1. Introduction

1. Immune response to cancer

The immune system is a complex network of cellular and humoral components that have the primary function of discriminating self from non-self-such as, pathogens or altered self, including tumours, and then mounting the appropriate response to destroy them.

About one hundred years ago Paul Ehrlich (1) predicted that the immune system could control the growth of some cancers; thus the ongoing debate over the immunologic control of cancer began a century ago. Over the next decades given the better knowledge of tumour immunogenetics and transplantation, Macfarlane Burnet and Lewis Thomas (2) reconsidered the concept of natural immune protection against cancer. Based on the presumption of immune tolerance Burnet suggested that tumour cell-specific neo-antigens may lead to an effective immunologic reaction resulting in eliminating of cancers (2, 3, 4). Thomas proposed that complex long-lived organisms must possess mechanisms to protect against cancers similar to those mediating homograft rejection (5). The ideas of Ehrlich, Burnet and Thomas supported by the functional demonstration of mouse tumour-specific antigens, helped the recognition of the cancer immunosurveillance hypothesis which stated that the sentinel thymus-derived T cells of the body constantly monitored host tissues for newly transformed cells (6). Over the next decades this hypothesis has been attacked by some authors (7,8) but despite these challenges, studies in the 1990s using new advanced techniques in mouse genetics and monoclonal antibody production provided much evidence in favour of the cancer immunosurveillance hypothesis (9,10, 11).

Based on the immunosurveillance hypothesis it could be predicted that immunodeficient subjects should experience much higher rates of all types of cancers as compared with the general population. In support of this suggestion, surveys of patients with primary and acquired immune deficiencies have demonstrated increased incidence of cancer but not substantiated an increased

risk of all cancer types (12). Indeed, immunosuppression leads to a higher incidence of virally triggered tumours or tumours of lymphohematopoietic or vascular origin, but interestingly does not influence the incidence of the more common tumours such as breast cancer, colon cancer, or lung cancer (13, 14).

Evidence favoring the existence of tumour immunity is the isolation from patients, especially melanoma patients, of T cell clones specific for antigens on the tumour and the identification of the corresponding major histocompatibility complex (MHC)-presented tumour antigens (15,16,17,18). The antigens generally are derived from self proteins that are overexpressed or inappropriately expressed in the tumour cells. The data obtained in experimental protocols showed that at least some tumour cells express antigens against which in many cases an immune response can be generated in vitro (19).

Together, all these observations suggest that the host immune response may, in some situations, control tumours, but in others, especially against peripheral solid tumours, the endogenous immune response is often not an effective barrier for tumour growth. Understanding why the endogenous immune response fails to control tumourigenesis is key to improving antitumour immunity.

1.1. Innate Immunity

The expression Innate Immunity refers to the ‘non–clontypic’ arm of the immune system whose primary role is to recognize and initiate response against microbes or substances produced in infections and eliminate them. It consists of cellular and biochemical defense mechanisms such as epithelial barriers, blood proteins, including members of the complement system, proteins called cytokines as well as effector cells, including neutrophils, mononuclear phagocytes, natural killer (NK) cells, NK T-cells (NKT) and dendritic cells (DCs). Although the recognition of pathogens by the mediators of natural immunity is characteristically described as non-clonotypic, each cell type carries a multitude of receptors and can recognize a host of different molecules. This differentiation between pathogen and host based molecules is mediated, amongst others, by

the members of the Toll-like receptor (TLR) family, which serve as pattern recognition receptors for a variety of microbe-derived molecules and stimulate innate immune responses to the microbes expressing them. TLRs are widely expressed by immature dendritic cells and macrophages but also by endothelial cells and mucosal epithelial cells. Ten different mammalian TLRs have so far been identified on the basis of sequence homology to Drosophila Toll, and can recognize a host of elements from most microbes such as gram-negative bacterial lipopolysaccharide (LPS), gram-positive bacterial proteoglycan, bacterial lipoproteins, the bacterial flagellar protein flagellin, heat shock protein 60, respiratory syncytial virus fusion protein, unmethylated CpG DNA motifs and double-stranded RNA.

Molecules produced during innate immune responses stimulate adaptive immunity and influence the nature of adaptive immune responses. Interferon (IFN)-γ is a crucial cytokine secreted by NK and NKT cells and cells of the adaptive immune system. It has a direct tumouricidal activity, anti-angiogenic properties and induces the secretion of chemokines by both the tumour cells and the surrounding normal cells. Adam et al. (20) show in their study that IFN-γ is necessary for activation of endogenous DCs and IL-12 production leading to CTL induction. Macrophages activated by microbes and by IFN-γ produce co-stimulators that enhance T-cell activation and IL-12, which stimulates IFN-γ production by T cells and the development of IFN-γ-producing effector T cells. NK-cell-DC cross-talk, on the other hand, may bypass the T helper arm in CTL induction against tumours expressing NKG2D ligands. Walzer et al. (21) state in their review that NK-cell/DC interactions are critical in situations where receptors allowing the recognition of the pathogenic agent are only expressed by one of the two subtypes. For instance the DCs could be the first cells activated by microbes, thanks to their expression of relevant innate sensors, TLRs, nucleotide-binding oligomerization domain (NOD) and thereby turn on the whole immune system. In the case of a tumour that does not cause overt inflammation but does express ligands for activating NK-cell receptors, NK cells would be the first cells to be activated and turn on the system (21). Once activated they are capable of directly killing those tumour cells which do not

express inhibitory MHC class I molecules, by the secretion of perforin and reactive oxygen and nitrogen species. In this case, activation of the whole immune system is dependent on the cellular cross-talk between the dendritic and NK cells which is believed to be the critical link between the innate and adaptive immune responses.

Cells of the innate immune system not only have the responsibility of surveying and “informing” the host of a breach in integrity, but also themselves possess intrinsic anti-tumour effector functions including lysis of tumour cells and the production of cytokines that inhibit tumour growth or block angiogenesis. NK, NKT and γδT cells express activating receptors such as NKG2D that recognize MHC class I chain-related (MIC) or ULI6-binding (ULBP) proteins that become up-regulated on tumour cells. Some NKT and γδT cells also express T-cell receptors with restricted diversity that recognize tumour through lipid or protein antigens presented in the context of non-classical MHC proteins (22). Summarizing all these findings, we come to the conclusion that the innate immune response may function as a major initiator of anti-tumour immunity.

1.2. Adaptive Immunity

Adaptive immunity is also called specific immunity because of its extraordinary capacity to distinguish among different, even closely related, microbes and molecules. Its defining characteristics are exquisite specificity for distinct molecules and an ability to ‘remember’ and respond more vigorously to repeated exposure to the same microbe. The adaptive immune system is subdivided into the humoral arm, mediated by B cells, and the cellular arm mediated by T cells. Antigen-specific response of the letter require the recognition of specific “non-self” antigens during a process called antigen presentation.

1.2.1. Antigen presentation/processing

The most potent antigen presenting cells (APCs) are the dendritic cells, which are classically defined as the sentinels of the immune system (21). At an ‘immature’ stage of development, DCs continuously uptake antigens in

peripheral tissues and migrate at a slow rate to lymph nodes (LNs). Under these steady-state conditions, DCs express low levels of MHC and costimulatory molecules and it has been suggested that their interaction with naive T cells is involved in peripheral T-cell tolerance (23). By contrast, encounter with microbial products or tissue damage in the periphery initiates DC maturation and their rapid migration to LNs (Figure 1). This activation program is due in part to the engagement of a complex set of innate sensors, such as the TLRs, which are able to recognize molecules and molecular patterns shared by various classes of microbes. The levels of MHC molecules expressed on mature DCs are very high, perhaps 60-fold higher than on macrophages, providing more peptide/MHC ligands for T-cell receptor engagement. They also express high levels of costimulatory molecules, which are critical for T-cell activation (23).

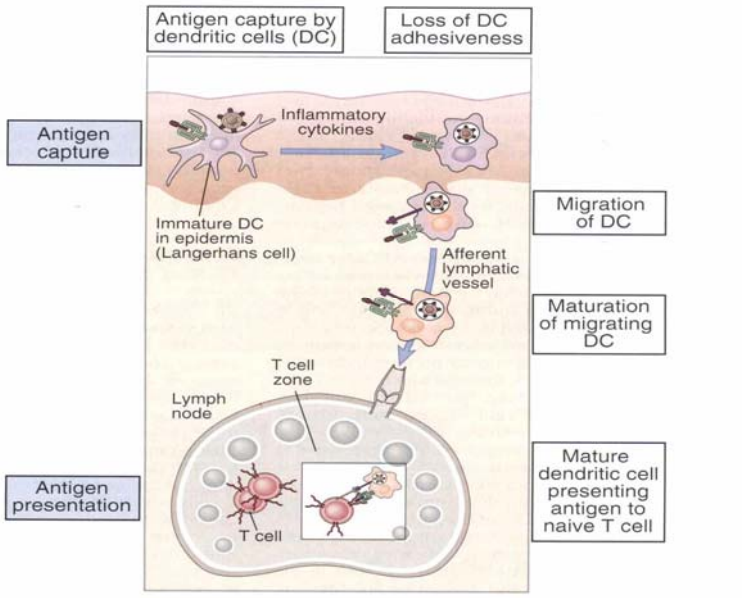


Figure 1. **Role of dendritic cells in antigen capture and presentation.** Figure taken from Abul K. Abbas and Andrew H. Lichtman (24).

Antigen processing is the conversion of native proteins into MHC–associated peptides. This process consists of the introduction of exogenous protein antigens into APCs or the synthesis of antigens in the cytosol, the proteolytic degradation of these proteins into peptides, the binding of peptides to MHC

molecules, and the display of the peptide-MHC complexes on the APC surface for recognition by T (25-27).

For MHC class I–associated antigen presentation, cytosolic proteins are proteolytically degraded in the proteasome, generating peptides with features that enable them to bind to class I molecules. For a peptide to be presented in a context of an MHC molecule the protein must first undergo processing through a number of steps, which vary considerably depending on the origin of the protein. The peptides that are presented bound to class I MHC molecules are derived from cytosolic proteins, such as those resulting from viral infection or oncogenic transformation, most of which are endogenously synthesized in nucleated cells. The degradation of endogenous protein take place within the cytoplasm and is mediated mainly by the proteasome, a large multiprotein enzyme complex with a broad range of proteolytic activity, found in the cytoplasm of most cells. Most proteins require ubiquitination to be presented by the class I MHC pathway. After ubiquitination, the proteins are unfolded, the ubiquitin is removed, and the proteins are ‘threaded’ through proteasomes. The proteasome has broad substrate specificity and can generate a wide variety of peptides from cytosolic proteins. IFN-γ enhances antigen presentation, changing the substrate specificity of the proteasome so that the produced peptides are 6 to 30 residues long, usually containing carboxyl terminal basic or hydrophobic amino acids. These peptides are delivered from the cytoplasm to the endoplasmic reticulum (ER) by an ATP-dependent transporter called TAP, a heterodimer comprised of TAP1 and TAP2, forming a pore between the cytosol and the ER. This complex does not indiscriminately take up peptides but rather it has been demonstrated that TAP has a preference for peptides of 8 to 16 amino acids that have hydrophobic or basic amino acids at the C-terminus. Within the ER, newly synthesized class I MHC-beta2-microglobulin dimers are attached to the TAP complex and receive peptides transported into the ER. Stable complexes of class I MHC molecules with bound peptides move out of the ER, through the Golgi complex, to the cell surface.

1.2.2. MHC Class I-restricted immunity

All nucleated cells are susceptible to viral infections and cancer-causing mutations. Therefore, it is important for anti-tumour immunity that the immune system be able to recognize cytosolic antigens harbored in any cell type. All nucleated cells can present class I MHC-associated peptides, derived from cytosolic protein antigens, to CD8+ T lymphocytes. This ubiquitous expression of class I molecules allows class I–restricted CTLs to recognize and eliminate virus-infected or possibly tumour cells. The CTLs are uniquely equipped with multiple mechanisms to induce the death of any cell that is displaying the unique target antigen recognized by its polymorphic TCR. CTLs kill targets that express the same class I-associated antigen that triggered the proliferation and differentiation of pre-CTLs and do not kill adjacent cells that do not express this antigen. This specificity of CTL effector function ensures that normal cells are not killed by CTLs reacting against infected cells. The cytolysis is mediated mainly by granule exocytosis, which releases perforin, a membrane pore-forming protein, and granzymes, which enter the target cell through the perforin channel and induce apoptotic death of the target cell. Other mechanisms by which CTL can kill target cells include via interactions between membrane molecules on the CTLs and target cells, such as Fas, and Fas ligand, which results in activation of caspases and apoptosis of targets.

The activated CTLs are able to induce apoptosis specifically of any tumour cell which presents the antigen for which their receptors are specific, whether through secretion of their cytotoxic granules or by stimulating the “death receptors” expressed by the tumour cells. In terms of cancer immunology, the eradication of established tumour burdens by adoptive transfer of CD8+ T cells has been reported for a mouse model (28) and has met with the best clinical success of any form of cancer immunotherapy (29,30,31). These findings highlight the kinetic battle between tumour growth and the production of a tumour specific response and have direct applications in effective adoptive immunotherapy.

1.2.3. Cross-presentation

The process of cross-presentation involves one cell type presenting antigens from another cell and priming T cells specific for these antigens. The classical MHC class I processing pathway is for the processing of endogenous antigens initiated by the infection of APC. However, the induction of a primary CTL response poses a special problem because the antigen may be produced by a cell type, such as virus-infected or tumour cell, that is not an APC. To be activated to proliferate and differentiate into effector CTLs, naive CD8+ T cells must recognize class I-associated peptide antigens and also additional stimuli. These additional stimuli, or second signal, may be provided by encounter with costimulators, such as B7 molecules, expressed on professional antigen-presenting cells (APCs) or signals, such as cytokines (IL-12), provided by $CD4^+$ helper T cells which stimulate the differentiation of naive T cells into effector cells. $CD4^+$ helper T cells may also activate APCs and prepare them to stimulate CTL development, for instance, by stimulating expression of B7 molecules (Figure 2).

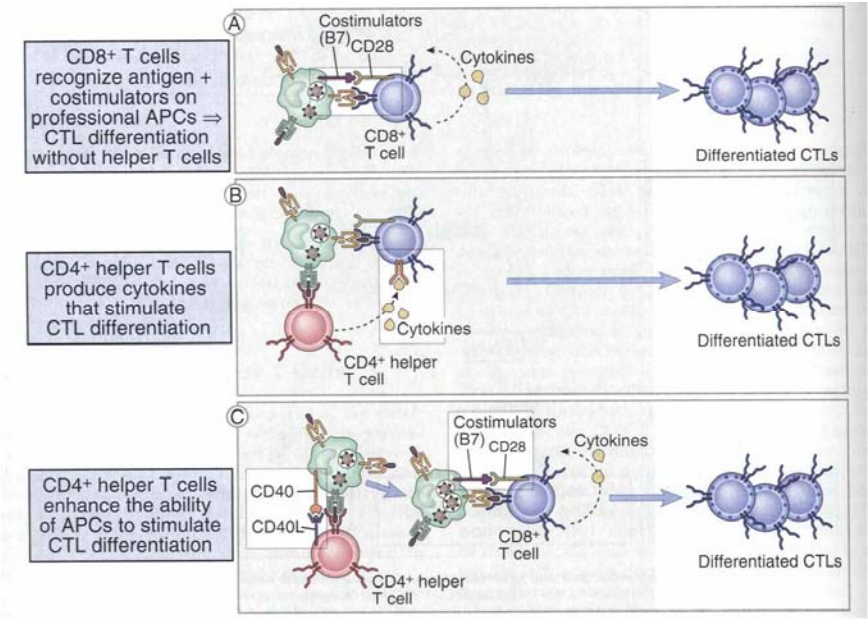


Figure 2. Role of costimulation and helper T cells in the differentiation of $CD8^+$ T lymphocytes. Figure taken from Abul K. Abbas and Andrew H. Lichtman (24).

