UNIVERSITY OF SOUTHAMPTON

CANCER SCIENCES DIVISION

FACULTY OF MEDICINE, HEALTH & BIOLOGICAL SCIENCE

SCHOOL OF MEDICINE

Identification of CD8+ T Cell-stimulating Shared Antigens That Are Uncovered in CT26 Vaccinated Mice in the Absence of CD25+ Regulatory T Cells

By

Ming-Hsin Yeh

Thesis submitted for the degree of Doctor of Philosophy

October, 2005

This work has been supervised by Professor Tim Elliott and Doctor Nick Murray.

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
CANCER SCIENCES

Doctor of Philosophy

By Ming-Hsin Yeh

There is evidence suggesting CD4+CD25+ regulatory T cells may impede generation of effective immunity against tumours as they were involved in maintaining tolerance to self antigen. CT26 tumour cells are rejected in BALB/c mice that have been *in vivo* depleted of CD25 regulatory T cells. In addition, the activated tumour immunity is also effective against tumours of different histological origins, such as A20, BCL1 and RENCA, suggesting that shared murine tumour-associated antigens were unveiled in the absence of CD25+ regulatory T cells. The characterization of such shared antigens would lead to increase in understanding the roles of CD4+CD25+ regulatory T cells in tumour immunity.

The identification of unknown tumour antigens essentially depends on the use of CD8+ T cells as probes for the screening of recombinant tumour cells cDNA libraries. Here, we generated from immunized BALB/c mice two tumour-reactive CD8+ T cell lines, from which, several CTL clones were isolated. These CTL clones were shown to be cross-reactive to several histologically different tumour cells with different H-2^d restriction. We also used somatic hybridization to establish a line of functional hybridoma cells generated by fusion of CTL clone 3 with BWZ36/CD8α cells that carry a construct of NFAT-LacZ gene.

Pools of cDNA from a CT26 tumour-derived cDNA library were transiently transfected into Cos-7 cells to establish an expression cloning system. Taking advantage of TNF/WEHI assay for CTL and CPRG assay for hybridoma cells, the CT26 cDNA libraries were screened for the identification of the CD8+ T cell-reactive tumour antigens.

Here, an endogenous murine leukemia virus (MuLV) *pol/env* antigen was identified as a shared tumour antigen operating in the cross-protection in CT26-vaccinated Treg depleted BALB/c mice.

List of Figures

List of tables

Acknowledgements

I would like to first thank my family, who have supported me and encouraged me along the way of my studies. Thank you to my wife Rita, who cooked delicious dinner for me everyday, smoothed my temper in encountering obstacles in works and prayed for me over the past three years.

I would like to thank Professor Tim Elliott, Dr. Nick Murray, Dr. Denise Golgher, Dr. Catherine Anne Bryant, Christopher, Nasia and all those working at Cancer Sciences Somers Building Southampton for their help and advice, especially Catherine for assisting with my animal experiments and ELISPOT. Thank you to Dr. Ruth French in Tenovus Research Institute Southampton and Dr. van den Eynde in Ludwig Institute for Cancer Research Belgium for giving me really good advice in designing my experiment.

I would like to thank Veteran General Hospital-Kaohsiung Taiwan and Yen Tjing Ling Medical Foundation in Taiwan for the funding of the tuition fee for my PhD study.

I would also like to thank CRUK for the funding they have provided for this project

Abbreviations

2-ME	2-mercapto-ethanol
ADCC	antibody-dependent cell-mediated cytotoxicity
CDC	activating complement-dependent cytotoxicity
CFSE	Carboxy Fluorescein Succinimidyl Ester
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CPRG	ChloroPhenol Red-β-c-Galactopyranoside
ELISA	Enzyme-linked Immunosorbent Assay
Elispot	Enzyme-linked immunospot
ERV	Endogenous retrovirus
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FCS/SSC	Forward scatter / Side scatter
HPV16	Humane papilloma virus type 16-derived
hTERT	Human reverse transcriptase telomerase
ICTM	Iscove's complete tumour media
IFN-γ	Interferon-gamma
IMDM	Iscove's modified Dulbecco's media
mAb	Monoclonal antibody
MACS	Magnetically Assisted Cell Separation
MHC	Major Histocompatibility Complex
MuLV	Murine leukaemia virus
MTT	(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NFAT	Nuclear Factor of Activated T lymphocyte
NF*k*B	nuclear factor Kappa B
NP40	Nonidet P40
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffer Saline
PE	Phycoerythrin
PEG	Polyethylene Glycol
RT-PCR	Reverse transcription-polymerase chain reaction
SAGE	Serial analysis of gene expression
SEREX	Serological analysis of recombinant cDNA expression libraries
TAA	Tumour-associated antigen
TCR	T-cell receptor
TAP	Transporter associated with Antigen Processing
T_H	Helper T ceil
TIL	Tumour-infiltrating lymphocytes
TNF	Tumour necrosis factor
TGF	Transforming growth factor
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor

1.1. General introduction.

Surgery and radiation ablation remain the mainstay for the treatment of primary cancer and are curative for a limited percentage of patients with very early stages of malignancies. Despite early detection of cancer followed by local ablation therapies provides favourable prognosis, some patients would still have locally recurrent tumour or distant metastasis. For this reason, many of the patients with advanced or recurrent tumours are offered with systemic adjuvant therapies, such as chemotherapy or hormone therapy. However, the majority of these patients will, with time, succumb to recurrent disease or metastasis. Alternative approach based on gene therapy and immunotherapy has focused attention in last decades. The potential for treating cancer patients by immunotherapy has held great promise for immunologists and cancer biologists. In addition, current treatment for advanced cancer relies on either chemotherapy or radiotherapy that could kill dividing cells or block cellular division. However, these therapies have severe side effects on normal proliferating and non-proliferating cells in cancer patients resulting in significant morbidity and mortality. On the contrary, by exploiting the naturally occurring defence system, immunotherapy could be a non-toxic method evoking tumour-specific immune responses, even against residual disease that is resistant to conventional anti-neoplastic agents, and favourably preserving most healthy normal cells.

