of recombinant vaccinia virus

Recombinant vaccinia virus (rVV) was constructed as previously described.[23,31] HLA-A*0201 restricted epitopes MAGE-$A4_{230-239}$, MAGE-$A10_{254-262}$, NY-ESO-$1_{157-165}$, and multi-MAGE-A were constructed within the viral genome in the I4L and A44L loci in the form of minigenes also including sequences coding for the adenovirus E3/19K signal peptide, driving the resulting fusion product in the endoplasmic reticulum,[31] as depicted in Figure 1. Each minigene was under the control of an individual viral promoter. The gene encoding CD80 co-stimulatory molecule was

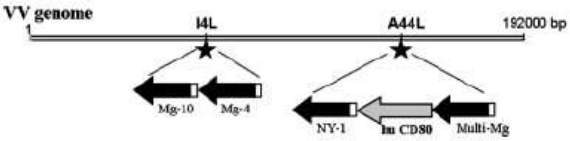

FIGURE 1 – Construction of the rVV encoding CTA derived epitopes. Sequences encoding HLA-A*0201 restricted epitopes MAGE-$A4_{230-239}$, MAGE-$A10_{254-262}$, NY-ESO-$1_{157-165}$, and Multi-MAGE-A were inserted in the I4L and A44L loci of viral genome as minigenes comprising sequences encoding adenovirus E3/19K signal peptide. Each insert is controlled by an individual promoter and the gene encoding CD80 co-stimulatory molecule has also been added.

also added. The rVV was made replication incompetent by psoralen/UV treatment as reported previously.[31]

Generation of antigen specific CTL

$CD8^+$ T cells were purified by magnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, D) from TIL expanded for 2 weeks. $CD14^+$ monocytes were isolated from peripheral blood mononuclear cells (PBMC) of each patient by magnetic beads. Immature dendritic cells (iDC) were generated from $CD14^+$ cells upon culture for 5–7 days in complete medium supplemented with 10% FCS (GIBCO, Paisley, UK), β-mercaptoethanol 0.004%, recombinant IL-4 (rIL-4, 1000 U/ml, courtesy of Dr. Lanzavecchia, Bellinzona, CH) and recombinant GM-CSF (rGM-CSF, 50 ng/ml, Laboratorio Pablo Cassarà, Buenos Aires, Argentina). Maturation of iDC was induced by a 8 hr exposure to LPS (abortus equi, Sigma, Buchs, CH) at 1 μg/ml concentration.[32]

Mature DC (mDC) were pulsed for 3 hr in the presence of antigenic peptides (50 μg/ml), washed and irradiated. Alternatively, mDC were infected for 1 hr at 37°C with the described rVV at 3 m.o.i. and cultured o/n prior to irradiation. In all cases, they were then added to autologous $CD8^+$ cells (10^6/ml) at a 1:10 ratio, in the presence of rIL-2 (20 U/ml). Cultures were stimulated weekly 3–5 times prior to testing for antigen specificity.

Multimers specific for the MAGE-A1, -A3, A10, multi MAGE and NY-ESO-1, HLA-A*0201 restricted epitopes under investigation were used to stain antigen stimulated $CD8^+$ T cells.[31] Cytotoxic activity was tested for all cultures, by using, as targets, ^{51}Cr labeled HLA-A*0201^+ T2 cells or a HLA-A*0101^+ EBV transformed cell line, upon pulsing with specific or control peptides at 10 μg/ml concentration for 2 hr. Specific lysis of target cells was calculated according to the standard formula.

Results

Clinico-pathological characteristics

The NSCLC under investigation included 17 squamous cell carcinomas, 12 adenocarcinomas and 4 large cell carcinomas (Table I). In 13 patients neither lymph node nor distant metastases were evident. Seventeen patients had lymph node but not distant metastases, whereas 3 patients had distant metastases without lymph node metastases.

Two tumors, both adenocarcinomas, were of grade 1, 13 of grade 2 and 17 of grade 3, while for 1 histological grading was not available (Table I).