The process that allows DCs and macrophages to take-up exogenous antigen and process these so that they are presented in the context of MHC class I is referred to as cross-presentation (Figure 3). Antigens that are to be cross-presented need to be first internalized by the APC. Restriction of presentation to a subset of APCs that uses passive acquisition rather than endogenous expression of antigens may prevent the dissemination of pathogens or the spread of cancerous cells through the lymphatic system (32).

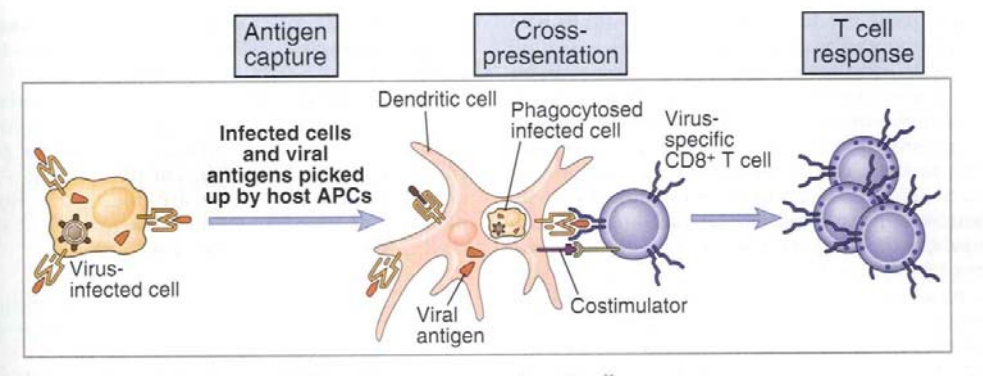


Figure 3. Cross-presentation of antigens to CD8$^+$ T cells. Figure taken from Abul K. Abbas and Andrew H. Lichtman (24).

Moreover, to test the concept that the immune system responds only to antigens perceived to be associated with dangerous situation such as infection, She et al (33) have identified uric acid as one of the principal endogenous immunological danger signals. It is constitutively present in cells and its concentration increases when cells are injured. When released from dying cells, it stimulates dendritic cells to mature and when co-injected with antigen in vivo augments the priming of CD8+ T cell responses to cross-presented antigen. Cross-presentation was first extensively documented in the 1970's in studies by Bevan (34, 35). The observation in the study of Carbon and Bevan (36) shows how a class I-restricted CTL response can be primed against a soluble protein antigen. This opened up the potential for the development of adoptive T-cell therapy. T cells can discriminate diseased cells based on subtle alterations in peptides displayed in association with MHC molecules at the cell surface. Recent success using the adoptive transfer of tumour-specific T cells has fueled

optimism that this approach may find a place as a targeted therapy for some human cancers. The ability to adoptively transfer T cells to treat cancer and infections has been demonstrated in rodent models, but the task of translating the principles established in animal studies into the clinic has not been easy (31,37,38,39).

1.3. Tumour antigens

The field of tumour immunology is based in large part on the demonstration that tumours can express antigens that permit immunologic recognition of malignant but not normal cells. Although historically much scepticism surrounded this idea, modern approaches facilitated by technological advances in cellular and molecular immunology have convincingly demonstrated that many human tumours do express antigens that can induce and be targets of cellular and humoral responses.

Since the discovery by van der Bruggen & Boon and colleagues of the first tumour-associated antigens (TAA) recognized by CTLs (16, 40), a monumental effort has been made to identify and define tumour antigens. Some tumour antigens have been found to be derived from viruses, with 10-20% of cancers expressing tumour antigens derived from infectious agents, including human papiloma virus (HPV) E6/E7 associated with cervical cancer, penile cancer and anal cancer (41). However, many tumour antigens identified so far are not virus-associated, but rather self or modifiedtumour-self antigens. Based on their tissue expression patterns, expression levels and mutations, these self-tumour antigens can be classified into five groups (42) as follows:

1. Cancer-testis like antigens (CT antigens): CT antigens include MAGE-1, MAGE-2, MAGE-3, MAGE-12, BAGE, GAGE, NY-ESO-1, and CML66 (43), and CML28 (44). Cancer-testis antigen mRNAs are expressed in a wide range of different cancers as well as normal testis (45), but generally are not expressed in most other normal somatic tissues, except testis (46). Since testis is an immune privileged site that does not express MHC class I or II molecules (47) and may express FasL to kill attacking lymphocytes (48), these antigens can

practically be regarded as tumour-specific and are highly desirable as targets for antigen-specific immunotherapy (40, 46, 49-58);

2. Differentiation antigens**:** Differentiation antigens include tyrosinase, TRP-1, TRP-2, gp100, MART-1, and MC1R. Since these differentiation antigens are expressed in differentiation stage-dependent and tissue-specific manners, immunotherapy based on these may not cause any autoimmune side-effects on other tissues. However, the disadvantage is that antigen-specific immune responses may be compromised by self-tolerance (50, 59);

3. Unique - tumour- specific antigens**:** antigens, specific to one individual tumour or patient and thought to arise after somatic mutation in the coding region of a ubiquitously expressed gene as a result of genetic instability of a neoplastic cell. They can arise as a single mutation, and include point mutations, frame-shift mutations, antisense transcripts, altered posttranscriptional translocations or fusion proteins induced by chromosomal translocations (60). Known antigens, detected within certain malignancies are B-RAF point mutation, k-ras oncogene and the BCR-ABL fusion protein.

4. Overexpressed antigens**:** Overexpressed antigens identified include proteinase 3 (myeloblastin) (47, 50), WT-1, MUC-1, normal p53, Her/neu, PAP, PSA, PSMA, and G250, CDK4, caspase-8, β-catenin, Bcr-Abl (47-50), mutated p21/ras, and mutated p53;

5. Oncofoetal antigens**:** The last group of antigens includes CEA, α-fetoprotein, 5T4, onco-trophoblast, and solid tumour-associated glycoprotein. Of note, most of these self-tumour proteins are non-mutated proteins (61).

Hybridoma technology (56) and the recent success of antibody-based immunotherapy (62, 63), such as Rituximab (anti-CD20) and immunotoxins which target tumour antigens expressed on the cell surface, such as HER2 (64) renewed the interest of immunologists in humoral antitumour responses. Tumour antigens have to be expressed on the tumour cell surface in order to be recognized by antibodies. Although some tumour antigens are expressed on the tumour cell surface (57, 65-67), most of tumour antigens identified so far are intracellular proteins (43,49) and may be defined as T-cell antigens.

Studies on identification of HLA-restricted T-cell-recognized epitopes of tumour antigens and T-cell based immunotherapy to tumours have also made significant progress (41). At the time of writing, more than 257 HLA class I-, and HLA class II-restricted T-cell epitopes have been identified. Since they are derived from various tumours, these T-cell antigen epitopes are very useful in diagnosis, prognosis, and immunotherapy of tumours. Furthermore, clinical studies of several formats of active immunization (recombinant viruses, naked DNA, dendritic cells pulsed with peptide, and peptides) in patients with melanoma showed that after two courses of immunization with the gp100, MART-1, or tyrosinase tumour antigens, up to 1-2% of all circulating CD8 T cells had anti-tumour activity, which is several hundred or thousand-fold higher than the frequencies of any given antigen-specific T cell in the normal T-cell repertoire. In combination with IL-2 therapy, the response rate could reach as high as 32% (41). A team at the Memorial Sloan-Kettering Cancer Center in New York reported on 14 patients with chronic myelogenous leukemia (CML) in a phase II study with Bcr-Abl peptides (a CML-specific fusion gene encoded by the Philadelphia chromosome) that were given 5 injections or 6 peptides over 10 weeks. A decrease in the percentage of Philadelphia chromosome + (Ph+) cells (a CML-specific fusion chromosome) was noted in 4 patients (68-70).
These examples have clearly demonstrated that identification of tumour antigens has potential for the development of antigen-specific immunotherapy of tumours.

1.4. Techniques for the identification of tumour antigens

Although many antibody-binding epitopes (about 15 amino acids) in self-antigens have been characterized, however, one does not have to map the antibody-binding epitopes before evaluation of antigen-specific antibody responses. In contrast, before examination of antigen-specific T cell responses, it is advantageous to know (a) whether the antigen of interest encodes HLA-restricted T-cell antigen epitopes (9-11 amino acids for HLA class I-restricted epitopes; 15-20 amino acids for HLA class II-restricted epitopes); (b) where the epitopes are located in the antigen sequence; and (c) what HLA allele

restrictions of the antigen epitopes are. In the following section, we have outlined the principles of several major techniques:

- T cell epitope cloning: Many antigens recognized by CD8+ T cells have been identified by transfecting cDNA libraries from tumour cells into target cells expressing the appropriate HLA molecule, and then using anti-tumour T cells isolated from tumour infiltrates (TILs) to identify the antigen epitopes presented by HLA on the surface of the transfected target cells (40, 71, 72) .

- HLA-bound peptide elution: Peptides eluted from cancer cells or from HLA molecules purified from cancer cells can be pulsed onto APCs and tested for reactivity with or sensitization of specific anti-tumour lymphocytes. Sequencing of these peptides can then lead to the identification of the parent protein antigens and allow the use of synthetic peptides for further experiments. (73, 74).

- SEREX: To identify tumour antigens recognized by the antibody repertoire of cancer patients, in 1995, Pfreundschuh's team developed a new method of molecular cloning called SEREX (75-78), which allows a systemic and unbiased search for antibody responses against protein antigens expressed by human tumours. Advantages of SEREX include rapid identification of multiple tumour antigens by screening the patient's antibodies to their own tumour products. (75). SEREX-defined tumour antigens are collected in a SEREX database maintained by the Ludwig Institute for Cancer Research (http://www2.licr.org/CancerImmunomeDB/). The relevance of these SEREX-defined tumour antigens to anti-tumour immune responses has been clearly demonstrated in several reports (49, 66, 67, 79-81).

In addition, since the overexpression of proteins in tumour cells may be the mechanism for the immunogenicity of non-mutated protein antigens, investigators have also searched for proteins or genes encoding the proteins overexpressed in tumours, detected by other techniques including differential display, serial analysis of gene expression (SAGE), or microarray, for tumour antigen candidates (82). Differential display, developed in 1992 (83), allows rapid, accurate and sensitive detection of altered gene expression. The key element is to use a limited number of short arbitrary primers in combination with

anchored oligo-dT primers to systematically amplify and visualize most of the mRNA in a cell. This method detects changes in mRNA profiles among multiple samples being compared without the need of any prior knowledge of genomic information. The SAGE method allows for a quantitative and simultaneous analysis of large number transcripts in any particular cells or tissues, without prior knowledge of the genes (84). This assay is based mainly on two principles, representation of mRNAs (cDNAs) by short sequence tags and concatenation of these tags for cloning to allow efficient sequencing analysis (85). By comparing the gene expression profiles derived from cancer and normal tissue of interest, a large number of genes were identified as tumour-specific. In microarray analysis (86) RNA is harvested from a cell type or tissue of interest and labeled to generate the target - the free nucleic acid sample whose identity or abundance is being detected. This is hybridized to the tethered probe DNA sequences corresponding to specific genes that have been affixed, in a known configuration, onto a solid matrix. Comparison of hybridization patterns enables the identification of mRNAs that differ in abundance in two or more target samples. Thus microarrays provide a powerful tool with which to screen biological specimens for alterations in the expression of mRNAs that accompany pathological changes. Microarray-based tests are superior to traditional DNA-based tests because of their unique ability to simultaneously measure the relative expression level of a large number of clinically-relevant genes. Currently a cancer-related microarray-based clinical test is on the market (MammaPrint breast cancer prognosis test, Agendia, The Netherlands), which activity confers information about the likelihood of tumour recurrence. Other cancer-related microarray-based tests dedicated for clinical use as diagnostic and/or prognostic tools are either in the process of FDA review (The AmpliChip p53 test, Pathwork™ Tissue of Origin Test) or under active development (87).

The T-cell antigen epitopes from these candidate antigens can be further characterized through the reverse immunology approach (88).

1.5. Reverse immunology

This method of epitope deduction, sometimes called “reverse immunology”, postulates that candidate peptide epitopes within selected molecules presented by HLA for stimulating T cells can be identified based on predicted binding affinities of peptide to MHC and scrutinized for immunogenicity based on the functional capacity of experimentally identified epitope-specific T cells (40, 71, 72, 88).

Reverse immunology becomes a useful alternative in characterization of antigen-specific T-cell responses for the large numbers of SEREX antigens, especially those tumours for which anti-tumour immunoreactivity in patients is weak (88).The identification of HLA class I-, and HLA class II-restricted T-cell epitopes from tumour antigens allows us to better examine antigen-specific T cells Moreover, immunodominant epitopes capable of eliciting marked CD8+ T cell responses would be expected to contribute decisively to the improvement of peptide-based immunization protocols for patients with tumours (89, 90) because cytotoxic CD8+ T lymphocytes can lyse tumour cells directly, and destroy large tumour masses in vivo. Furthermore, CD4+ T cells have been shown to play an important role in CML remission induced by donor CD4+ T cell infusion for the relapsed CML after allogeneic bone marrow transplantation (91). Also, HLA class II-restricted CD4+ T cells have been demonstrated to recognize mutated tumour antigens (91). Additionally, in the meantime the well-established epitope based antigen-specific T-cell assays including MHC tetramer assays (59, 92, 93), and ELISPOT (98) are also available for routine clinical use for the evaluation of tumour antigen-specific T cell responses in addition to the “traditional” immunotechniques that are used in detect the antigen-specific antibody responses in patients with tumours (43,44).

1.6. Manipulating the immune response

Manipulating the immune system to mediate tumour regression is well demonstrated in experimental and clinical settings. Several factors hamper the effectiveness of anti-tumour immune responses involve mainly the generation/stimulation of immune effector CTL and NK cells according to

number, avidity, migration and tumour infiltration capacity. On the other hand, tumour cells themselves have evolved to escape the immune attack by altering/downregulating MHC class I and tumour antigen expression, dysregulating expression of adhesion/accessory molecules by tumour and/or antigen-presenting cells, and secreting factors that modulate immune effector function (95). Thus, cancer cells may produce immunomodulating cytokines such as IL- 10 and transforming growth factor β1 (TGF-β1) for example that suppress IFN-γ expression, downregulate effector function of CTLs, Th1 and NK cells (96-98). NK function could be also blocked by soluble MIC (MICA, MICB) released by tumour cells (99). The microenvironment of the tumour is another aspect to be considered, acting as a barrier through which effector cells must establish contact with targeted malignant cells.