The concept that the immune system can recognize and eliminate primary developing tumour in the absence of external manipulation has existed for over 100 years. The concept was initially developed in 1909 [1]. However, the scientific and medical

attitudes toward the notion of cancer immunotherapy fluctuated from time to time over a century. The validity of this concept has been difficult to establish, because so little was known at that time about the molecular and cellular basis of immunity. It was not until the past decades, with the advancement in knowledge and technologies in immunology and the overwhelming amount of compelling data from animal models as well as from human patients, that the concept of tumour immunosurveillance became more convincing. Over the past ten years, cancer immunotherapy has received renewed attention.

The molecular identification of numerous tumour-associated antigens (TAA) in humans [2] further supported concept that cancer immunosurveillance can occur in patients with cancers and led to new possibilities for the development of effective cancer immunotherapies. Although some tumour-associated antigens derive from mutated genes, most are products of non-mutated genes that encode cellular proteins commonly expressed by autologous cancer cells. Administration of anti-cancer vaccines directed to those newly discovered TAAs has been shown to induce antigen-specific cytotoxic T lymphocyte (CTL) responses and protect from subsequence tumour challenge in some cases [3, 4].

The discovery of the first human tumour antigen recognized by CD8+ CTLs in patient with melanoma [2] has opened new possibilities for the development of effective immunotherapy for patients with cancer. This and other advancement in the knowledge of tumour-specific cellular immune response have triggered enthusiasm for the development of immunotherapeutic regimens against various malignancies. Unlike most vaccines for infectious disease, cancer vaccines are generally therapeutic in intent, involving attempts to activate immune responses against antigens of the

tumour to which the immune system has already been exposed. Various approaches included immunization directed to target the identified common tumour antigenic peptides, proteins, recombinant DNA or RNA alone and in the forms of dendritic cell-based vaccine, DNA vaccine, etc. Other individualized immunotherapies include immunization with autologous tumour cell products or with genetically modified autologous tumour cells; common vaccines in combination with hapten conjugation, and other adjuvant administration; in situ immunization by intratumour injection of dendritic cells, adjuvant or viruses. Cytotoxic T cells reactive against some unknown or known tumour antigens can be generated ex vivo or isolated from tumour-bearing patients and used in adoptive cellular immunotherapy to treat autologous cancer patients, such as in cases of advanced malignant melanoma [5]. In the past years, a wide range of cancer vaccines have been tested and shown promise in animal models. To date, varieties of different vaccine strategies have been or are being evaluated in preclinical and clinical settings for the treatment of various human malignancies (see figure 1.1).

In order to develop efficient immunotherapy for human cancer, it is important to have an better understanding in the following issues: 1) The nature of the immune networks against autologous tumour cells, 2) the identity of tumour antigens and strategies to monitor immune response in patients, 3) the mechanisms that tumour cells utilize to escape from host immune system and the strategies to overcome them, 4) the development of efficient immune interventions to eliminate cancer cells.

However, in current clinical trials, only a limited number of patients with certain types of malignancies (such as malignant melanoma, renal cell carcinoma, EBV-associated lymphoma, and haematological malignancies after allogeneic bone marrow

transplantation) have been observed with significant clinical responses in cancer immunotherapy [6, 7, 8]. Anticancer trial employing immunological approach appears to reach a plateau of results in the clinical setting, and currently no vaccination regimen is considered as a standard therapy. There may be several reasons to explain why the clinical therapeutic manipulation by various cancer vaccine strategies is unable to eradicate tumour cells. First, in most cases, these clinical trials were performed in patients with far advanced metastatic disease or in patients who have been treated with chemotherapy or radiotherapy. In either situation, the patients are frequently associated with a profound immunocompromised status. In addition, the rapid growth and spread of tumours in late stage may overwhelm the capacity of the immune system to eradicate tumour cells. Second, many tumours have specialized mechanisms for evading host immune system. Third and the most important, vast majority of the TAAs described to date are self-antigens or altered self antigens. They may either be ignored by the immune system or fail to provoke a sufficient immunological anti-tumour response in vivo. The latter has been suggested by the observation that, in a large number of cancer patients, in spite of the presence of spontaneously occurring tumour-specific T cell repertoire, these reactive cytotoxic T cells are either not expanded in vivo or anergic to tumour antigens and therefore insufficient to mediate tumour rejection [9].

Recently, a unique naturally occurring lineage of CD4+ T cells that suppress T cell effectors' functions has attracted much attention [10]. These so-called regulatory T cells possess potent suppressive activities in immune responses both in vivo and in vitro [11, 12]. It has long been described that these naturally occurring T regulatory cells are crucial in the maintenance of peripheral tolerance to self antigens and prevention of autoimmune disease. Because most of the TAAs are self antigens or altered self

antigens, it has been suggested that CD4+CD25+ T cells might be involved in the suppression of immune responses to tumour antigens. This notion has been supported by various animal experiments, in which the depletion of CD4+CD25+ T cells could effectively provoke rejection of syngeneic tumour cells transplant in mice [13, 14, 15]. Thus, these and other observations raised the hypothesis that CD4+CD25+ T cells may be involved in tumour escape from the immune surveillance as they are involved in the prevention of autoimmune disease, and that depletion of CD25+ regulatory cells could preserve the immune function against tumours.

Earlier in our laboratory [16], we successfully set up an animal model, in which *in vivo* depletion of CD25+ regulatory T cells in BALB/c mice before immunization with living CT26 tumour cells has induced a long-term immunity against subsequent challenge of parental CT26 tumour and a broad range of other tumours with different histological origins. Surprising, there is no observed or pathological evidence of autoimmune disease found in these immunized mice. Furthermore, it has been shown that both CD8+ and CD4+ T cells are involved in this model of tumour immunity. However, there still are more questions about the relationship between CD25 regulatory T cells and CD8+/CD4+ effectors cells in this and other model of tumour immunosurveillance that need to be elucidated in the future. In the current stage, we are going to follow the immunization procedures in this animal model, aiming to isolate and culture *ex vivo* the emerging tumour-specific CD8+ cytotoxic T cells and use CTL as a probe to define the CD8+ T cell-reactive antigens in this system.