Expression of CTA genes in NSCLC

The expression of MAGE-A1, -A2, -A3, -A4, -A10, -A12 and NY-ESO-1 genes in NSCLC was studied by quantitative real time PCR. At least one CTA gene was expressed in 15/33 samples (45%). MAGE-A10 and -A12 were the most frequently (10/15 and 12/15, respectively) and MAGE-A1, -A4 and NY-ESO-1 the least frequently expressed genes (6/15, 6/15 and 4/15, respectively). In 10/15 positive cases at least 4 CTA genes were concomitantly expressed (Fig. 2, Panel *a*). In particular, 5/12 (42%) adenocarcinomas, 8/17 (47%) squamous and 2/4 large cell carcinomas were positive with concomitant expression of at least 4 CTA genes in 3/12, 5/17 and 2/4 cases, respectively. In 3 tumors (LT 8, 14 and 24), a strong level of expression of at least 1 CTA gene, *e.g.* >10% of the relative expression of β-actin gene, was detectable.

Interestingly, expression of CTA genes was not correlated with previous administration of neo-adjuvant treatment.

Immunohistochemical detection of cancer/testis antigens in lung cancers

In 19 cases, we had access to sufficient amounts of material to perform immunohistochemical studies (Fig. 2, Panel *b*). In 15 staining with anti NY-ESO-1 mAb D8.38 or mAb 57B recognizing multiple MAGE-A CTA yielded results fully compatible with quantitative real time PCR data. In 4 of them, however, IHC data did not match gene expression profiles. In LT 11, immunohistochemistry was negative in the presence of a weak MAGE-A12 gene expression, whereas in LT 8 and LT 13 a focal NY-ESO-1 specific staining was observed in the apparent absence of specific gene expression. Similarly, in LT 32, a MAGE-A specific staining was also observed in the apparent absence of CTA expression. These discrepancies are not surprising considering that CTA expression is frequently focal and limited to defined tumor areas.[13]

Remarkably, in some NSCLC samples, CTA specific staining extended to virtually all tumor cells, and both nuclear and cytoplasmic positivity was observed. Figure 3 shows representative examples of 57B and D8.38 staining in NSCLC specimens.

Expression of IFN-γ gene in NSCLC specimens

TIL freshly derived from surgically excised tumor specimens have been suggested to be anergic and unable to produce factors, such as IFN-γ, typically induced in effector cells upon T cell receptor (TCR) triggering.[33] To preliminarily assess the activation status of TIL from NSCLC, we tested by qPCR the expression of genes encoding CD8α and IFN-γ. In 23/26 samples, evidence of CD8α gene expression was indeed detectable, whereas IFN-γ transcripts could only be amplified from 5/26 specimens. No significant correlation between CTA and IFN-γ gene expression could be observed.

Generation and phenotypic characterization of lung tumor infiltrating lymphocytes

Single cell suspensions were obtained from surgical specimens by a combination of mechanical and enzymatic treatments. In a number of cases ($n = 14$) phenotypic characterization of freshly derived cells was attempted. Only a tiny minority (<3% of the total) of cells expressed T cell (CD3, and CD4 or CD8), NK cell (CD16/CD56) or monocyte/macrophage (CD14) markers. In particular, the low percentage of total $CD8^+$ cells (0.7 ± 0.8 %) precluded an *ex vivo* analysis of tetramer staining.

TIL were successfully expanded from 26/33 tumor derived cell suspensions. Failure to expand TIL was mainly related to contamination (5/7 cases), as expectable, considering the anatomical origin of the specimens. In 2 cases, however, no obvious explanation for defective TIL expansion could be provided.

Expanded TIL were almost exclusively (95 ± 3%) $CD3^+$ and prevailingly (68 ± 13%) $CD4^+$ with substantial percentages of $CD8^+$ cells (26 ± 11%) while numbers of NK cells were limited to <3% of the total.