Concerning the generation of tumour-specific CTLs, it is of importance to consider the immunogenicity of TAAs which derive from diverse sources (see above) in the whole context of protein expression, intracellular processing leading to a defined peptide sequence and finally peptide presentation by the MHC. In this respect, Rohrer et al. (100, 101) have shown that epitopes present on the TAA peptide OFA/iLRP recognized by cytotoxic CD8+ T cells were distinct from those recognized by non-cytotoxic ones. On the other hand, initiating IFN-γ secreting CTLs or IL-10-secreting regulatory-suppressor CD8+ T cells, depended on the presence of relevant epitopes. This example shows the necessity for analyzing the exact peptide sequence in order to obtain an adequate tumour vaccine. Furthermore, the mechanisms involved in the immune T-cell unresponsiveness to self TAA peptides (i.e. antigen ignorance, peripheral anergy, tolerance-immunosuppression, central deletion) have to be considered. There are indications that treatment using IL-12 could reverse TAA peptide unresponsiveness (102,103). Moreover, the data reported by Speiser et al. (108) showed that not only an effective induction of TAA specific CTLs, but also maintenance of their sustained activity is necessary for mediating tumour regression. A question frequently arising in cancer immunotherapy is related to the ratio of effectors to target cells. In fact, the number of immune effector cells generated by endogenous manipulation is usually far below those applied in

cytotoxicity assays in vitro. This feature underlines the necessity in improving not only the quality but also the quantity of immune effector agents.

Although the host immune system may often eventually be unable to control tumour growth, the presence of identifiable tumour antigens in most tumour cells, the identification of a detectable but ineffective host response to many tumours, and an improved understanding of the mechanisms by which tumour cells evade immunity suggest that it may be possible to manipulate and amplify the immune system to promote tumour eradication. The challenge for immunotherapy is to use advances in cellular and molecular immunology to develop strategies that effectively and safely augment antitumour responses.

2. Renal cell cancer

Renal cell cancer (RCC) is a disease with an approximate annual incidence of 10 per 100.000 with a peak incidence in the sixth and seventh decades and a 2:1 male: female predominance. It accounts for around 3% of human malignances and its incidence increases by 2-3% per year. The highest rates of disease are seen in North America, Australia/New Zealand, and Europe (105). Classical renal cell cancer originates from within the renal cortex and is responsible for 80% to 85% of primary renal tumours (106). 60% of the cases are detected because a renal mass is incidentally identified on radiographic examination, because there are no typical early symptoms and the RCC masses remain non-palpable until they are advanced. The facultative classic presentation of renal cell carcinoma includes flank pain, hematuria and a palpable abdominal mass. Other common presenting features may be nonspecific, such as fatigue, weight loss, or anemia. Most cases of RCC are sporadic, with risk factors such as obesity, diet, hypertension, with cadmium exposure and tobacco exposure posing the greatest relative environmental risks, increasing with amount of exposure and decreasing relative to time since exposure. Further risk factors are acquired cystic kidney disease associated with an end-stage renal disease as well as hereditary RCC syndromes (<3%), which also have major clinical implications. Renal cell cancer is described as a

heterogeneous disease consisting of different histological types, highly resistant to chemotherapy and radiation. RCC is a diagnosis which encompasses a broad spectrum of histological subtypes, and an appreciation of the unique attributes of each type has become increasingly important for predicting response to therapy. The identification of families with a predisposition to the development of renal neoplasia has made possible the identification of the different genes contributing to susceptibility to these cancers.

To date, there are four well-described familial renal neoplasia, including von Hippel-Lindau (VHL) disease, hereditary papillary renal carcinoma (HPRC), Birt-Hogg-Dube´ Syndrome (BHD), and hereditary leiomyomatosis and renal cell cancer (HLRCC). The dominant subtype of RCC bears a strikingly high frequency of mutations in the von Hippel- Lindau (VHL) gene, a classic 2-hit tumour-suppressor gene (107,108). The VHL tumour susceptibility gene resides on chromosome 3p24-25, which is a region of the genome commonly deleted in sporadic kidney cancer (109,110). Somatic mutations of MET, a proto-oncogene, encoding a receptor tyrosine kinase, normally activated by hepatocyte growth factor (HGF), occur in 13% of sporadic papillary type-1 RCC. BHD syndrome is a genodermatosis, and the BHD gene encodes folliculin, without known function. BHD somatic mutations are very rare in sporadic RCC, but hypermethylation is observed in ~30% of all RCC histological types (111).

Immunotherapies for RCC can be divided into 5 categories: cytokine administration, vaccines, antibodies directed at immune response modulators, immune-cell transfer, and allotransplantation. Based on the single agent activity of VEGF-targeted and immune-based therapy, integrated strategies are undergoing active investigation. The current mainstay of cytokine treatment is IL-2. IL-2 and IFN-α were first reported to have antitumour activity in the 1980s (112). Although IL-2 can induce sustained remissions in a small subset of patients, it is highly toxic, and is not available in all treatment centers. IFN-α, exhibits modest activity in the treatment of RCC, using various doses and routes of administration (113).

The hypothesis that tumour rejection during IL-2 treatment is mediated by tumour-specific T cells leads to the approach of utilizing vaccination to enhance

the endogenous antitumour T-cell response either in combination with IL-2 or as an alternative to IL-2 (114). The evolution of immunotherapy from traditional cytokine-based therapy to more sophisticated and tumour-specific therapy has become an alternative immunotherapeutic strategy in the mainstream treatment of this disease (115). Recent meta-analyses from the earlier vaccine studies have documented the development of tumour-specific CD4+ and CD8+ T cell responses following vaccination (116, 117). In Brazil, a dendritic cell-tumour cell hybrid vaccination strategy achieved objective responses in three of 22 patients (14%), including one complete response, with a median TTP of 5.7 months with no significant toxicities noted (118-120).

There are new methods of vaccinating being advocated. For example minimal peptide epitopes, DNA, recombinant viruses engineered to express not only the target antigen but other costimulatory molecules, dendritic cells, modified tumour cells and heat shock proteins chaperoning tumour antigen epitopes were all shown to be potential vaccine approaches in animals (114). Currently, for vaccines against cancer, the issues of inadequate T-cell activation (i.e., 'ignorance') and peripheral inactivation of T cells recognizing self-antigens (tolerance) remain unresolved obstacles (114).

The Goal and Tasks

Recently, an HO-1-derived peptide found in the tumour tissue of a female patient diagnosed with renal cell carcinoma was isolated (121). The sequence of the peptide was determined to be APLLRWVL originating from the enzyme HO-1 positions 265-272. This peptide showed a binding motif for HLA-B*08, one of the HLA alleles of the patient. For a peptide to have any potential clinical application in anti-cancer immunotherapy, it is important to determine if it demonstrates immunogenicity in vitro.

The purpose of the present study was to test the immunogenicity of the isolated HO-1-derived peptide and to attempt to generate a specific T cell population that could recognize HO-1-derived epitopes.

To accomplish this goal the following tasks were set at the beginning of the investigation:

1. To generate specific T-cell lines from PBMC of healthy donors, using synthetic HO-1 peptide.
2. To screen the established T-cell lines for HO-1-specific cytokine release.
3. To measure antigen-specific IFN-γ production by T lymphocytes at the single cell level.
4. To evaluate the antigen-specific cytolytic activity of the generated peptide-specific CTL line.

2. Material and Methods

1. Isolation of PBMC

For the isolation of peripheral blood mononuclear cells (PBMC) either buffy coats or 100 ml heparinised peripheral blood from HLA-B8-positive healthy consenting donors (University Clinic Tuebingen, Department for Transfusion Medicine), were obtained. Blood was then diluted 1:1 (buffy coats 1:2) with Hank's Buffered Salt Solution (HBS) and isolated on a Ficoll/Hypaque discontinuous density gradient (p=1,007 g/ml). Briefly 35 ml blood/HBS is carefully layered over 15 ml Ficoll and centrifuged at 900 g for 20 minutes. PBMC are then harvested from the interphase layer and washed in HBS three times. Granulocytes and erythrocytes contained in the pellet are discarded. Following HLA typing (see below) cells can then be used directly for DC generation or frozen and stored in liquid nitrogen for future use. Cells are frozen in 1 ml RPMI containing 20% FCS and 10% DMSO at a concentration of 1×10^{7} ml, as described below.

2. HLA typing

PBMC and cell lines were typed for human leukocyte antigen (HLA) class I and II alleles. Standard molecular methods were used to establish the HLA-A and B types, as well as HLA-DR, DP and DQ. The HLA laboratory of the Second

Department of Internal Medicine, Tuebingen (Prof. C.A. Müller) carried out this work.

3. Synthetic peptides

HO-1-derived synthetic peptide APLLRWVL was used in T-cell sensitisation experiments and for assessing the peptide-specific activity of these T lymphocytes. This peptide was found to be present exclusively in the tumour tissue of a 73 year old female patient diagnosed with a clear cell carcinoma (pT1) of the right kidney (121).The HLA-type of the patient was A*01, A*33, B*08, B*44. The sequence of this peptide was determined to be APLLRWVL originating from the protein heme oxygenase (HO-1) (Swiss-Prot: P09601) positions 265-272 (121). This peptide showed a binding motif for HLA B*08, one of the HLA alleles of the patient. The peptide was synthesized by FMOC solid phase strategy on a MilliGen 9050 continuous flow synthesizer (Millipore, Bedford, MA). After RP-HPLC purification, purity was determined by LC-MS to be greater than 90%. In addition to the HO-1–derived peptide APLLRWVL, an irrelevant HLA-B8–binding ribosomal protein-derived peptide (YLKVKGNVF) was also used for pulsing autologous PBMC or HLA-B8-positive B-cell line MGAR (source: 10^{th} International Histocompatibility Workshop; cell number 10W9014).

4. Cultivation of cell lines

B cell lines were cultivated in RPMI 1640 medium supplemented with Glutamax and the broad-specificity antibiotic, Gentamicin. The medium was also supplemented with 10% FCS. Cell lines were cultivated at 37%°C in 5% CO2 and at 95% humidity and routinely controlled for cell concentration and contamination by means of reverse-phase microscopy. Before cell lines were cryopreserved as a large stock they were tested for mycoplasma contamination. Adherent cell lines were seeded in 75 cm^2 plastic tissue culture-treated flasks at approximately 2×10^5 cells/flask, in 15ml of culture media and growth to confluence. Cells were then washed twice with HBS and removed from the flask

surface by incubating with Trypsin/EDTA for one minute at 37°C. Trypsin was inactivated by the addition of serum-containing media and the cells were pelleted by centrifuging at 300g for 5 minutes and washed once further with HBS. Cells were then resuspended in 150 ml media and transferred to a triple-flask (×-cm^2) for further cultivation, until the cells were required for further experimentation or cryopreserved.

Suspension cells were seeded at a concentration of 1-5×10^5 per ml and maintained in the described culture medium until a concentration of approximately 1-2×10^6 per ml was obtained. Cells were then split and further cultivated maintaining a concentration of approximately 1×10^6 per ml until needed for further experimentation or cryopreserved.

4.1. Quantification of cell number and viability

Cells were counted and controlled for viability by means of a Neubauer-haemocytometer. 10µl of cells in suspension were diluted 1:1 with 0.4% Trypan-blue, a dye that determines membrane integrity, and applied to the haemocytometer. Cells were analysed with a phase-contrast microscope and cells present within one quadrant, comprised of 16 subunits with a total volume of approximately 0.1µl, that were not stained with the dye were counted and applied to the equation bellow to determine total-cell number per ml.

Cell number per ml = cell count × dilution factor × 10,000

4.2. Cryopreservation of cells

For long-term storage, cells were cryopreserved at –196°C in liquid nitrogen. Cells in culture were washed twice with HBS and cell pellet, containing a maximum of 1×10^7 cells, was resuspended in 1 ml of RPMI 1640 media comprising a final concentration of 20% FCS and 10% DMSO, and transferred to cryopreservation ampoules. DMSO, although cytotoxic, is added to protect cells from damage caused by the formation of ice crystals. Due to its cytotoxicity

a diluted solution is pre-chilled to 4°C prior to use. Cells were frozen in a – 70°C freezer overnight prior to transfer to liquid nitrogen.

For the thawing of cryopreserved cells the following protocol was strictly adhered, to enable the recovery of cell with highest degree of viability. Cryopreservation ampoules were transferred directly into a 37°C water bath until thawed and immediately diluted 1:1 with RPMI 1640 by means of drop wise addition, and subsequently further diluted to a final concentration of 1:10. Cells were pelleted at 300g for 5 minutes and washed twice prior to counting and resuspension in the appropriate media.

5. Dendritic cell generation

Dendritic cells were generated from healthy donor PBMC by inducing the differentiation of CD14+ monocytes with a cytokine cocktail. Monocytes were isolated and enriched from PBMC by means of plastic adherence. PBMC were plated in 6-well plates (Greiner, Nuertingen, Germany) at a concentration of 1×10^{6}/well in 3 ml of X-Vivo 15 medium and incubated for 2 hours at 37°C, 5% CO2. In this way, plastic adherence of monocytes allowed their separation from lymphocytes, which were removed by washing with RPMI and frozen for future use as responder cells. Isolated monocytes were cultured in 3 ml of X-Vivo 15 (BioWhittaker, Walkersville, MD, USA), supplemented with 800 U/ml GM-CSF (Leucomax 300, Novartis, Nuernberg, Germany) and 500 U/ml IL-4 (Novartis, Basel, Switzerland) at 37°C in 5% CO2 (Day 0). After 24 hours 2 ml of fresh medium was added containing 1600 U/ml GM-CSF and 1000 U/ml IL-4 (per ml of total culture media) (Day 1) and the cells were incubated for further 2 days. On day 3, 2.5 ml of medium was removed and replaced with fresh medium containing GM-CSF /IL-4 as on day one. Cells were then incubated for two more days. On day 5, 1 ml of medium was removed from each well and replaced with 1 ml fresh medium containing 50 ng/ml TNF-alpha (10 ng/ml total culture media) and the cells were returned to their incubation conditions. DCs were harvested from the plate on day 7, washed with RPMI, counted and the phenotype was determined by FACS analysis. If sufficient DCs were obtained, a proportion of mature DC was cryopreserved for future use as stimulator cells.

6. Generation of T cell lines

For T-cell sensitisation experiments, the protocols described by Müller et al (122) and Knights et al (123) were applied. Every restimulation was monitored with the help of ELISA or ELISpot assays as well as by CD107a mobilisation assays to detect degranulation.

6.1. Cytokine-modified bulk-culture protocol

Peripheral blood mononuclear cells of a HLA-B8-positive healthy donor at $1x10^6$ cells/ml were stimulated with 10 µg/ml of synthetic HO-1 peptide in 2-ml cluster plates. X-Vivo 15 serum-free culture medium was employed with addition of different cytokines, as follows: 800 U/ml GM-CSF, 500 U/ml IL-4 and 1 ng/ml IL-15 (R&D Systems, Abingdon, UK). After 7 days, and every two days thereafter, IL-2 (20 U/ml) was added to the cultures. The stimulated T lymphocytes were rechallenged every 7-9 days once with $1x10^6$/ml irradiated autologous PBMC together with 10 µg/ml HO-1 peptide and secondly with $7.6x10^5$/ml HLA-B8 positive B-cell line (MGAR) cells, that had been pulsed for 4 hours with the synthetic peptide HO-1.