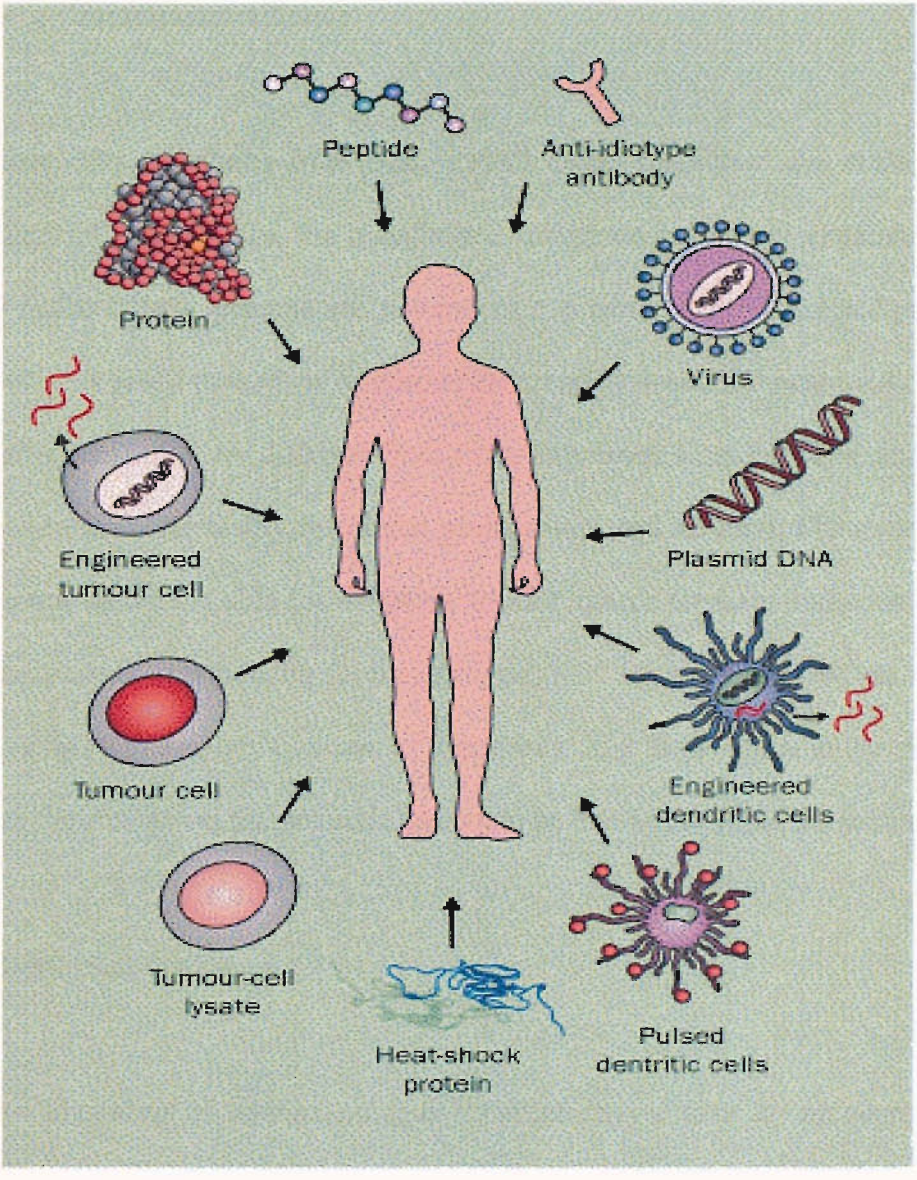


Figure 1.1. Proposed strategies in cancer immunotherapy

Several immunotherapy strategies have been or are being attempted separately to induce antitumour responses in patients (copy from Simone Mocellin, *et al.* Vaccines for solid tumours. *The Lancet Oncology 2004; 5: 681-689*).

1.2. Cancer immunosurveillance

As tumour cells transform from host normal cells, proliferate and metastasis in replacement of normal cells, the transformed malignant cells must experience some changes in the cellular genome and patterns of expression. These changed properties of tumour cells result in the loss of normal differentiation and the expression of some abnormal proteins that are different from those expressed in parent cells, and overall these lead to the abnormality in cellular proliferation and survival. Theoretically, the host immune system is able to recognize any foreign or aberrant antigenic proteins and destroy organisms bearing foreign antigens that are not expressed on host self cells. Taking together, this raises a hypothesis that host immune system does recognize the changes in tumour cells and could be boosted to eliminate tumour cells.

1.2.1. Historical review

The classical concept of vaccination against cancer derived from the pioneer study by a New York City surgeon, Dr. William B. Coley [17], about one hundred years ago. He was interested in the area of cancer immunotherapy stemming from an observation that a cancer patient who had a complete remission of the tumour following two attacks of erysipelas caused by acute infection with bacteria *Streptococcus Pyogenes*. Subsequently, Coley took extracts of pyogenic bacteria, *Streptococcus pyogens*, and injected them into cancer patients to stimulate anti-tumour response [17]. During the next 43 years Coley treated almost 900 cancer patients with this bacterial preparation. In his preliminary report, over 10% of the treated patients were "cured" by this so-called Coley's vaccine. Despite these successes, "Coley's toxin" was not widely accepted by the scientific and clinical communities of the time, possibly due to the

severe site effects, such as high fever, induced by the treatment and the perceived low cure rate. Nevertheless, Coley's early studies led to the use of bacilli Calmette-Guerin (BCG) for cancer immunotherapy, with this treatment being used to the present day as one of the most effective therapies against superficial bladder cancer.

Over the following nearly a hundred years, scientific attitudes toward cancer immunotherapy fluctuated from time to time. The validity of this concept has been difficult to establish, because so little was known about the molecular and cellular basis of immunity. Later in 1909, Ehrlich [1] first proposed a role for host immune system in defence against spontaneous tumour. This idea was subsequently refined in the 1960s and led to the formal hypothesis of "immune surveillance" by Thomas L. (1959) [18] and Burnet F. M. (1970) [19]. The hypothesis was based on an emerging understanding of cellular basis of transplantation and tumour immunity. In addition, Burnet and Thomas had predicted that lymphocytes were responsible for eliminating continuously arising, nascent transformed tumour cells. This idea was possibly tested later in animals other than human, such as mice and rat.

A variety of experiments has taken up Stutman's animal model [20], in which some kinds of tumour (such as sarcoma) could be induced in CAB/H strain athymic nude mice by treating with a chemical carcinogen, methylycholanthrene (MCA). More importantly, these chemically induced tumours could be transplanted between inbred strains of mice that bear congenic MHC molecules on cellular surface. Transplanted tumours exhibit progressive growth in recipient mice and eventually kill the host. Among these animal experiments, the immunological findings obtained from the following several landmark studies [21, 22] provided evidence regarding the existence of tumour-specific immunity. In these animal studies (see figure 1.2 below), if mice are

injected with irradiated or inactivated tumour cells, these mice wound survive and some could be protected against subsequent challenge with a normally lethal dose of live parental tumour cells, in contrast to naïve mice which succumb to a similar challenge. Moreover, the protection could not be generated against distinct tumours. These observations were interpreted that the transplanted tumours expressed specific surface molecules that can become targets of murine host immune system.