Expansion of antigen specific CTL from lung tumor infiltrating lymphocytes

$CD8^+$ T cells were purified from expanded TIL populations. Staining with CTA peptide specific multimers was virtually undetectable (0.37 ± 0.15%) in these cells. Subsequently, cells were repeatedly stimulated with autologous mDC pulsed with CTA

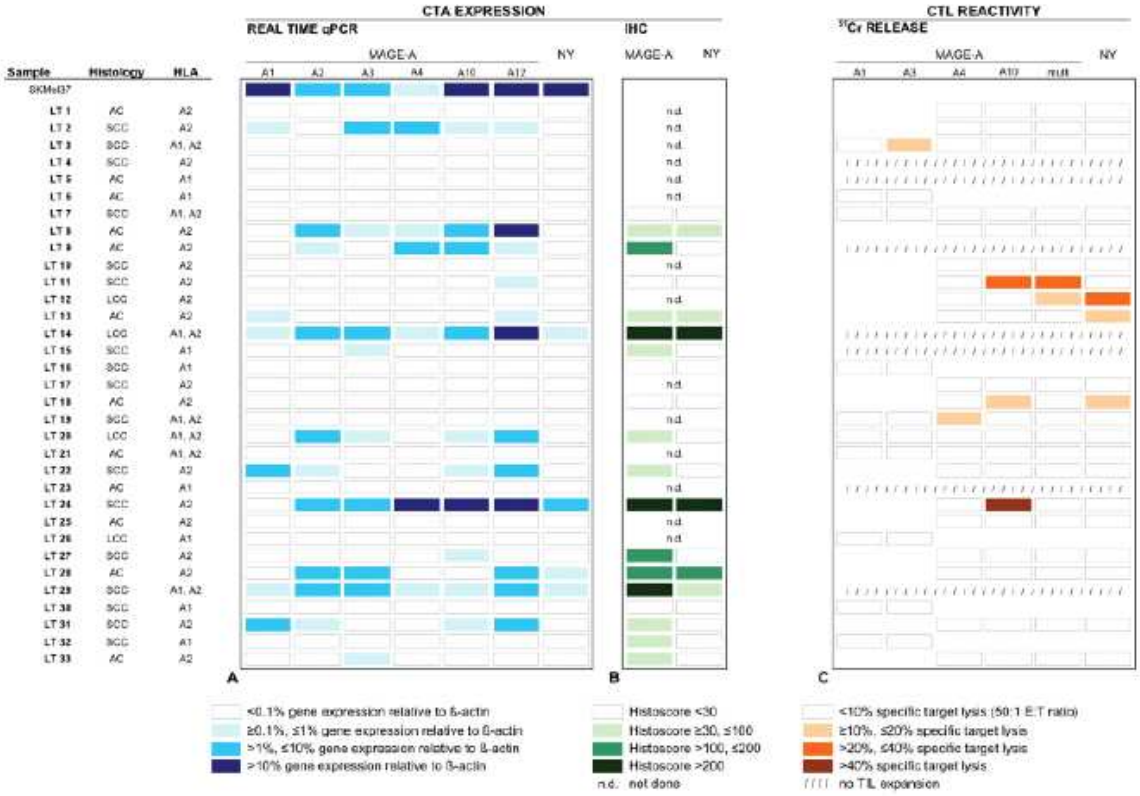

FIGURE 2.