6.2. Dendritic cell sensitisation

Mature DCs were pulsed with the indicated concentration of peptide for 4 hours at 37°C, followed by a wash step. $1×10^5$ DCs were co-cultured with $2×10^6$ autologous PBMC in 2 ml serum-free X-Vivo 15, in 24 cluster plates for one week. On day 7 frozen autologous DCs were thawed and pulsed (as previous) for 4 hours followed by washing. $1×10^5$ DCs were added per well in 1ml of fresh medium. Following 24 hours co-culture, 60 IU/ml of IL-2 was added per ml media. Medium was exchanged and fresh IL-2 added every third day. On day 14, cells were restimulated with $5×10^5$ peptide-pulsed, irradiated (30 Gy) autologous PBMC, and IL-2 added as previously. T-cell lines were tested for their specificity on day 21.

7. Inactivation of cells used as antigen presenting cells

Cells used in culture experiments as antigen presenting cells were first exposed to controlled levels of gamma-irradiation as a means of inhibiting their proliferative capacity. The level of exposure to the radioactive source was controlled, by means of previous experimental determination, so that sufficient DNA damage was inflicted to result in inhibition of proliferative capacity but not further cellular function detrimental to their role as antigen presenting cell. The following doses were used:

PBMC: 30Gy

B-cell line: 80Gy

8. Enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA was implemented to monitor the secretion and concentration of various cytokines (IFN-γ and GM-CSF) from activated T cells following challenge with peptide pulsed target cells. A kit comprised of cytokine specific antibody pairs was purchased from PharMingen and used according to the manufacturer´s directions. Briefly, 2-5×10^4 T cells were incubated with the appropriate target or presenting cells, in a final volume of 200 µl serum-free medium. Whenever possible, triplicate wells were set for all control and experimental wells to enable the determination of a mean value. After a period of co-incubation, cell-free supernatants were obtained. Prior to sample testing the microtitre plates were coated with the capture anti-cytokine antibody (1 µg/ml, Pharmingen, Hamburg) overnight at 4°C followed by washing 4 times with PBS/Tween. The non-specific binding was blocked by incubation for 30 min at RT with blocking buffer (1% BSA in PBS). After 3 washings (PBS/Tween) the supernatants along with serial dilutions of the recombinant control protein were applied to microtitre plates and incubated 4 hours at RT. Thereafter plates were washed (3x with PBS/Tween) and biotin-labelled secondary-antibody (0.5 µg/ml, Pharmingen, Hamburg) added for 1 hr at RT. After a further wash step (6x with PBS/Tween), avidin-peroxidase (Sigma, Deisenhofen) was added to the plates (30 min) followed by washing (8x with PBS/Tween) and adding the

enzyme substrate (ABST) for 30 min for colour development. Colour reaction was stopped with stopping solution. The colorimetric determination of cytokine concentration was performed at 405 nm with a spectrophotometric microtitre plate reader. The final cytokine concentration was determined by comparison of the OD values from the experimental wells with the standard curve derived from the recombinant-protein.

9. IFN-γ ELISpot assay

The ELISpot (Enzyme Linked Immuno-Spot) assay provides an effective method of measuring antigen-specific cytokine production by T-lymphocytes at the single cell level. As a first step, anti-IFN-γ capture monoclonal antibody is immobilized on a solid phase (microtitre plates with a nitrocellulose filter base). In the second step, the T cell lines to be investigated are added to the wells in presence or absence of stimuli (target cells) and incubated for a relevant time period to allow cytokine production. The secreted IFN-γ resulting from cell stimulation will immediately bind to the capture antibody in the vicinity of the producing cells. After removal of the cells by washing, anti-IFN-γ enzyme labelled antibody, reactive with a distinct epitope of the target cytokine is added to detect the captured cytokine. The detected cytokine is then visualized using a precipitating substrate. The coloured end product (a spot) typically represents an individual cytokine-producing cell. The spots can be counted with an automated ELISpot reader system or manually, using a stereomicroscope.

The test was performed using MAHA S4510 filter plates (Millipore) according to the manufacturer's protocol. Briefly, plates were coated overnight at 4°C with a primary antibody (anti-human IFN-γ, mAb1-D1K, Mabtech, Sweden, 10µg/ml PBS) followed by 2 x washing (PBS) and blockage for 2 hrs at 37°C with X-vivo 15 media. Then 1-5×10^4 T cells were co-incubated with an optimal number of irradiated autologous PBMC (1-1.5x10^5) or HLA-B8-positive B-cell line pulsed with the HO-1–derived peptide APLLRWVL or the control HLA-B8–binding ribosomal protein-derived peptide (YLKVKGNVF) for 40 hours in a humidified 37°C CO_2 incubator. Following this incubation period, cells were discarded and the plate washed (4×PBS/0.05% Tween 20) before addition of a secondary

biotinylated antibody (anti-human IFN-γ, biotinylated, Mab 7-B6-1, Mabtech, Sweden; 2μg/ml PBS) and incubation for 2 hrs at 37°C. The plate was washed (6×PBS/0.05% Tween 20), an avidin-biotinylated enzyme complex (Vectastain ABC kit, Vector Labs., USA) was added followed by incubation for 1 hour at room temperature. After a final wash, the substrate (AEC) was added and left to develop for approximately 4 minutes before stopping the reaction by washing with pure water. The number of IFN-γ spot-forming cells was counted the next day by an automated ELISPOT reader (Aelvis GmbH, Hannover, Germany) and analysed using AELVIS V3.3 software. Spots measured in the presence of APC pulsed with the control peptide were considered as non-specific background (negative control). Samples were considered to contain peptide-specific T cells when the number of spots in test triplicates exceeded control triplicates at least two-fold.

10. Effector-cell degranulation assay

This assay is based on the expression of CD107a (lysosomal-associated membrane protein-1 - LAMP-1) molecule, a membrane component of the vesicles within cytotoxic cells that contain perforin and granzyme-B, which becomes transiently mobilised to the cell surface during the process of cell killing as a result of degranulation. Therefore this test allows the rapid identification of antigen-specific cytolytic T cells. As this molecule is only temporarily expressed on the surface, the anti-CD107a antibody is incubated with the cells from the point of co-incubation to allow for its binding and subsequent internalisation and labelling of the cytotoxic T cells (Fig.4). To prevent reduction of fluorescence of FITC-labeled monoclonal antibody that is internalized into an acidic endosomal and lysozomal compartment, monensin is added to neutralise the pH within the endosomes and lysosomes.The HLA-B8-positive B-cell line MGAR was used as target cells and loaded with either the specific HO-1 peptide or a control peptide, pulsed for 2 hours and washed. The T cell lines generated were used as effector cells at 2×10^{6} cells/ml ($1x10^{5}$ cells/well). The E/T ratio used was 1:1. To each well 1μl of 2 mM monensin in 100% ethanol and 1μl of CD107a-FITC antibody were added. The

plate was centrifuged at 300g for 2 minutes and then placed into an incubator for 5 hours at 37°C and 5%CO2. After incubation, the plate was centrifuged at 500g, supernatants were removed and cells were washed with PBS supplemented with 0.02% sodium azide, 0.1% BSA and 0.5mM EDTA. Thereafter, samples were stained with CD3-PE and CD8-PerCP for 30 minutes and then fixed with 2% paraformaldehyde. Three-colour flow cytometric analysis was performed using a FACScan flowcytometer. The results were then analysed using CellQuest software. To control for non-specific binding or internalisation of the FITC-CD107a antibody, a FITC-labelled isotype control was used.

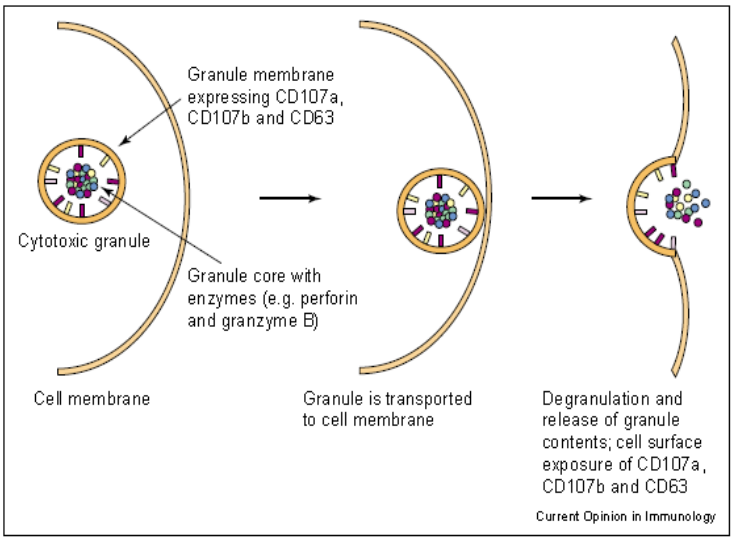


Figure 4. Diagrammatic representation of the principle underlying the CD107 degranulation assay. CD107a, CD107b and CD63 are expressed on the inner leaflet of granule membranes, and are thus transiently exposed on the cell surface upon granule fusion with the plasma membrane. The presence of antibodies to CD107 in the stimulation culture allows for labeling of these transiently exposed molecules. Taken from Suni MA et al (124).

3. Results

1. Generation of specific T-cell lines from PBMC of healthy donors, using synthetic HO-1 peptide

To address the immunogenicity of an HO-1 derived peptide, detectable exclusively from the cancer tissue of a patient with renal cell carcinoma, we performed in vitro T-cell sensitisation assays. As described in Materials and Methods this peptide showed a binding motif for HLA B*08, one of the HLA alleles of the patient and its sequence was found to be APLLRWVL. In order to generate specific T-cell lines we used in our experiments PBMC from HLA-B8-positive healthy donors and a synthetic peptide with sequence APLLRWVL.

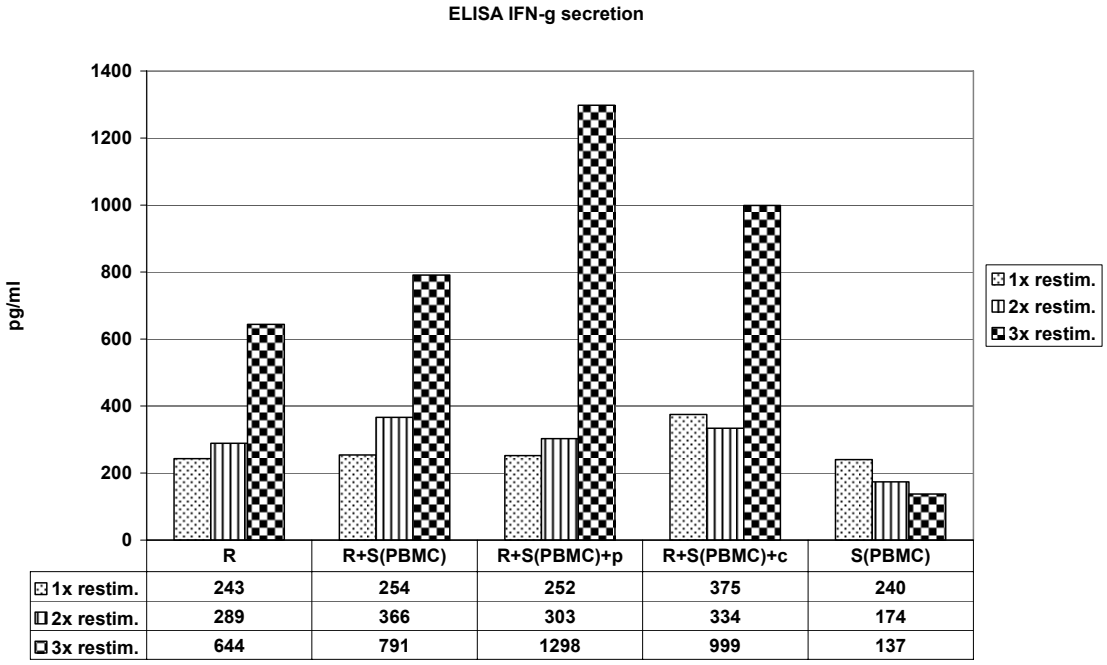
Cell cultures were re-stimulated weekly as described for several rounds and subcultured when necessary. The established T-cell lines were screened after every re-stimulation for HO-1-specific cytotoxic activity and HO-1-specific cytokine release (IFN-γ and GM-CSF), which are secreted by both CD8+ CTL as well as T-helper cells of the Th1 phenotype upon activation.

2. Screening the established T-cell lines for HO-1 specific cytokine release by ELISA

As already mentioned these T-cell lines were cultured with autologous irradiated PBMC or an HLA-B8-positive B-cell line (MGAR) in the presence or absence of HO-1-derived peptide or a control HLA-B8-restricted peptide. In order to determine the concentration of cytokines released by these T cells into the cell-free supernatants, cytokine ELISA was utilized. Cell culture supernatants were taken after 24 hours and both IFN-γ and GM-CSF levels determined. The cytokine secretion was considered specific if the cytokine level in the presence of the HO-1 peptide-challenged cells was approximately 2-fold that in the presence of the irrelevant peptide-challenged cells after subtraction of cytokine values without added peptide. After the third re-stimulation we found a very high background (644 pg/ml) in the responder cells. We detected also a certain stimulation of the responder cells in the presence of stimulator cells (PBMC) without any peptide added (791pg/m). In the assay with T cells pulsed with the irrelevant peptide we found 999 pg/ml IFN-γ release and 1298 pg/ml when pulsed with the HO-1 peptide. After subtraction of the high background (R+S) the cytokine secretion of the T cells sensitized with HO-1 peptide (507 pg/ml)

was more than 2-fold (2.44) that of those sensitized with the irrelevant peptide (208 pg/ml).

Figure 5 indicates that significant specific IFN-γ release could be seen after the third re-stimulation. Although in this example only IFN-γ secretion is shown a similar secretion pattern was also observed for GM-CSF.



	R	R+S(PBMC)	R+S(PBMC)+p	R+S(PBMC)+c	S(PBMC)
1x restim.	243	254	252	375	240
2x restim.	289	366	303	334	174
3x restim.	644	791	1298	999	137

Figure 5: IFN-γ secretion ELISA by HO-1–sensitised T-cell line following challenge with peptide-pulsed autologous PBMC. IFN-γ secretion by T cell lines following first, second and third re-stimulation was measured by means of cytokine ELISA. Cell-free supernatants were harvested 24-hrs after co-culture. R - responder T cells, S - APC, irr- control irrelevant peptide, HO-1 - relevant HO-1 peptide.

3. Measurement of antigen-specific IFN-γ production by T lymphocytes at the single cell level by ELISpot assay

The T-cell lines were tested after third re-stimulation by ELISpot assay, which allows detection of individual IFN-γ secreting cells. Figure 6 shows positive and negative ELISpot results. In Figure 7 ELISpot results after the fourth re-stimulation are presented. Greater numbers of cytokine-producing T cells (spots) were observed after the fourth re-stimulation (Fig. 7) in samples

containing T cells sensitized with HO-1 peptide and re-stimulated with peptide-pulsed HLA-B8 positive B cell line cells compared with non-stimulated samples, or after re-stimulation by B cells in the presence or absence of control peptide.