The validity of immunotherapy originated from such a carcinogen-induced tumour vaccination/challenge setting was questioned because of the usual lack of immunogenecity in spontaneous murine tumours models, that mimicked more closely human cancer compared to these chemically induced tumours. However, a number of later studies demonstrated immune recognition of spontaneous tumours. Modification of non-immunogenic tumours by viruses was shown by Kobayashi and colleagues in the 1970s [23] to induce a strong anti-tumour reaction against the virally infected tumour cells and, importantly, also against the parental cells. Boon T. and colleagues in the 1980s [24] showed that, even in spontaneous mouse leukaemia, protective immune responses against non-immunogenic parental tumours can be induced following the application of chemically mutated variants. These observations implied that the spontaneous tumours were potentially immunogenic and their expressed antigens were recognized by the immune system, however, for some unknown reason, an effective response had not heretofore been generated.

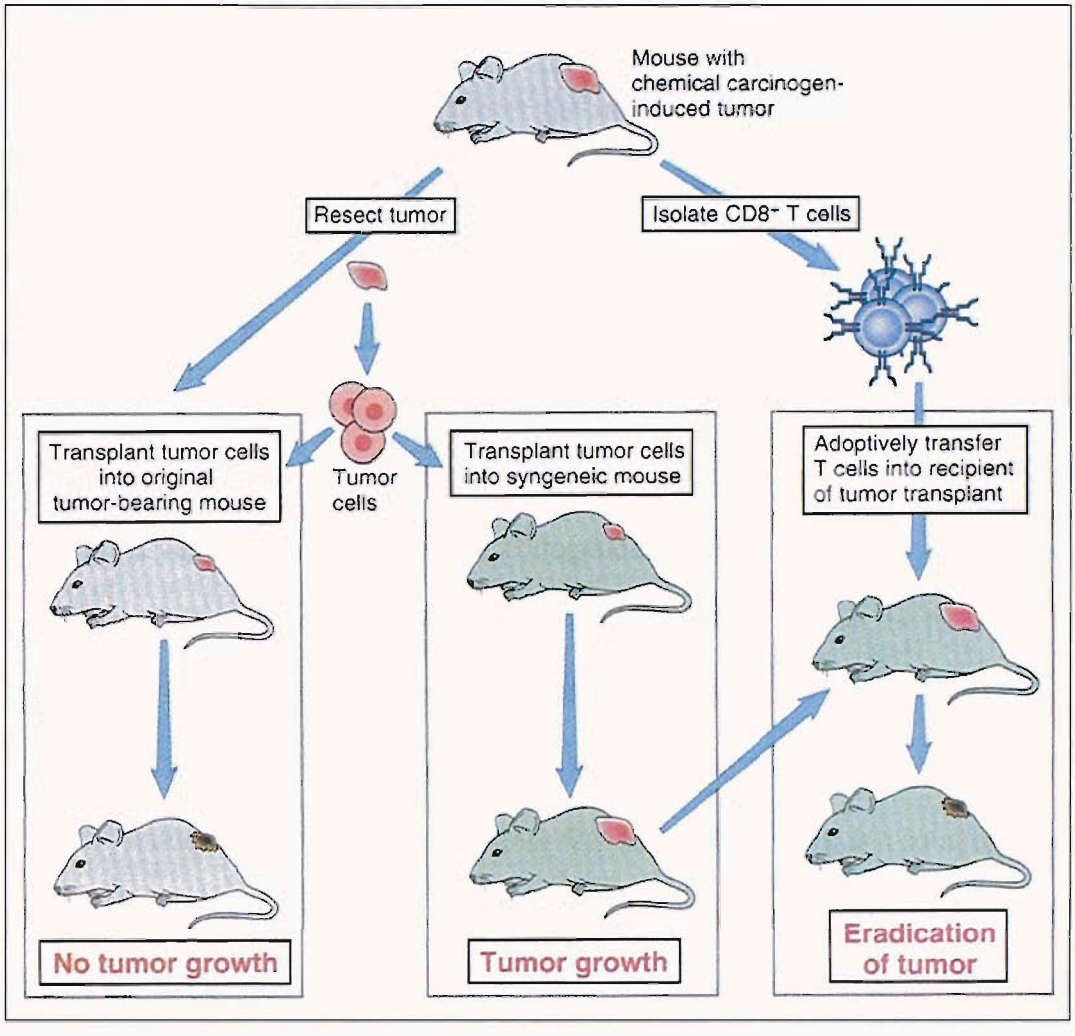


Figure 1.2. Experimental demonstration of tumour immunity

Mice that have been surgically cured of a chemical carcinogen (methylycholanthrene)-induced tumour could reject subsequent transplants of the same tumour, whereas the transplanted tumour grows in normal syngeneic mice. The tumour is also rejected in normal mice that are given adoptive transfer of T lymphocytes from the original tumour-bearing animal.

(Copy from *Cellular and Molecular Immunology* fourth edition, by Abul K. Abbas, Andrew H. Lichtman and Jordan S. Pober)

More recently, taking advantage of the development of gene targeting and transgenetic mouse technologies and the capacity to produce specific blocking

monoclonal antibodies (mAb) against particular immune components, a large number of works have accumulated to support the model of cancer immunosurveillance in murine models. These improved *in vivo* cancer models have proved strong and more convincing data and provided a deeper probe into the relationship between tumours and various immune components.

Two sets of studies incited a renewed interest in the field of cancer immunosurveillance in 1990s. First, endogenous interferon-γ (IFN-γ), an important soluble cytokine of innate immunity system, was shown to protect the host against the growth of transplanted tumours and the formation primary chemically induced tumours [25]. Second, mice lacking perforin (pfp-/-) were found to be more susceptible to MCA-induced and spontaneous tumour formation [26]. Perforin is a component of the cytolytic granules of cytotoxic T cells (CTL) and natural killer (NK) cells that plays a crucial role in lymphocyte-mediated killing. Therefore, these findings of the capacity of endogenous IFN-γ and perforin to regulate tumour cell immunogenecity have led to a broad search for the physiologically important immune components in cancer immunosurveillance.