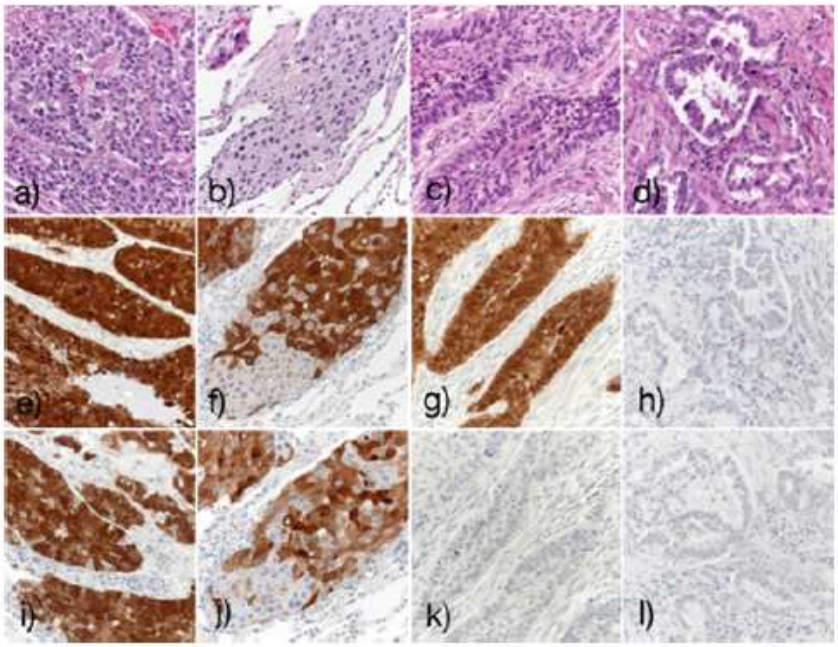

FIGURE 3.

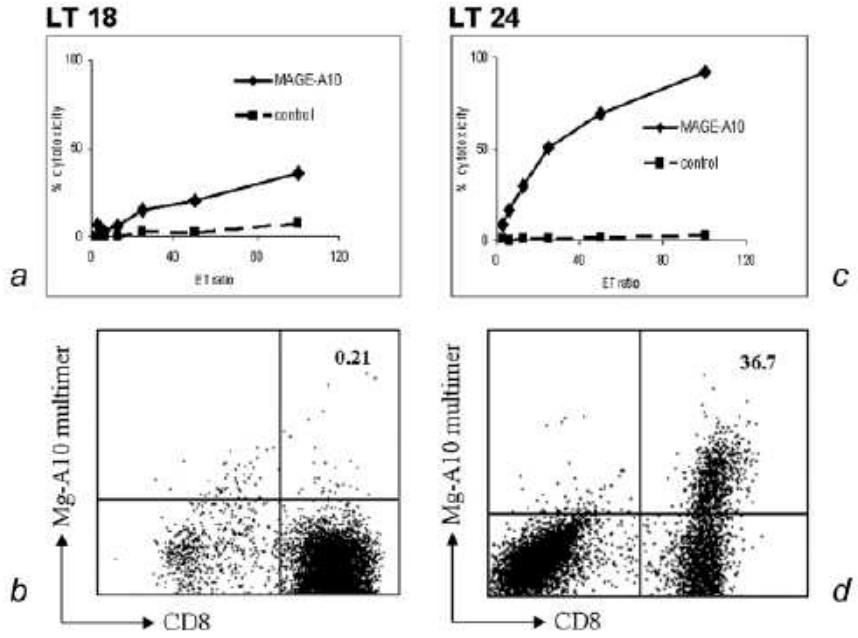

FIGURE 4 – Comparative evaluation of CTA specific cytotoxicity and multimer staining in NSCLC derived TIL populations. Cultured TIL were repeatedly stimulated with irradiated autologous mDC pulsed with the indicated peptides or infected with rVV. Cells were then used for the assessment of specific cytotoxic activities (panels *a* and *c*) and for staining with FITC labeled anti CD8 and PE labeled multimers specific for the same epitopes in the context of the restricting HLA determinants (panels *b* and *d*). Representative examples refer to weakly positive cultures (LT 18, panels *a* and *b*) and to a strongly positive culture (LT 24, panels *c* and *d*). Numbers in the upper left quadrants refer to percentages of total $CD8^+$ cells stained by specific multimers.