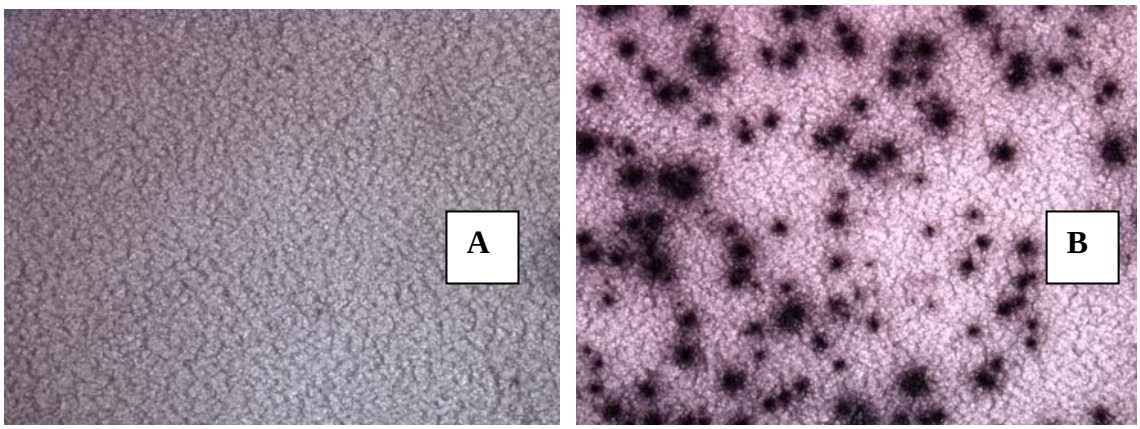


Figure 6. Images from non-reactive (A) and reactive (B) samples in ELISpot assay

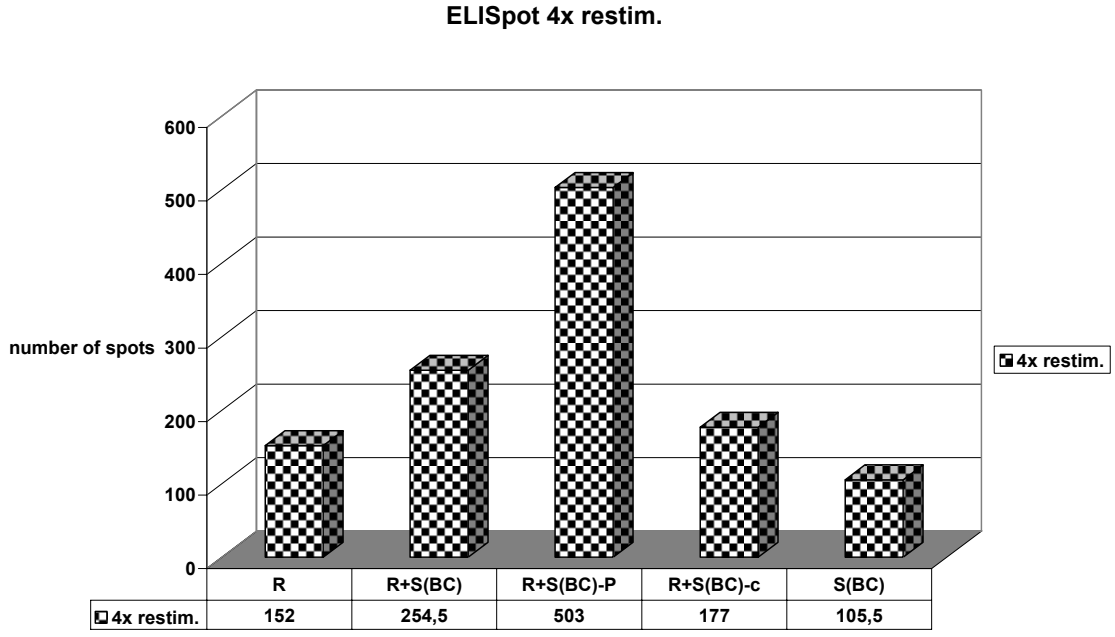


Figure 7. Results of ELISpot assay after the fourth re-stimulation showing the number of IFN-γ spots produced by unstimulated T cells (R) or re-

stimulated with: B cells S(BC) without any peptide added [R+S(BC)]; BC loaded with HO-1 peptide [R+S(BC)-P] or BC pulsed with irrelevant control peptide [R+S(BC)-c].

Following the fifth re-stimulation (Fig. 8) the analysis showed again a very high level of cytokine secretion by the T cells cultured with autologous PBMC or HLA-matched B-cells (MGAR) loaded with the specific HO-1 peptide and a very low number of spots in the absence of HO-1 peptide.

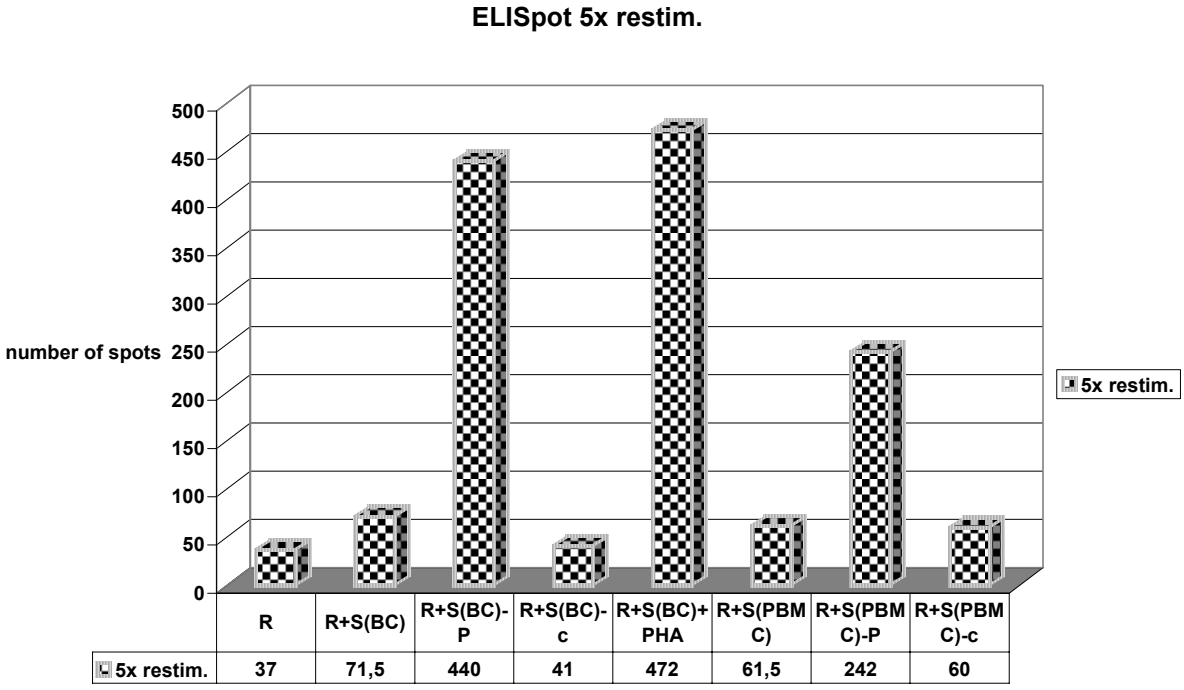


Figure 8. Results of ELISpot assays after the fifth re-stimulation showing numbers of IFN-γ spots produced by unstimulated T cells (R) or restimulated with PBMC without any peptide added [R+S(PBMC)]; with addition of HO-1 peptide [R+S(PBMC)-P] or with addition of irrelevant control peptide [R+S(PBMC)-c]; with B cells without any peptide added [R+S(BC)] or loaded with HO-1 peptide [R+S(BC)-P]; restimulated with BC pulsed with irrelevant control peptide [R+S(BC)-c] or with the mitogen PHA as a strong positive control [R+S(BC)+PHA].

After the sixth re-stimulation (Fig. 9) the secretion of large amounts of IFN-γ by T cells cultured with autologous PBMC or an HLA-B8-positive B-cell line (MGAR) loaded with HO-1-derived peptide was still higher in contrast to only a low number of cytokine-producing T cells cultured with MGAR cells pulsed with the control HLA-B8-restricted peptide.

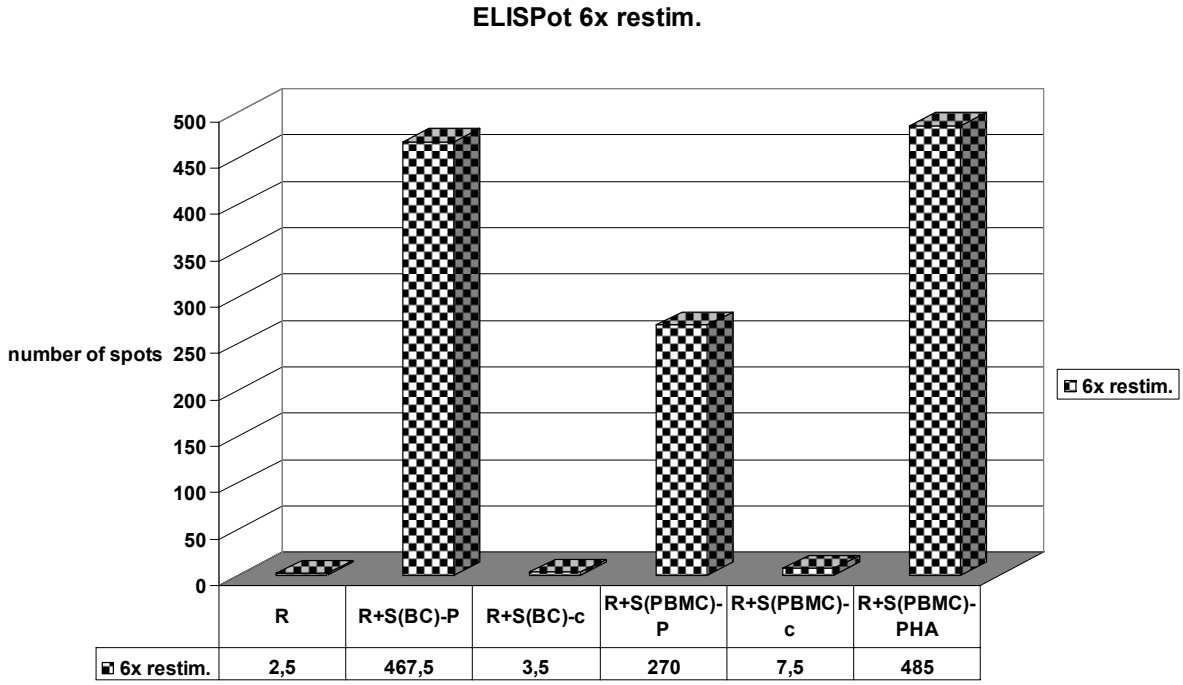
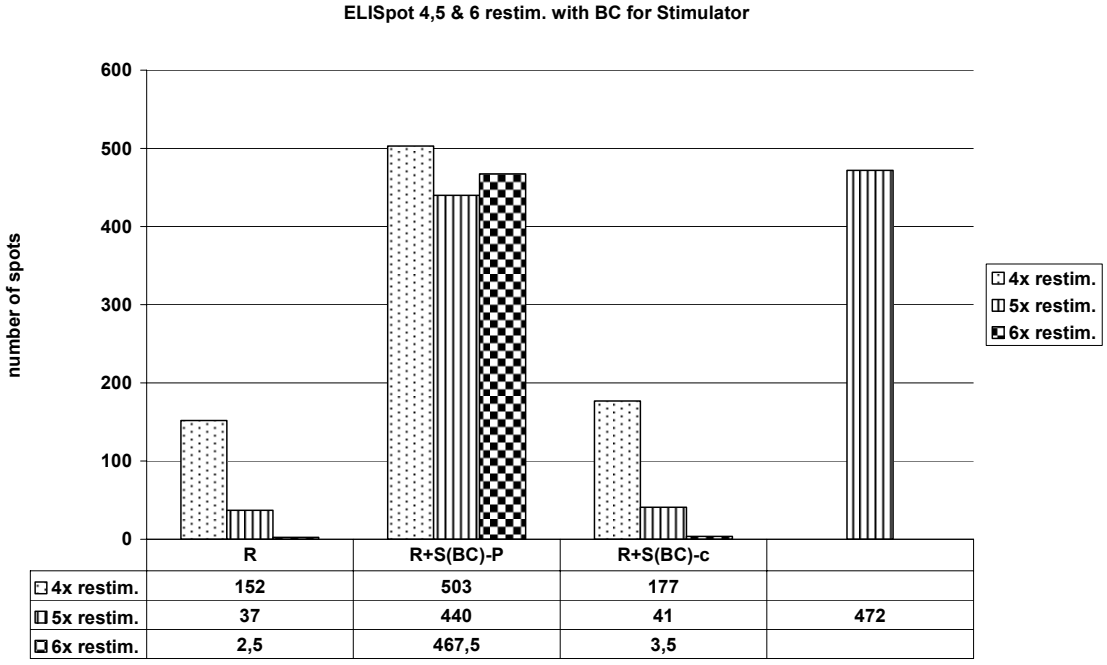


Figure 9. Results of ELISpot assay after the sixth restimulation showing number of IFN-γ spots produced by unstimulated T cells [R] or restimulated with BC loaded with HO-1 peptide [R+S(BC)-P] and BC pulsed with an irrelevant control peptide [R+S(BC)-c]; restimulated with PBMC with addition of HO-1 peptide [R+S(PBMC)-P] or of an irrelevant control peptide [R+S(PBMC)-c] and with the mitogen PHA as a strong positive control [R+S(PBMC)-PHA].

Further, we analysed the effect of repeated stimulations on specific peptide recognition by T-cell lines. No significant changes in the number of IFN-γ-producing T cells were observed after the fourth, fifth and sixth rounds of re-stimulation by either autologous PBMC or HLA-B8 positive B-cell line pulsed

with HO-1 peptide (Fig. 10, 11). However a greater numbers of spots were noticed at the 4th re-stimulation in un-stimulated responder cells or when cultivated with stimulator cells without any peptide added or loaded with the control peptide compared to the next restimulations. So, in order to better assess the level of the specific IFN-γ production by the HO-1 generated T-cell lines the background values of the irrelevant peptide-challenged cells were subtracted from the values of the relevant peptide-challenged cells. Using this approach a trend for increase of IFN-γ-producing T cells was observed after several rounds of re-stimulation (from 4th to 8th re-stimulation) when responder cells were stimulated by either B-cell line cells or autologous PBMC pulsed with HO-1 peptide (Table 1). In addition, higher values of IFN-γ-producing T cells (spot numbers) were observed when re-stimulation was with B cells in comparison to re-stimulation with PBMC, but the difference did not reach statistical significance (t=2,23; p>0,05).



	R	R+S(BC)-P	R+S(BC)-c	
4x restim.	152	503	177	
5x restim.	37	440	41	472
6x restim.	2,5	467,5	3,5	

Figure 10: Overview of the results of ELISpot assay for HO-1-specific T cells (R) challenged with peptide-pulsed HLA-B8 positive B-cell line (S) after the 4, 5 and 6 re-stimulation; (P - HO-1 peptide; c - control peptide, PHA - positive control).