.

These aforementioned studies support the existence of a cancer immunosurveillance in mice; does a similar process exist in human beings? There is ample evidence suggests that cancer immunosurveillance indeed occur in human. To speak generally, there are three lines of evidence imply this notion. First of all, spontaneous regression of a variety of malignancies did happen in a few immunocompetent hosts, while increased frequency of nonviral cancers was noted in populations of aged individuals and immunocompromised transplant recipient [27]. Secondly, taking advantage of the improved technologies in cellular and molecular immunology, spontaneous

occurrence of adaptive and innate immune responses against autologous tumours can now be detected directly in many cancer patients. Thirdly, the immune system often appears cognizant of tumours, as seen in that accumulation of various immune cells was found within the tumours, and the presence of tumour-infiltrating lymphocyte can correlate with an improved prognosis of patient survival [28, 29].

There is also ample evidence that both innate immune system and adaptive immune system play roles in tumour clearance in human being [30, 31, 32]. This may provide an explanation for the anti-tumour properties of the aforementioned Coley's toxin used in cancer patients in that bacterial DNA, which is one component of Coley's toxin, is immunostimulatory through bacterial specific CpG motifs which are mostly recognized by the host innate immune system [33, 34, 35].

1.2.2. T lymphocytes can recognize specific antigens on human tumours

Other studies have begun to shed light on the specific lymphocyte subsets that are involved in cancer immunosurveillance. In the aforementioned animal studies [21, 22] (see figure 1.2 above), in which mice are injected with irradiated or inactivated tumour cells, these mice would survive and some could be rendered protected against subsequent challenge with a normally lethal dose of live parental tumour cells. These protective effects are not seen in T-cell deficient mice but can be conferred by adoptive transfer of T cells from those immunized mice, showing the need for T cells to mediate the immune protective effect. Later on, several other studies have documented increased tumour incidences of MCA-induced sarcoma or spontaneous tumours in mice lacking T cells [36, 37]. Taking together, these studies implied the importance of T cells in cancer immunosurveillance.

T cells are by far the largest component of mononuclear tumour infiltrates in all human tumour [38]. The presence of tumour-infiltrating lymphocytes has been shown to be associated with improved patient survival in various types of human cancer, such as colon, breast and oral carcinoma [28, 29, 38]. In addition, it is now possible to isolate T cells from PBMC or tumour tissue of tumour-bearing patients, expand in culture and test their antitumour functions *in vitro* [29]. More recently, some experiments using peptide/MHC tetramers to stain T cell and analysis by flow cytometry have directly demonstrated the presence of tumour peptide-specific T cells in the circulation of cancer patients [39, 40]. These findings, as well as the recent identification of numerous TAA expressed on various tumour cells, which are able to induce cellular immune responses both *in vivo* and *in vitro*, strongly support the notion that T cells could recognize the presence of tumour cell and respond to it.

Based on the advancement in immunology, the mechanisms of the host adaptive immune system in responding to foreign antigens have been relatively clearly defined. During the invasion of foreign pathogens, such as bacteria and virus, antigen-presenting cells (APCs) are able to capture and process antigenic proteins from these pathogens, and migrate to nearby secondary lymphoid organs where they present antigens by surface major histocompatibility complex (MHC) molecules to either CD4+ or CD8+ T cells via their T cell receptors. Afterwards, naïve T cells proliferate and differentiate into effector cells after antigen encounter. To become activated, naïve T cells simultaneously have received the antigen signal in combination with certain costimulatory signals, which can be provided properly by some antigen-presenting cells like dendritic cells.

The activation of various antigen-specific T cells leads to a broad set of specificities

against various specific antigens. These activated T cells then enter the host circulation and migrate to peripheral tissue, where they recognize the target cells through the engagement specific T cell receptor (TCR) with specific antigen/MHC molecules complex on the surface of target cells. As a result, these T cells could kill the antigen-bearing target cells through different mechanisms, such as direct cytotoxicity by perforin/granzyme; cytokine secretion (IFN-γ, TNF-α) that can arrest proliferation of targeted pathogens and prevent angiogenesis necessary for target cells growth; or a ligand-to-ligand receptor interaction that induced cell apoptosis.

On the other hand, for the occurrence of adaptive immune responses against a load of different foreign antigens, T cells expressing TCR with correct biding specificity have to be present. The peptide biding specificity of TCR$\alpha\beta$ T cells is determined by variations in the structure of the TCR $\alpha\beta$ heterodimers. The rearrangements of TCR α- and β-chain genes occur at defined stages of T cell maturation in thymus and are led by *RAG1* and *RAG2* gene expression. In addition to combinational diversity of rearrangement among multiple variable (V), diversity (D) (β chain only) and joining (J) genes, addition or deletion of nucleotides in the complementary determining region (CDR3) during intrathymic development also contributes to shape the variability of TCR.

Both CD8+ cytotoxic T cells and CD4+ helper T cells are shown to be involved in anti-tumour immune responses. Among which, CD8+ T cells may play a central role in direct tumour clearance. Removal of CD8+ T cells *in vivo* caused enhanced tumour growth [41]; contrarily, the adoptive transfer of antitumour CD8+ T cells result in delayed tumour growth [42, 43, 44]. The antitumour effect of CD8+ T cells is presumably mediated through the ability of these cells to lyse tumour cells by perforin/granzyme

and cytokines secreted upon recognition of tumour antigens.

The tumour rejection antigens are peptides of tumour cell-derived proteins that are presented to CD8+ T cells by MHC class I molecules, therefore constitute stimulations for the adaptive immune system against tumours, just as the same as antigens from pathogens or viral antigens in infection disease. These peptides, which are derived from intracellular proteins digested by the proteasome complex, are usually 8-11 amino acid long with 2-3 primary anchor residues that interact with the MHC class I molecules and 2-3 amino acid residues which bind to TCR. These rejected tumour antigens could become the targets of antigen-specific T lymphocytes because they are not displayed on the surface of normal cells, or at least not at sufficient level on normal cells to be recognized by T cells. Adaptive immune responses to tumour are mediated by CD8+ T cells recognizing tumour-derived antigens bound to self-MHC class I molecules expressed on APCs, which internalize and process tumour antigens. Tumour cells themselves may serve as APCs, although low expression of MHC class I molecules and absence of costimulatory molecules on tumour cellular surface makes this an insufficient process [45].