peptides alone ($n = 15$) or, at alternate weeks, with DC pulsed with peptides or infected with the rVV encoding the corresponding HLA-A*0201 restricted epitopes ($n = 11$). Antigen specificity was evaluated by ^{51}Cr release assays and multimer staining. CTA specific cytotoxic activity could be observed in 7/26 TIL populations (Fig. 2, panel *c*) and in 3 of them epitopes from 2 different CTA were targeted. In 6/10 antigen stimulated cultures, CTL only showed a minor cytotoxic activity not exceeding 20% specific lysis at 50:1 E:T ratio. In 3/10 antigen stimulated cultures specific cytotoxic activity ranged between 20% and 40% at a 50:1 E:T ratio (Fig. 4, panel *a*). In all these cases, no specific multimer staining was observed (Fig. 4, panel *b*) and detection of antigen specific CTL did not obviously correlate with expression of specific CTA in the original tumor specimens.

Most importantly, in one case where high CTA expression was detectable at the gene and protein level (LT 24), a high cytotoxic activity, against MAGE-$A10_{254-262}$ HLA-A*0201 restricted epitope was observed (Fig. 4, panel *c*) following stimulation of TIL with rVV infected antigen presenting cells (APC). Furthermore, while upon anti CD3/CD28 stimulation only 0.45% of $CD8^+$ cells from these TIL could be stained with MAGE-$A10_{254-262}$ HLA-A*0201 specific multimers, a high percentage (>35%) of T cells positive for the specific multimers was detectable following the subsequent antigen stimulation (Fig. 4, panel *d*).

Discussion

CTA represent attractive targets for active specific immunotherapy of cancer, since they are expressed in tumors of different histological origin,[2,3,10] while normal spermatogonia, that also express CTA, are HLA class I negative and, therefore, cannot be recognized by specific CTL. Lung cancers, and, in particular, NSCLC have been shown to frequently express CTA.[14,15] Importantly, their expression in these tumors has been shown to correlate with poor prognosis.[13,34]

FIGURE 2 – CTA gene and protein expression, and TIL specificity in NSCLC. (*a*) Total cellular RNA was extracted from 2 pieces of each NSCLC specimen, reverse transcribed and amplified by real time qPCR in the presence of specific primers and probes. Average results from the duplicate assays were normalized to the expression of reference β-actin house keeping gene as observed in the same samples. SK-Me137 cell line expressing all genes under investigation was used as positive control. (*b*) Sections from paraffin embedded specimens were stained with 57B mAb, recognizing multiple MAGE-A antigens or D8.38 mAb, recognizing NY-ESO-1. Data are expressed as ''Histoscore'' as detailed in ''Material and methods''. (*c*) TIL from NSCLC were repeatedly stimulated in the presence of autologous mDC and specific peptides, or rVV infected mDC. Cytotoxic activity was tested by using peptide pulsed ^{51}Cr labelled cells as targets. Reported data refer to specific killing as observed at a 50:1 E:T ratio. LT, lung tumor; AC, adenocarcima; SCC, squamous cell carcinoma; LCC, large cell carcinoma; IHC, immunohistochemistry; NY, NY-ESO-1.

FIGURE 3 – Immunohistochemistry. Sections from paraffin embedded tumor samples LT 14 (panels *a, e, i*), LT 8 (panels *b, f, j*), LT 27 (panels *c, g, k*) and LT 18 (panels *d, h, l*) were stained with anti MAGE-A 57B mAb (panels *e–h*), with anti NY-ESO-1 D8.38 mAb (panels *i–l*) or with HE (panels *a–d*). LT 14 large cell neuroendocrine carcinoma displays a pattern of strong tumor specific positivity to either mAB, whereas unstained tumor cells are visible in LT 8 solid adenocarcinoma. LT 27 squamous cell carcinoma is characterized by an intense tumor specific staining by the anti MAGE-A mAb but not by the anti NY-ESO-1 reagent. LT 18 acinar adenocarcinoma is negative to both. Magnification ×200.