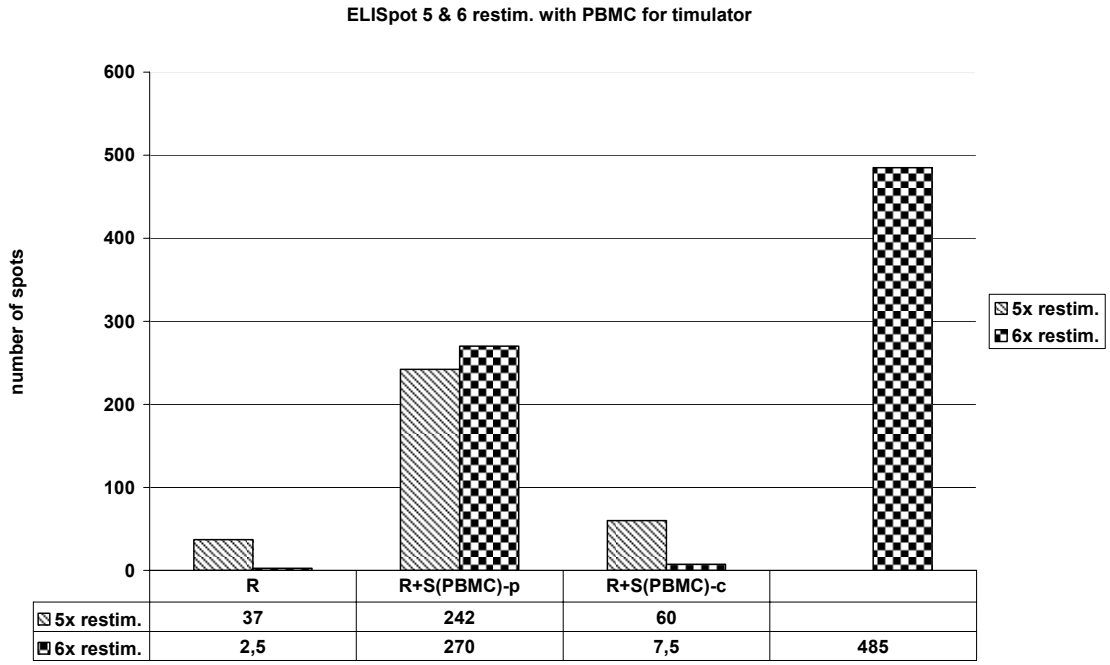


Figure 11: Overview of the results of ELISpot assay for HO-1 specific T cells (R) challenged with peptide-pulsed autologous PBMC (S) after the 5 and 6 re-stimulation; (P - HO-1 peptide; c - control peptide; PHA - positive control).

Table1. Number of specific IFN-γ-producing T cells after several rounds of re-stimulation

Restimulation	[R+S(BC)-P] – [R+S(BC)-c)	[R+S(PBMC)-P] - [R+S(PBMC)-c]
4	326	n.d.
5	399	182
6	464	263
8	518	445
X ± σ	426.8 ± 82.9	298.0 ± 136.9

R – responder T cells; S – stimulator cells; BC – B cells; P – HO-1 peptide; c – irrelevant control peptide ; R+S(BC)-P - T cells re-stimulated with BC loaded with HO-1 peptide; R+S(BC)-c - T cells re-stimulated with BC pulsed with

control peptide; R+S(PBMC)-P - T cells re-stimulated with autologous PBMC with addition of HO-1 peptide; R+S(PBMC)-c - T cells re-stimulated with PBMC with addition of control peptide.

Further, we assessed HO-1 specificity data using a peptide-specificity index (pSI) as described by Müller et al. (122):

$$PSI = \frac{(R+S+HO\text{-}1) - R}{(R+S+c) - R}$$

where R is the responder, S the stimulator, HO-1 indicates the presence of HO-1 peptide and c indicates the presence of control peptide. As a whole, our results revealed an increase in the degree of HO-1 specificity with increasing numbers of restimulating cycles. For example, the ELISA results expressed as a pSI were 0.06 after the first restimulation, 1.01 after the second and 2.5 after the third (Fig. 5). For ELISpot assay the pSI of the T-cell line challenged with autologous PBMC was 8.9 after the fifth restimulation and increase to 53.5 after the sixth (Fig. 11). The pSI for T-cell stimulation with MGAR B cells was 14.0 after the fourth, 100.8 after the fifth and reached a value of 465 after the sixth restimulation (Fig.10).

4. Evaluation of antigen-specific cytolytic activity of the generated peptide-specific T-cell line by CD107a mobilisation assay.

In order to investigate the cytolytic activity of HO-1-specific T-cell lines, the CD107a mobilisation assay was performed. In addition to CD107a staining, cells were also labelled with anti-CD3 and anti-CD8 antibodies and the CD8+ population gated. This flow cytometric analysis enables direct identification and quantification of the CD3+CD8+ potentially cytotoxic population. Thus, the CD107a mobilisation assay is a surrogate for direct cytotoxicity assays, and provides a quantitative value as well as information on effector cell function. In this assay HLA-matched B cells (MGAR) pulsed with either the relevant or irrelevant HLA-B8 binding peptides were used as target cells. Figure 12 shows

that a T-cell line, sensitised with HO-1-pulsed autologous DCs and restimulated four times, demonstrates peptide-specific cytotoxicity when challenged with B cells pulsed with the relevant HO-1 peptide. In this example, 29% of the CD3+CD8+ T-cell population (where the CD8+ fraction represents 74% of the gated lymphocyte population) degranulated upon challenge and hence shows cytotoxicity against B cells pulsed with HO-1 peptide, compared to 11% when the B cells presented a control HLA-B8 binding peptide.

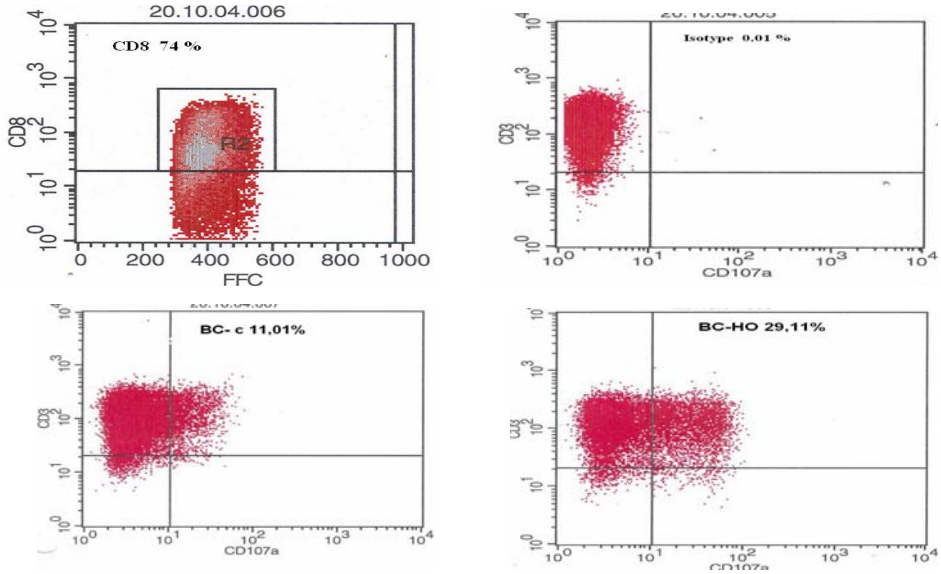


Figure 12: CD107a degranulation assay with HO-1 specific CTL, challenged with peptide -pulsed HLA-B8 positive B-cells (MGAR) after the 4th restimulation.

Degranulation of CD3+CD8+ T-cells in an HO-1 specific T-cell line on challenge with B cells pulsed with the relevant HO-1 peptide was determined by staining for CD107a surface expression. HO-1 specific T cells, used as effector cells, and HLA-B8-positive B-cell line (MGAR) used as target cells pulsed with HO-1 peptide or with a control HLA-B8-restricted peptide (c) were co-cultured at a 1:1 ratio in the presence of either anti-CD107a-FITC antibody or the isotype control for 5 hours at 37°C. Following washing, they were stained with anti-CD3-PE and anti-CD8-PerCP mAbs for 30 min. at 4°C. CD8+ cells were gated along with the FSC/SSC lymphocyte gate. Quadrants were set using the FITC-isotype control.

The CD107a degranulation assay after the 5th restimulation is shown in Figure 13 and after the 7th restimulation in Figure 14 In this example, around 53% of the CD3+CD8+ T-cell population after the 5th and the same number after the 7th restimulation degranulated on challenge and hence exhibit cytotoxicity against B cells pulsed with HO-1 peptide, compared to 10% after the 5th and 1% after the 7th restimulation when the B cells present a control HLA-B8 binding peptide. In this experiment, of the gated lymphocyte population the lines contained 95% CD8+ cells after the 5th and 97% after the 7th restimulation.

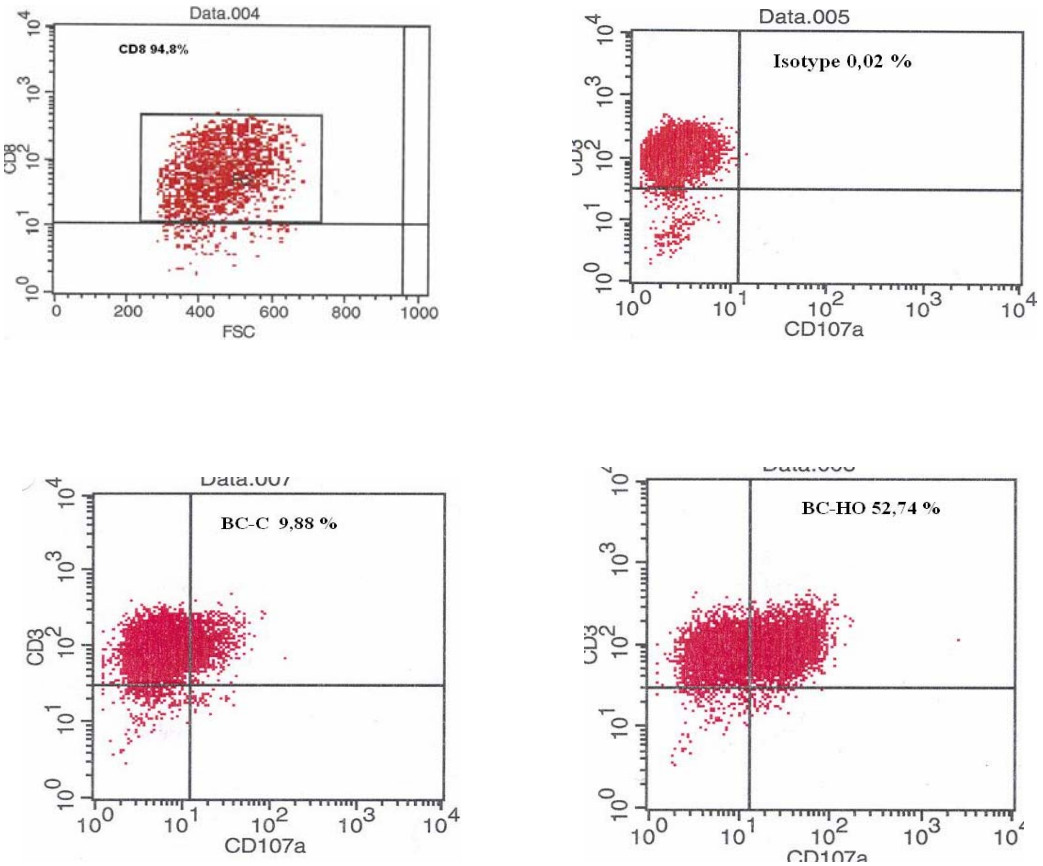


Figure 13: CD107a degranulation assay with HO-1 specific CTL, challenged with peptide-pulsed HLA-B8 positive B cells (MGAR) after the 5th restimulation.

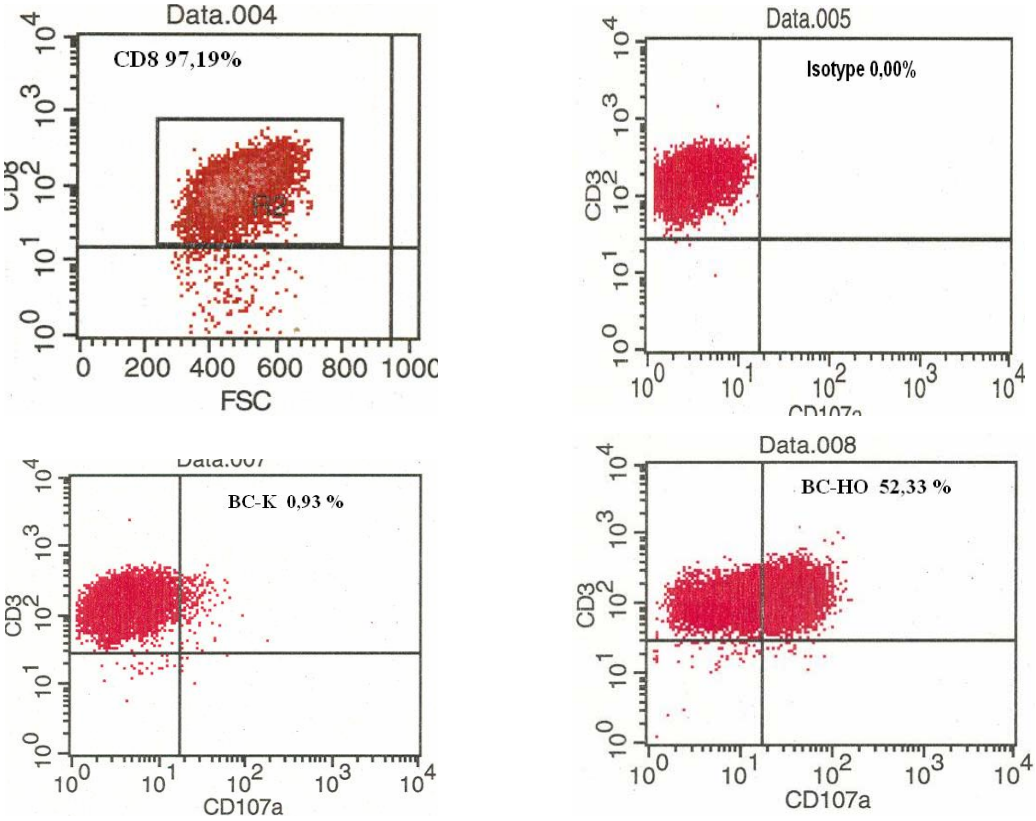


Figure 14: CD107a degranulation assay with HO-1 specific CTL, challenged with peptide-pulsed HLA-B8 positive B cells (MGAR) after the 7th restimulation.

Thus, an increase of the CD8+ cell fraction within CD3+ T-cell population as well as an enhancement of the cytolytic activity (CD107a expression) was observed following every round of peptide-specific restimulation (Table 2). However, the recognition of B cells pulsed with the irrelevant control HLA-B8-binding peptide resulted in a higher level of cytotoxicity after the fourth and fifth restimulations than one would expect. In addition, higher levels of CD107a expression by effector cells only was also noticed after the fourth restimulation. Taking into consideration this observation, the background values of unstimulated CD107a-expressing CD8+ cells and the values for the recognition of B cells pulsed with a control peptide were subtracted from the values for the recognition of B cells loaded with the HO-1 peptide to give the fraction of T cells exhibiting peptide-specific recognition. As a result of this approach, the

increase of the percentage of CD8+ T cells mediating a cytotoxic response towards the peptide-pulsed B cells after several rounds of restimulation was more evident (4th restimulation – 13.8%; 5th restimulation – 42.8%; 7th restimulation – 49.2%).