In addition, it has become apparent in recent years that CD8+ T cytotoxic cells are heterogeneous. Like CD4+ helper cells, CD8+ cytotoxic cells fall generally into two subpopulations based on the cytokine secretion. Type 1 CD8+ T cells (Tc1) secrete IFN-γ and TNF-α, whereas type 2 CD8+ T cells (Tc2) secrete IL-4, IL-5 and IL-10 [46, 47]. The precise roles of the two CD8+ T cells subpopulations in tumour immunity remain undefined. One study indicates that both types of CD8+ T cells could kill target cells *in vitro*, however, only tumour-specific Tc1 cells can deliver protective antitumour immunity *in vivo* [48]. Whereas, another study suggests that Tc1 and Tc2

cells immunotherapy mediate long-term tumour immunity by different mechanisms [49].

In the other hand, the role of CD4+ helper T cells in tumour immunity is relatively less clear. CD4+ helper T cells may play a role in antitumour responses by providing help to CD8+ T cells. The immunologic functions of CD4+ T cells are presumably mediated by the multiple cytokines secreted by CD4+ helper T cells, such as IL-2, IFN-γ and TNF-α of Th1 cytokines, which enhance cell-mediated immunity; and TGF-β, IL-4, IL-5 and GM-CSF of Th2 cytokines, which support humoral immunity. Most effective antitumour immune responses in preclinical and clinical models depend on the efficient generation of Th1-type immune response and thus on CTL-mediated tumour killing [50, 51, 52, 53]. As suggested, the Th1-type cytokines secreted by CD4+ T helper cells upon the engagement of TCR by specific antigens can induce antigen-presenting cells to express costimulatory molecules (such as CD40) that may provide a secondary signals needed for the differentiation and proliferation of CD8+ T cells into antitumour CTL. CD4+ helper T cells can also upregulate the expression of MHC class I molecules on tumour cells. Thus, they overall increase sensitivity of tumour cells to the lysis by CTL. These and other findings have suggested that the *in vivo* persistence and maintenance of tumour-specific CD8+ effector cells may depend on the presence of endogenous CD4+ helper cells [52, 53].

CD4+ T cells respond to antigenic peptides that are presented by MHC class II molecules on antigen-presenting cells. The peptides presented by MHC class II are longer in size, usually 10 to 34 amino acids, and derived from exogenous proteins that are mobilized into intracellular endosome-lysosome compartments by endocytosis or phagocytosis. Most tumour cells are not derived from professional antigen-presenting cells (APCs) and therefore do not express the class II molecules. A likely possibility is

that tumour cells or their antigens are picked up by host APCs, and then processed inside the APCs. Thus, these professional APCs express both class I and class II MHC molecules that may present internalized tumour antigens and activate both CD8+ and CD4+ helper T cells. This process is called cross-priming.

1.2.3. Humoral immunity in anti-tumour responses

In addition to CD4+ helper and CD8+ cytotoxic T cells, there is ample evidence that various other components of the innate and adaptive immunity system also participate in the process of immune responses against tumours. Depending on the mechanism of tumour transformation, the anatomic location, the origins of tumour cells and the stage in immune recognition, cancer immunosurveillance may be a multivariable process requiring the orchestrated actions of a variety of immune effectors and soluble factors.

There is evidence that tumour-bearing host may spontaneously produce antibodies against various tumour antigens: and the occurrence of humoral immune response is associated with a favourable clinical outcome in patients with breast carcinoma and malignant melanoma [54, 55]. Antibodies may specifically bind to antigens on tumour cells and kill tumour cells by activating complement-dependent cytotoxicity (CDC) or by antibody-dependent cell-mediated cytotoxicity (ADCC), in which Fc receptor bearing macrophage or NK cells are recruited and mediate killing. However, the precise role of antibodies in elimination of tumour cells *in vivo* is largely unclear. Although in preclinical models, vaccine-mediated tumour regression in generally associated with CTL activation, TAA-specific antibodies or antibodies targeted tumour surface receptors are widely used in treatment of malignancies, such as

Rituximab (a chimeric mAb against CD20) for B-cell lymphoma, Bevacizumab (a humanized murine mAb against vascular endothelial growth factor (VEGF)) for colon and breast carcinoma, and Trastuzumab (a humanized mAb against Her-2/neu) for Her-2/neu overexpressed breast cancer [56, 57, 58].

The finding of tumour-specific antibodies has opened up new possibilities in the field of tumour serology, where it has long been hoped to develop antibody-based strategies for general use in monitoring, diagnosis, treatment and prognosis of cancer. In recent years, many investigators are enthusiastic in utilizing the anticancer antibodies as a targeted therapy of a variety of cancer patients. Taking advantage of the high specificity and affinity of these mAb against specific defined tumour antigens, these administered antibodies could accurately identify and bind to the malignant cells bearing the target antigens and create antitumour effects by complement-dependent, cell-mediated cytotoxicity or by the delivered function when they are engineered to conjugate with a broad of cytotoxic agents, such as radioisotopes, drugs and cellular poison. Therapeutic antibodies have been increased used in clinical oncology owing to their specificity, by which they would not affect or produce toxicity in non-tumour surrounding cells [59].

1.2.4. Innate immunity in anti-tumour responses

Natural killer (NK) cells (with phenotype of CD3-CD56+), which are innate effector cells that contain perforin-rich as well as granzyme-rich granules; represent 8% to 10% of lymphocytes in the peripheral circulation. NK cells usually serve as the first line of immunological defence against invasion pathogens in healthy human being. Nowadays, it has been shown that NK cells are crucial in tumour immunosurveillance,

which indirectly is suggested by the fact that higher incidences of tumour development were found in mice transgenically lacking NK cells [32]. Furthermore, it has been demonstrated that NK cells can kill various tumour cell lines *in* and *ex vivo* [60, 61, 62]. The presence of NK cells in the peripheral circulation can kill tumour cells and prevent the establishment of distant metastasis [63]. Under most physiological situations, however, the dominant signal received by NK cells is inhibitory particularly because of the recognition of syngeneic MHC molecules by the inhibitory receptor on NK cells. It has been proposed that, for tumours with downregulated expression of MHC class I molecules, NK cells may be the predominant effector mechanism of defence [60, 64]. Recent data has suggested, in addition to the direct perforin-mediating cytotoxic activity against malignant cells, NK cells constitutively express several ligands of the TNF family, which are capable of inducing apoptosis of tumour cell targets [65]. Furthermore, NK cells are believed to play a crucial role in facilitating APC-T cells interaction in the early phase of adaptive immunity, thus providing a key link between innate and adaptive immunity [66].