Clinical trials based on the administration of synthetic peptides, recombinant proteins or exosomes loaded with CTA derived peptides or recombinant suggest that specific T cells can indeed be induced by immunization.[35–37] Evidence of existing CTA specific pre-sensitization, possibly induced by tumor cells, may provide a strong incentive to the development of vaccination protocols able to boost immune responses for therapeutic purposes in defined types of cancer. Thus, for the first time, here we systematically investigated whether CTL recognizing specific CTA epitopes could be expanded from lymphocytes infiltrating NSCLC in non vaccinated patients.

To provide optimal cellular reagents, we updated classical TIL stimulation protocols.[38] TIL were expanded in the presence of mitogenic anti CD3 and anti CD28, and mDC were used as APC for peptide specific T cell stimulation.[37,39]

We confirmed CTA expression in NSCLC at both gene and protein level. Most importantly, we show that concomitant expression of different CTA genes is frequently detectable in the same specimen.[34] These data suggest that the use of multi antigen vaccine preparations[31,40,41] of CTA could be proposed in subgroups of NSCLC patients.

Detectable levels of CTA epitope specific CTL activity could be demonstrated in 7/26 expanded TIL populations. However, in 6/7 cases cytotoxicity was weak and no specific multimer staining was observed, suggesting that these effector cells were endowed with low functional avidity.[42] Furthermore, in the original tumor specimens, either no CTA was expressed (4/7 cases) or some CTA were indeed expressed, but not those encoding the specifically recognized epitopes (2/7 cases). Still unclear is whether this constellation of data relates to immunoediting of the antigenic phenotype of the tumors by specific CTL responses[43] or if the detection of weak specific cytotoxicity could rather be attributed to primary *in vitro* sensitization.

In one case, however, a high level of CTA specific CTL response was concomitantly detectable by cytotoxicity assays and multimer staining. MAGE-A10 gene encoding the target epitope was highly expressed in the original tumor, and MAGE-A specific staining was evident in a large majority of tumor cells.

Stimulation of melanoma TIL with differentiation antigens frequently results in expansion of specific CTL.[38] In contrast, our present observations from experiments relying on repeated stimulation of TIL with a panel of well defined peptides and highly effective APC, suggest that CTA specific CTL sensitization in NSCLC is not as frequent. In this respect, our data are consistent with a number of previous reports mostly focused on individual CTA specificities. For instance, frequency of CTL precursors specific for a MAGE-A3 peptide in the blood of healthy donors or melanoma patients prior to specific immunotherapy has been shown to be very low.[44,45]

Most interestingly, the expression of a number of CTA has recently been shown to take place also in the thymus.[46] As a consequence, tolerance towards CTA may indeed exist and impair vaccine mediated induction of specific CTL. Importantly, efficient expansion of CTA specific CTL *in vivo*, has been shown to require long immunization courses or the repeated administration of peptide pulsed mDC.[37,47]

On the other hand, our results indicate that TIL from lung cancers may indeed recognize antigens from autologous tumors, thus confirming data from others who reported specific immune responses targeting determinants other than CTA.[48–50] Taken together these findings suggest that other antigens undetected so far, or epitopes from known TAA, restricted by HLA alleles other than HLA-A*0101 or A*0201 may exist in NSCLC.

Also, since we have only explored the specificity of TIL, we cannot formally exclude that peripheral blood T cells may display comparatively enhanced reactivity, possibly targeting a wider range of epitopes. However, our previous experience in melanoma suggests that cultured TIL are indeed at least as powerful, as effector cells, as autologous peripheral lymphocytes.[38]

In conclusion, the frequent, concomitant expression of multiple CTA in NSCLC and the existence of natural CTL responses in these cancers, as demonstrated by our study, support the development of specific vaccination protocols. However, the therapeutic induction of CTL recognizing CTA might be difficult, and require peculiar immunization procedures. It is tempting to speculate that a multi-CTA recombinant virus similar to the one described in this work might prove useful in the implementation of clinically effective antigen specific immunotherapy in NSCLC.[51]

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