Table 2. CD107a degranulation assay after repeated restimulations

Restimulation	Isotype control (%)	CD8+ cells (%)	CD107a expressing cells (%)			
			Effectors (Es) only	Es + BC-c	Es + BC-HO1	Es + BC-HO1 (background subtracted)
4	0.01	74.0	15.34	11.01	29.11	13.8
5	0.02	94.8	0.82	9.88	52.7	42.8
7	0	97.2	2.18	0.93	52.33	49.2

Es – effector T cells; BC-c – B cells pulsed with irrelevant control peptide; BC-HO1 – B cells loaded with HO-1 peptide

Although this assay does not actually measure the direct killing of the target cell population, it is assumed that once CTLs are in contact with their target cells and are activated resulting in the release of the contents of their cytotoxic granules, the target cell will undergo the process of cell death. So, the CD107a degranulation assay applied to analyse the HO-1 sensitised T-cell line demonstrates that this T-cell line can mediate cytotoxicity towards target cells presenting this peptide.

4. Discussion

If we type “tumour antigen” as a key word in August 2009, we could easily find more than 80,000 publications and 9,500 reviews listed in the Pub Med database. Therefore studies on identification of tumour antigens, their immunogenicity and antigen–specific immunotherapies have made significant progress and entered the mainstream of current immunological research and cancer research (41, 72, 89, 125, 126).

So far, the identification of such peptides has been hindered by the presence of only a small number of tumour-specific peptides amongst several thousands of different MHC-peptides on each tumour cell. Further, the identification of human tumour T-cell antigens has to a great extent relied on the use of specific T lymphocytes recognizing the tumour, either based on a gene expression approach (16) or on the mass spectrometric sequencing of isolated MHC-peptides (75). At the time of writing, the public SYFPEITHI database (www.syfpeithi.de) lists about 200 identified human MHC class I peptides, classified as “cancer-related”. Most of these peptides were identified by experiments based on their recognition by tumour-specific T lymphocytes (127). The general drawback of using T cells as a primary screening tool is the limitation in the generation and expansion of different T-cell clones from an individual cancer patient. An alternative T cell-independent approach is based on epitope prediction from known tumour antigens and subsequent screening for the predicted MHC-peptides by LC-MS (128). However, due to the heterogeneity of tumours, the choice of the appropriate tumour antigen for the prediction step is difficult and in some ways arbitrary. To address this, improvement of this method on the basis of differential gene expression profiling between cancerous and normal tissue of individual cancer patients has been proposed. Genes overexpressed in the tumour tissue then form the basis for searching for predicted MHC-peptides by LC-MS in the tumour MHC-peptide pool (129). However, the presence of the messenger RNA does not reliably predict presentation of an MHC-peptide on the cell surface, due to the involvement of many variables including translation, post-translational modifications and MHC-class I processing. Additionally, the repertoire of presented MHC-peptides on the cell surface becomes even more complex because certain MHC-peptides are spliced before their presentation, as recently described for epitopes from an RCC (130) and a melanoma cell line (131). Thus, the most promising differential approach to examine differences between cancer and benign tissue is probably performed at the MHC-peptide level, as this is the molecular key to recognition by tumour-specific T cells (132). This approach led to the identification of two MHC-peptides (from beta-catenin and

ribosomal protein L24), which were approximately 3-fold over-presented in the cancerous tissue. Using this approach Flad and coworkers (121) compared the dominant MHC-peptides of a renal cell carcinoma biopsy with healthy kidney tissue from the same patient after nephrectomy using three different mass spectrometric techniques. Two dominant peptides of monoisotopic masses ((M+H)+) 973.43 u and 967.59 u were found to be present exclusively in the tumour sample. One of these was identified as deriving from heme oxygenase, a protein involved in induction of apoptosis resistance, immunosuppression and neoangiogenesis and reported to be up-regulated in various cancer types (133, 134). Heme oxygenase (HO) made its debut onto the scientific stage in 1964, when Wise and coworkers first demonstrated the in vitro degradation of heme to biliverdin (135). Thus, heme oxygenase catalyzes the rate-limiting step in heme degradation, resulting in the formation of iron, carbon monoxide, and biliverdin, the latter of which is subsequently converted to bilirubin by biliverdin reductase. Recent attention has focused on the biological effects of product(s) of this enzymatic reaction, which have important antioxidant, anti-inflammatory, anti-apoptotic, anti-proliferative as well as cytoprotective functions and to protect against diseases as diverse as atherosclerosis and sepsis. Two major isoforms of the HO enzyme have been described: an inducible isoform, HO-1, discovered in the late 1980s and expressed under physiological conditions in the liver and spleen; and the constitutively expressed isoform, HO-2, which is mainly expressed in brain and testis. A third isoform, HO-3, closely related to HO-2, has also been described, whose mRNA has been detected in many organs including spleen, thymus, liver, prostate, heart, brain, kidney and testis (136). Several stimuli implicated in the pathogenesis of renal injury, such as heme, nitric oxide, growth factors, angiotensin II, cytokines, and nephrotoxins, induce HO-1. Induction of HO-1 occurs as an adaptive and beneficial response to these stimuli, as demonstrated by studies in renal and non-renal disease states (133,137)**.** Remarkably, the activity of this enzyme results in profound changes in cells' abilities to protect themselves against oxidative injury (138).

The sequence of the peptide isolated by Flad et al. (121) from RCC was determined here in Tübingen to be APLLRWVL originating from the protein HO-

1 (Swiss-Prot: P09601) positions 265-272. This peptide showed a binding motif for HLA B*08, one of the HLA alleles of the patient. For a peptide to have any potential clinical application in anti-cancer immunotherapy, it is important to determine if it can be used to raise an immune response. It is first pertinent to show this in an in vitro setting, in which the potential effect of a suppressive host environment induced by the tumour can be eliminated. In addition, these experiments should be initially performed with healthy donor-derived PBMC as the antigen-presentation capacity may be reduced in cancer patients. Studies have shown that in vitro generated monocyte-derived DCs from CML patients have a reduced capacity to stimulate T cells (139) and an impaired capacity to migrate, capture, and process antigen (140). So, the purpose of the present study was to test the immunogenicity of the isolated HO-1 derived peptide and to attempt to generate a specific T-cell population that could recognize HO-1-derived epitopes.

To address the immunogenicity of the isolated HO-1 derived peptide, we performed in vitro T-cell sensitisation experiments. The first step was to stimulate PBMC from HLA-B*08-positive healthy donors with a synthetic HO-1 peptide as described for several rounds of restimulation. The next step was to determine if the peptide is immunogenic in healthy donors and peptide-specific T-cell lines were generated following sensitisation experiments in vitro. To establish whether the generated T-cell lines could specifically recognize the HO-1 peptide, specific cytokine release by T cells and cytotoxic activity were determined following several re-challenges with autologous PBMC or B-cell line cells pulsed with either the relevant peptide (APLLRWVL) or an irrelevant HLA-B8–binding ribosomal protein-derived peptide (YLKVKGNVF). For evaluation of cytokine secretion, IFN-γ and GM-CSF were chosen as these cytokines are produced by both CD8+ CTLs as well as T-helper cells of the Th1 phenotype after activation. In addition, it is well known that IFN-γ is an essential mediator of an effector T-cell response. Although the effect of GM-CSF on T cell function is still under investigation, it is suggested that specific subpopulations of activated T cells produce significant amount of GM-CSF, which is critical for the

function of T cells (141). In addition, the T cell derived GM-CSF can further enhance the function of APC (141).

Using ELISA, increased secretion of IFN-γ and GM-CSF by the T cells sensitised to the HO-1 peptide was demonstrated after the third re-stimulation in contrast to the lower levels of cytokines released by T cells in non-stimulated samples or after re-stimulation by autologous PBMC in the presence or absence of control peptide. To confirm these results we decided to assess the peptide-specific T-cell response with a more sensitive assay than ELISA such as ELISpot. ELISpot assays employ a technique very similar to the sandwich ELISA (142). The advantage of the ELISpot is that this technique allows visualization of a secretory product of individual activated or responding cells. Each spot that develops in this assay represents a single reactive cell. Thus, the ELISpot assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information. With detection levels that can be as low as one cell in 100,000, the ELISpot is one of the most sensitive cellular assays available. In fact, ELISpot displays a similar sensitivity as an RT-PCR but detects the secreted protein instead of the mRNA. For cytokine analyses this is advantageous since many cytokines are translationally regulated. ELISpot analyses are also less impaired by binding proteins and protease activity, because the analyte is bound to the capture antibody immediately after secretion. Due to its high sensitivity, the ELISpot has proven particularly useful when studying small populations of active cells such as those regularly found in specific immune responses. As for ELISA, IFN-γ production was chosen for an analyte in the ELISpot since this cytokine secreted by activated CTL and Th1 polarized T cells has a crucial role in anti-tumour immunity. In this assay, high numbers of IFN-γ-secreting T cells cultured with autologous PBMC or the HLA-B8-positive B-cell line (MGAR) in the presence of HO-1-derived peptide were observed. Furthermore, only low numbers of cytokine-producing T cells were found in non-stimulated samples or after restimulation by autologous PBMC or HLA-matched B-cells in the presence or absence of control peptide. These findings clearly reveal a peptide-specific immune response and in vitro immunogenicity of the HO-1 peptide. In addition,

a trend for increase in the numbers of IFN-γ-producing T cells was observed from the fourth to eight re-stimulation by both autologous PBMC and B-cell line cells pulsed with HO-1 peptide. It should be noted that higher values of IFN-γ-producing cells were observed by stimulation with B cells in comparison to autologous PBMC pulsed with the relevant peptide. Thus, autologous PBMC stimulated T cells to an appreciable extent, but always at a lower level than HLA-B8-positive B cells. These data suggest that MGAR B cells may have a greater capacity to present antigen and to stimulate T cells. Results for IFN-γ release in terms of a pSI revealed an increase in the degree of HO-1 specificity with increasing numbers of restimulating cycles. The most impressive were the data for B-cell line as stimulator where the pSI increased almost 30-fold from the fourth to the sixth restimulation. This probably reflects enrichment of HO-1-specific T cells in the line during repetitive restimulation.

To our knowledge, with the exception of our publication (121) there are few studies reporting the use ELISpot assay for testing of HO-1 (HO212 QLFEELQEL) peptide–specific IFN-γ production by circulating T cells from HLA-A2–positive cancer patients (143). Unfortunately, the authors have not been able to expand HO-1–specific T cells successfully in vitro.

We could conclude from this part of the study that using both ELISA and a more sensitive ELISpot assay we established high HO-1-specific cytokine production by the generated peptide-specific T-cell lines. The specific cytokine-producing cells appear after multiple rounds of re-stimulation. Similar observations were reported by other authors (123, 144, 145). In addition, ELISpot assay demonstrate stable HO-1-specific IFN-γ release and indicate that the established T-cell lines have not lost their peptide specificity following multiple rounds of re-stimulation.

An important goal of the present study was to evaluate the antigen-specific cytolytic activity of the generated peptide-specific CTL line using CD107a flow cytometric assay for degranulation. This assay is based on the principle that CTLs mediate cytotoxicity through the release of cytolytic molecules perforin and granzyme-B. The LAMP-1 (CD107a) molecule contained within the membrane of the cytolytic granules is transiently expressed on the cell surface.

The exogenous addition of an anti-CD107a antibody at the point of T-cell challenge with target cells enables the fluorescently labelled monoclonal antibody to bind to CD107a as it is incorporated into the plasma membrane and the cells remain labelled as the molecule is internalised. Degranulation is a relatively rapid event and after T-cell activation CD107a becomes detectable on the cells surface within 30 minutes (146), enabling a fast and simple surrogate method for determining cytotoxicity. In addition, this assay is able to quantify the CD3+CD8+ cytotoxic population thus providing quantitative values as well as information on effector cell function. So, we performed CD107a flow cytometry test in order to monitor the cytotoxic activity of HO-1 specific T-cell lines. HLA-matched B-cell line (MGAR) pulsed with either the relevant or irrelevant HLA-B8 binding-peptide was used as target cells. Our data show that a higher proportion of the CD3+CD8+ T-cell population degranulates upon challenge and hence exhibits cytotoxicity against B cells pulsed with HO-1 peptide, compared to the B cells presenting a control HLA-B8 binding peptide. Thus, we demonstrated that T-cell line sensitised with HO-1 pulsed autologous DCs and restimulated several times shows peptide-specific cytotoxicity when challenged with B cells pulsed with the relevant HO-1 peptide. However, we observed higher background levels of CD107a expression than would be expected in the un-challenged CTL and for effector cells in the presence of target cells pulsed with the irrelevant control peptide at the 4th restimulation. Moreover, similar data were obtained for IFN-γ production. Summarizing these results we conclude that a background degree of cytotoxicity and cytokine release may be observed in responder cells alone and after challenge with APC pulsed with an irrelevant peptide. Our results are consistent with the observations of other investigators assessing the immunogenicity of WT1 peptide (122,123). These observations are difficult to be explained. It could be speculated that a small population of other T-cell clones with unknown specificity may expand as „let-over“ activity from the previous stimulation On the other hand a peptide-independent stimulation by APC pulsed with a control peptide could not be excluded. Another explanation could be that the presence of CTL populations that recognise target cells pulsed with the control peptide or release cytokines

after challenge with APC pulsed with an irrelevant peptide may result via a process of cross-recognition. Finally, the significant decrease of these peptide-unspecific cell populations after subsequent repeated peptide-specific stimulations may be related to overgrowth of these small cell subsets by the specific T-cell clone.

Although CD107a mobilisation assay does not actually measure the direct killing of the target cell population, it is assumed that once CTLs are in contact with their target cells and are activated resulting in the release of the contents of their cytotoxic granules, the target cell will undergo the process of cell death. So, the CD107a degranulation assay applied to analyse the HO-1 sensitised T-cell line demonstrates that this T-cell line can mediate cytotoxicity towards target cells presenting this peptide. In addition, we observed a high proportion of the CD8+ fraction (% of the gated CD3+ population) in the T-cell line sensitised with HO-1-pulsed autologous DCs and an increase of this percentage from the fourth to seventh restimulation (from 74% to 97%), suggesting a high enrichment of the CD8+ T-cell population after several rounds of peptide-specific restimulation. Thus, our experiments indicate that the capacity of HO-1 specific T-cell line to mediate cytotoxic response toward target cells pulsed with that peptide is greatly increased after repeated re-stimulation and correlates with the increase of CD8+ T-cell subset.

Comparing our results from ELISA, ELISpot and CD107a degranulation assays used in this study for assessing the HO-1 specificity of the generated T-cell lines it could be concluded that:

- HO-1-specific cytokine production by T cells could be detected after the third stimulation and increased after several rounds of re-stimulation of PBMC from healthy HLA-B8-positive donors by both MGAR B-cell line cells and autologous PBMC pulsed with HO-1 tumour-derived peptide with the sequence APLLRWVL;
- The specific IFN-γ production remained high and stable after repeated stimulations;

- The specific CD107a degranulation assay demonstrated that a high proportion of the T cells which recognize HO-1 peptide in the context of HLA-B*08 and are present following several restimulations are CTL;
- An enrichment of the CD8-positive T-cell subset at every round of peptide-specific re-stimulation was documented.