Another category of innate effector cells, NKT cells (with phenotype of CD3+CD56+) have been found to accumulate in peripheral circulation and tumour in tumour-bearing patients, and are believed to be capable of eliminating tumour target *in vivo* [67]. NKT cells co-express TCR (the characteristic of T cells) and the NK1.1 receptors (the characteristic of NK cells). These cells have been considered a remnant of an evolutionary primitive form of innate immune system. They can recognize a limited array of peptides and non-peptides (lipid or glycolipid) antigens presented by the nonpolymorphic MHC-like molecule CD1, which is widely expressed in most APCs and several tissues. After antigen-CD1 recognition, NKT cells immediately produce large amounts of type 1 and type 2 cytokines [32]. In many respects, NKT cells resemble

NK cells, and together, these cells are considered to be responsible for lymphokine-activated killer cell (LAK) activity [32].

Other soluble factors in innate immune system are known to participate directly and indirectly in tumour immunosurveillance. Interleukin-2 (IL-2), the most powerful stimulator of NK cells, has been successfully used alone or in combination with different vaccination regimens in human [68, 69]. However, the precise mechanism of IL-2 in contribution to the antitumour effect is difficult to be investigated. Moreover, the toxicity associated with the systemic administration of IL-2 has hampered its clinical utilization. Other NK cells activators (e.g., IL-12, IL-15, IL-18 and IL-21) have been used alone or as an adjuvant manipulation of other immunotherapeutic strategies in preclinical cancer models, some studies have been reported to be promising [61, 70, 71, 72].

Cytokine modified autologous tumour vaccines by gene transfer have been tested in preclinical and clinical trials for several years. The introduction of granulocyte-macrophage colony-stimulating factor (GM-CSF) into tumour cells has produced active antitumour immunity in clinical trials. Promising examples were seen in vaccination with irradiated autologous melanoma cells engineered to secrete human GM-CSF in patients with metastatic melanoma [73]; and in vaccination with irradiated allogeneic pancreatic tumour cells transfected with GM-CSF gene for the treatment of pancreatic adenocarcinoma [74].

1.2.5. Tumour-reactive cytotoxic T cells could be isolated and expanded *ex vivo* for adoptive cellular immunotherapy

As mentioned in numerous studies, T cells with antitumour reactivity could be isolated from tumour-bearing patients, normally from peripheral blood, lymph nodes, or tumour tissue of patients, and successfully survive in e*x vivo* culture with or without the presence of cytokine, such as IL-2, IL-7 or IL-15. These *in vitro* growing lymphocytes have been found to retain their antitumour cytotoxicity. After propagation, a sufficient number of autologous T cells could therefore be administered back to tumour-bearing patients for adaptive cellular immunotherapy.

The history of adoptive cellular transfer of immunity can be traced back several decades. At that time many researchers found that it is possible to eliminate the host immune function in recipient animal by ionizing irradiation from X-ray or γ-ray before attempting to restore immune function by adoptive transfer and allows the effect of the adoptively transferred cells could be studied in the absence of other host lymphoid cells. James Gowans and colleagues (1966) [75] originally used this model and proved the roles of the lymphocytes in immune responses. He reported that all active immune responses could be transferred to an irradiated recipient by adoptive transfer of lymphocytes from an immunized donor. Such a cellular transfer must be manipulated between genetically identical donors and recipients, such as in members of the same inbred strain of mouse, so that the transferred lymphocytes are not rejected by the recipient and do not attack the recipient's tissue.

Adoptive cellular immunity using activated T cells has long been successfully used in many clinical applications, such as employing defined antiviral CTL in infectious disease or as an adjuvant in bone marrow transplantation. In contrast to vaccine therapies, where reagents are usually more readily available, adoptive cellular therapy using antigen-specific T cell clones may be relatively labor- and time-intensive.

However, adoptive cellular immunity provides more rigorous control on the specificity and phenotype, and more augmentation of the intended immune response through the improvement of the general and specific functions and phenotypes of the transferred T cells by previous genetic modification or adjuvant treatment. The frequency of responding T cells in patient circulation could be increased and maintained to a sufficiently high level, whereas it is typically less than one percent of total PBMC in patients, even after multiple cycles of vaccination. In addition, immune responses could be easily and precisely monitored by tracking the transferred immune effectors *in vivo*. Most importantly, this approach does not require immunocompetent patients. Taken together, the feasibility of adoptive cellular immunotherapy is promising and has been considerably investigated to treat patients with cancers.

In recent decades, the advances in the field of immunology have provided opportunity for the adoptive cellular treatment of malignancy by manipulation of T cells *in vitro*. Numerous animal studies have shown that transplanted tumours were rejected in normal mice receiving adoptively transferred T cells isolated from mice that have been surgically cured of the same chemical carcinogen-induced tumour [21, 22]. Nowadays, high-dose chemoradiotherapy, which results in severe bone marrow ablation, followed by rescue of the resulting leukopenia with allogeneic hematopoietic stem cells transplant (HSCT) has become a standard treatment for many hematological malignancies. A life-threatening problem with this therapy is graft-versus-host disease (GVHD) that results from allogeneic donor T cells injuring the "foreign" host tissue. However, malignant cells that survive chemoradiotherapy are also "foreign" to donor T cells. As a result, patients who develop GVHD have lower relapse rates because of an associated graft-versus-tumour (GVT) effect [76]. Other applications using adoptive allogeneic T cells to mediate antitumour responses

including CD8+ T-cell specific to defined antigens for the treatment of patients with metastatic malignant melanoma, renal cells carcinoma [5, 43, 77, 78] and EBV-associated post-transplant lymphoproliferative disease (PTLD) [79, 80]. In most of these trials, autologous tumour-reactive T cells were harvested from patients, expanded and activated *in vitro*, and then were administered back into patients' circulation.