Together, these data provide evidence that the above-mentioned peptide could be considered as a new target for tumour immunotherapy.
The concept of T-cell mediated cancer therapy is based on selective tumour destruction in the absence of the systemic toxicity that is associated with conventional cancer treatments. Currently, three main approaches in the immunotherapy of human solid cancers can be distinguished: non-specific immunomodulation, active immunization (cancer vaccines) and adoptive cell transfer. The non-specific immunomodulation is mainly based on the administration of the T-cell growth factor IL-2 in order to activate endogenous tumour-reactive cells in vivo which in turn to cause cancer regression. More recently, antibody mediated blockade of a cell surface inhibitory molecule (CTLA4), has resulted in objective clinical responses in 10–20% of patients with metastatic melanoma or renal cancer (147, 148). Investigations are underway to evaluate other general immune modulators such IL-15, anti-TGFβ and anti-PD-1 antibodies (31). Active immunization approaches are based on immunizing cancer patients against their autologous tumours using either whole cells, proteins, peptides or a wide variety of immunizing vectors. Unfortunately, cancer vaccines aiming to induce a therapeutic response by the patient's immune system have so far met with little success. Moreover, results of a number of larger trials in the past few years have indicated that vaccination therapy can also have a detrimental effect and be associated with worse outcome (149). The adoptive cell transfer-based immunotherapies involve the administration of large numbers of T cells that have been activated ex vivo to exhibit antitumour effector functions (39). While adoptive regimens can ensure the availability of highly selected cells with high avidity for tumour antigens, the cancer bearing host does not offer a supportive environment for adoptively

transferred T cells. Conditioning the host before adoptive transfer to promote the survival and function of transferred T cells represents key step in improving the efficacy of adoptive cell transfer (31, 39). Studies of adoptive cell therapies have clearly demonstrated that the administration of highly avid anti-tumour T cells directed against a suitable target can mediate the regression of large, vascularized, metastatic cancers in humans (31). A need exists for the generation of T cells with broad reactivity against shared cancer-associated antigens present on multiple tumour types. Thus, many proteins that are aberrantly expressed or overexpressed by tumours in comparison with the normal tissue counterparts might be candidate targets for T cell therapy. In this context, although HO-1 is expressed at low levels in some normal tissues and its expression could be enhanced by stress-inducible stimuli (150), overexpression of this protein in a constitutive manner has been also reported in solid tumours (151-156) and in leukemic cells (138, 157). In this investigation we demonstrated that an HLA-B-restricted HO-1-derived peptide, isolated exclusively from tumour tissue of a patient with RCC, exhibits immunogenicity in vitro in PBMC from HLA-matched healthy donor and specific CTLs were generated. Although it was hypothesized that HO-1 may have important roles in tumour progression and formation of metastases as well as resistance to anticancer therapy (158,159), the data obtained in this study encourage us to consider that the above mentioned peptide may be of interest as a potential tumour-specific T-cell target epitope. However, further investigations would be necessary to support this hypothesis.

As it has been already mentioned, adoptive cell therapy is a treatment that uses a cancer patient's own T lymphocytes with anti-tumour activity, expanded ex vivo and reinfused into the patient with cancer (31). With few notable exceptions, adoptive T cell therapy of human cancer has focused on the generation of a tumour-reactive repertoire of CD8+ T cells (38). Since in this study we succeeded in generating HO-1-specific CTLs from a healthy donor, it will be pertinent to test whether patients' PBMC can be sensitized by the HO-1-derived peptide in vitro. Further, attempts should be made to isolate HO-1-specific T cells from patients and to test their activity and phenotype in ex vivo

experiments. If the specific CTLs are exhausted the adoptive transfer of ex vivo activated and expanded T cells may be a promising therapeutic approach (160). More recently it has been reported (143) by in vitro experiments that CD8+ T cells, specific for a single epitope from HO-1 (an HLA-A2–restricted peptide epitope HO212) and isolated from PBLs from patients with MM, RCC and breast cancer, inhibited the antigen-specific cytokine release, proliferation, and cytotoxicity of T cells. These HO-1–specific T cells appear to have a rather naive or central memory-like phenotype and may represent a new subset of regulatory T cells (Tregs). It is likely that the same antigenic peptides can stimulate either effector cells or Tregs depending on antigen dose, peptide-MHC avidity, and presence of costimulatory molecules and cytokines (161, 162). Such antigens may be proteins that are expressed in the late phase of inflammatory reactions to inhibit or terminate inflammation, such as HO-1 (143).

It will be also important to test whether HO-1-reactive T cells may recognize RCC as it is often reported that peptide-specific lines do not recognize native tumour and that the infusion of large numbers of T cells or T-cell clones specific for TAA failed to completely eradicate tumours in the majority of patients (38). The later observation could be in part due to the short persistence of the transferred T cells in vivo. In addition, the results from mouse and human adoptive cell therapies revealed that the relative sensitivity of a T cell to antigen influences its ability to recognize tumours, and that high avidity T cells are required for efficient antitumour immunity (163). TCR affinity and other factors (co-receptor molecules, transcription factors, etc.) may also influence on the recognition of T-cell targets (163).

In summary, the results obtained in this study with the synthetic HO-1 peptide (APLLRWVL) indicate that this peptide is immunogenic in the context of HLA-B*08 and gives rise to peptide-specific T cells. Using a supplementation of priming cultures with cytokines (GM-CSF and IL-4) known to be important growth and differentiation factors for DCs allowed successful generation of HO-1-specific T-cell lines from HLA-matched healthy donors. The T cells obtained under such cytokine-modified priming conditions (121, 122) showed high-level cytokine release on re-challenge with synthetic HO-1 peptide. In addition, using

autologous DC in T-cell sensitization experiment, higher proportion of HO-1-specific CTLs was generated. It could be speculated that the HO-1-derived peptide APLLRWVL may be used for in vitro generation of CTLs, which may be considered as a potential adoptive cell therapy for tumours expressing the HO-1 peptide.

5. Abstract

Studies to identify tumour antigens, test their immunogenicity and develop antigen–specific immunotherapies have made significant progress and entered the mainstream of current immunological research and cancer research over the last decade. So far, the identification of such peptides has been hindered by the presence of only a small number of tumour-specific peptides amongst several thousands of different MHC-peptides on each tumour cell. The identification of human tumour T-cell antigens has to a great extent relied on the use of specific T lymphocytes recognizing the tumour, either based on a gene expression approach or on mass spectrometric sequencing of isolated MHC-peptides. At the time of writing, the public SYFPEITHI database (www.syfpeithi.de) lists about 200 identified human MHC-class I peptides, classified as “cancer-related”.

Heme oxygenase-1 (HO-1) is an inducible stress protein and catalyzes the degradation of heme to carbon monoxide (CO), biliverdin and iron. More importantly, it is now well known that expression of high levels of HO-1 occurs in various tumours, and that HO-1 has an important role in rapid tumour growth because of its antioxidative and antiapoptotic effects. HO-1 was thus considered to be a key molecule for tumours to resist attack by host immunity or treatment by chemotherapy and radiotherapy by protecting the tumour cells from oxidative insults. All these effects taken together suggest a potential association of HO-1 with cancer. Recently, two dominant peptides of monoisotopic masses ((M+H)+) 973.43 u and 967.59 u were found to be present exclusively in the tumour sample from a patient with renal cell carcinoma. One of them was identified as originating from HO-1 and the sequence of this peptide was determined to be APLLRWVL.

The purpose of the present study was to test the immunogenicity of the isolated HO-1 derived peptide and to attempt to generate a specific T-cell population that could recognize HO-1-derived epitopes.

To accomplish this goal the following tasks were set at the beginning of the investigation and have been successfully achieved:

5. To generate specific T-cell lines from PBMC of healthy donors, using synthetic HO-1 peptide.
6. To screen the established T-cell lines for HO-1-specific cytokine release.
7. To measure antigen-specific IFN-γ production by T lymphocytes at the single cell level.
8. To evaluate the antigen-specific cytolytic activity of the generated peptide-specific CTL line.

The results obtained in this study with the synthetic HO-1 peptide (APLLRWVL) indicate that this peptide is immunogenic in the context of HLA-B*08 and gives rise to peptide-specific T cells. Using a supplementation of priming cultures with cytokines (GM-CSF and IL-4) known to be important growth and differentiation factors for DCs allowed successful generation of HO-1-specific T-cell lines from HLA-matched healthy donors. The T cells obtained under such cytokine-modified priming conditions (121, 122) showed high-level cytokine release on re-challenge with synthetic HO-1 peptide. In addition, using autologous DC in T-cell sensitization experiment, higher proportion of HO-1-specific CTLs was generated. It could be speculated that the HO-1-derived peptide APLLRWVL may be used for in vitro generation of CTLs, which may be considered as a potential adoptive cell therapy for tumours expressing the HO-1 peptide.

6. Zusammenfassung

Studien, die Tumor-Antigene identifizieren, ihre Immunogenität testen und antigenspezifische Immuntherapien entwickeln, machten grosse Fortschritte und schufen die Grundlage für die gegenwärtige Immunologie- und Krebsforschung in dem letzten Jahrzehnt. Bis jetzt wurde die Identifikation solcher Peptide von der Präsenz nur geringer Anzahl an tumor-spezifischen Peptiden, unter einigen Tausenden unterschiedlichen MHC-Peptiden auf jeder einzelnen Tumorzelle, verhindert. Die Identifikation menschlicher Tumor T-Zellantigene beruht zum grössten Teil auf der Anwendung spezifischer T-Lymphozyten, welche die Tumorzellen erkennen. Dies kann entweder durch die Vorgehensweise der Genexpression oder durch massenspektrometrische Sequenzierung von isolierten MHC-Peptiden erfolgen. Zur Zeit der Forschungsarbeit hat die öffentliche SYFPEITHI Datenbank (www.syfpeithi.de) ungefähr 200 unidentifizierte menschliche MHC-Klasse-I-Peptide aufgelistet und klassifiziert als „krebsbezogen“.

Hemeoxygenase-1 (HO-1) ist ein induzierbares Stressprotein, welches Häm zu Eisen, Biliverdin und Kohlenstoffmonoxid abbaut. Wichtiger ist, dass hohe Niveaus von HO-1 in unterschiedlichen Tumoren zu finden sind, sowie das HO-1 aufgrund dessen antioxidativen und antiapoptotischen Effekt eine ausschlaggebende Rolle für das schnelle Tumorwachstum hat. HO-1 ist ein Tumorenschlüsselmolekül, weil es sowohl den Angriffen des eigenen Immunsystems, als auch chemo- oder radiotherapeutische Behandlung durch eine Protektion der Tumorzellen vor oxidativem Insult widersteht. Kürzlich wurden zwei dominante Peptide von monoisotopischen Massen ((M+H)+) 973.43 u und 967.59 u ausschliesslich nur in Tumorbiopsie von Patienten mit Nierenzellkarzinom gefunden. Eines von den Peptiden wurde als vom HO-1 abstammend identifiziert und seine Sequenz wurde als APLLRWVL determiniert.

Ziel der vorliegenden Studie war das Testen der Immunogenität des aus dem vom HO-1 stammenden isolierten Peptides sowie der Versuch, eine spezifische

T- Zellpopulation zu generieren, die der HO-1 entstammten Epitopen erkennen kann.

Um dieses Ziel zu erreichen wurden am Anfang der Forschung folgende Aufgaben gesetzt, die erfolgreich erfüllt wurden.

1. Eine spezifische T- Zelllinie aus PBMC von einem gesunden Spender durch Verwendung synthetischer HO-1 Peptide zu generieren.
2. Die Prüfung der HO-1 spezifischen Zytokinfreisetzung der erzeugten T-Zelllinien.
3. Messung der antigenspezifischen IFN-γ Produktion der T- Lymphozyten auf einem einzelnen Zellniveau.
4. Bewertung der antigenspezifischen zytolitischen Aktivität der generierten peptidspezifischen ZTL-Linie.

Die aus dieser Studie gewonnenen Ergebnisse zeigen, dass das synthetische HO-1-Peptid (APLLRWVL) in dem Kontext von HLA B*08 immunogen ist und zur Gründung peptid-spezifischer T- Zellen führt. Hinzufügung von Zytokinen (GM-CSF und IL-4) zu den angesetzten Kulturen, die wichtige Wachstums- und Differenzierungsfaktoren für dendritische Zellen darstellen, erlaubt die erfolgreiche Erzeugung von HO-1 spezifischen T-Zelllinien aus HLA-kompatiblen gesunden Spendern. Die unter solchen zytokinmodifizierten Ansetzungsbedingungen erzeugten T Zellen, haben ein hohes Niveau an Zytokinfreisetzung auf der Restimulierung mit synthetischem HO-1 Peptid aufgezeigt. Zudem führte die Anwendung von autologen dendritischen Zellen im Rahmen des T- Zellsensibilisierungsexperiments zur Generierung eines erhöhten Anteils an HO-1 spezifischen zytotoksischen T-Lymphozyten. Es kann darüber diskutiert werden, ob das abgeleitete HO-1 Peptid APLLRWVL bei der in vitro Generierung zytotoksischer T-Lymphozyten angewendet werden kann. Dies könnte bei Patienten mit Tumoren, welche HO-1 Peptide exprimieren, als eine potentielle adoptive Zelltherapie berücksichtigt werden.

7. List of Abbreviations

APC-	Antigen Presenting Cells
CD-	Cluster of Differentiation
CML-	Chronic Myelogenous Leukemia
CTL-	Cytotoxic T Lymphocytes
DC-	Dendritic Cell
ELISA-	Enzyme- Linked Immunosorbent Assay
ELISpot-	Enzyme- Linked Immunospot
ER-	Endoplasmatic Reticulum
FACS-	Fluorescence Activated Cell Sorter
FCS-	Fetal Calf Serum
FITC-	Fluoresceinisothiocyanat
GM-CSF-	Granulocyte Macrophage- Colony stimulating Factor
HLA-	Human Leucocyte Antigen
IL-	Interleukin
INF-	Interferon
LN-	Lymph Nodes
MHC-	Major Histocompatibility Complex
MICA/B-	MHC class I Chain-related protein A/B
mAb-	monoclonal Antibody
mMDC-	mature Monocyte-derived DC
MLR-	Mixed Lymphocyte Reaction
NK-	Natural Killer
NOD-	Nucleotide-binding Oligomerization Domain
PBMC-	Peripheral Blood Mononuclear Cell
PBL-	Peripheral Blood Lymphocyte
RCC-	Renal Cell Cancer
SEREX-	Serological analysis of Recombinant cDNA Expression libraries
TAA-	Tumour Associated Antigen
Th-	T helper
TGF-	Transforming Growth Factor

TIL- Tumour Infiltrating Lymphocytes

TLR- Toll-Like Receptor

ULBP- UL16- Binding Protein

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Wissenschaftliche Interessen

Molekulare-, Zelluläre- und Tumorimmunologie

Publikationen

Thomas Flad, Ludmila Mueller, Hassan Dihazi, Veneta Grigorova et al**.** T cell epitope definition in by differential massspectrometry : identification of a novel, immunogenic HLA-B8 ligand directly from renal cancer tissue. Proteomics 2006; 6 (1): 364-374.

Sprachkenntnisse

Bulgarisch – Muttersprache
Deutsch – Verhandlungssicher
Englisch – Verhandlungssicher
Italienisch – fließend in Wort und Schrift