The propagation and manipulation of T cells *in vitro* with maintenance of the specificity and function, previously a hurdle to cellular immunotherapy research, has been largely overcome. Generally, the *ex vivo* culture of functionally active tumour-specific T cells for adoptive therapy requires sources of responder T cells (peripheral blood lymphocytes PBMC or tumour-infiltrating lymphocyte TIL), antigen-presenting stimulator cells (such as dendritic cells, activated B cells or inactivated autologous tumour cells), accessory feeder cells and cytokines. The purpose of *in vitro* re-stimulations by specific antigens is not only to positively select the tumour-reactive T cells, but also to propagate the specific T cells, which are present at extremely low frequency in peripheral blood. During repeated stimulation, cells other than reactive T cells presumably die because of lacking activation through TCR.

The optimal method for stimulation and propagation of the antigen-specific T cells *ex vivo* remains to be defined. In general, during T cell preparation, antigen-presenting cells (APCs) pulsing with defined tumour antigens are used to trigger reactive T cells initially. Alternatively, inactivated autologous tumour cell can be used directly to stimulate the specific T cells. Afterwards, the viable reactive T cells can then be nonspecifically stimulated by antibodies against CD3 and/or CD28. Following *in vitro* stimulation, the reactive T cells requires being screened for their reactivity and

selected for higher-affinity tumour-specific T cells recognizing autologous tumour cells since the majority of the T cells elicited in this manner are likely to have lower affinity. Supplemental cytokines, such as IL-2 and Il-15, are necessarily included in cell culture to support the proliferation, survival and differentiation of lymphocytes; and to overcome the lack of co-stimulatory molecules that required in normal immune responses *in vivo*. With this approach, it is possible now to expand the tumour-reactive T cells to a large number *ex vivo*, above several hundred- to thousand-fold. Re-infusion of billions of specific T cells could therefore achieve *in vivo* frequencies beyond those attainable with current vaccine regimens without the overt toxicity.

However, despite the high *in vivo* frequencies of tumour-specific effector cells achieved by adoptive cellar transfer, only a limited fraction of patients respond, pointing to the existence of additional hurdle. A clear requirement is that the infused cells must retain effective functions in terms of the ability of migration to tumour microenvironment and the functional avidity.

In vitro experiments have shown that T cells display differential expression of adhesion molecules (such as CD62L) and chemokine receptors (such as CCR7), which allow them to extravagate and migrate into non-lymphoid tissue and respond to target cells at peripheral tissue site [81]. The fate of T cells was determined by the expression of adhesion and chemokine molecules. As a result, CCR7 ^{high} and CD62L ^{high} T cells are found in lymph nodes; whereas T cells with CCR7 ^{low} and CD62L ^{low} are found in extranodal sites such as liver and lung. .

"Functional avidity" of T cells represents a cumulative effects of the binding affinity of its TCR for their cognate peptides displayed on target cells; the expression levels of

TCR, adhesion and costimulatory molecules; and the redistribution of these molecules on T-cell and their subsequent recruitment of intrinsic signaling molecules [82, 83, 84].

In other hand, the inclusion as adjuvant of some growth factors or cytokines, which aim to promote T cell activation, proliferation and tumour killing activity, may augment clinical outcomes for adoptive cellular immunotherapy. Among these, interleukin-2 (IL-2) has long been used for the treatment of malignant melanoma and, more recently, was also included as an adjuvant in adoptive cellular immunotherapy [85].

Nowadays, tumour-infiltrating T lymphocytes (TIL-T) are the major sources of tumour-reactive T cells that are used in adoptive cellular immunotherapy against various tumours. However, numerous studies have indicated that TIL-T, isolated from patients with melanoma, renal cell carcinoma, colorectal cancer and oral cancer are functionally deficient. The deficiency is manifested by either reduced expression of a signal transduction molecules associated with the TCR complex, ζ chain [86, 87, 88]; or by suppressed activation of nuclear factor Kappa B (NF*k*B), a transcription factor regulating expression of a number of immune and inflammatory genes [89, 90]; as well as by reduced proliferation in response to anti-CD3 antibody and increased frequency of apoptosis *ex vivo* [86, 87, 88, 91]. Furthermore, some other studies also documented that the signaling abnormalities, functional impairment and apoptosis seen in TIL-T are likely to present in autologous peripheral blood T lymphocyte (PBL-T) [92, 93].

It has been shown that the antitumour effects of adoptive cellular transfer were even more pronounced in the absence of host lymphocytes, with an objective response rate of 50% in patients with solid metastatic tumours [94, 95]. This strategy resulted in the

most consistent and dramatic clinical responses observed in the treatment of metastatic cancer [94]. The specific mechanisms that contribute to this enhanced effect of adoptive cellular immunotherapy in lymphocyte-depleted patients remain poorly understood. However, the recent insights pointed in three fields: the elimination of host cellular competition for homeostatic γc-cytokines, such as IL-7, IL-15 and possibly IL-21, which activate and expand tumour-reactive T cells *in vivo*; the impairment of regulatory T cells that suppress tumour-reactive T cells; and the induction of tumour cell death and antigen release with subsequently antigen-presenting cells activation [96].

1.3. Tumour-associated antigens

Transformation of normal cells into malignant cancer involves a number of changes in cellular genome and their expressions, including chromosomal translocations, axon deletion, and gene point mutation and so on. These changes, which overall result in deregulation of the regulatory circuits of the cellular proliferation and survival, would lead to profound changes in the antigenic composition of tumour cells. The altered proteins that may be a mutated antigenic peptide or protein aberrantly expressed or overexpressed on tumour cells are generally referred as tumour-associated antigens (TAA). Most of these tumour antigens are not expressed or expressed at very low level on normal host cells. Theoretically, they can be recognized by host immune system.

The criteria of an ideal tumour-associated antigen include: 1) the antigen is expressed widely in the great majority of human cancer; 2) the antigen is induced even at the earliest stages of malignant transformation; 3) the antigen plays an essential role in tumourigenesis; 4) the antigen contains numerous immunogenic epitopes with high affinities for the most common HLA alleles; 5) the antigen can be recognized by a T-cell repertoire.

1.3.1. Identification of tumour-associated antigens is important for the development of tumour vaccines

For designing a cancer immunotherapy, the vaccine can be targeted to multiple tumour proteins without knowing the information of antigenic peptides and defined T cell epitopes. Examples of this approach are seen in vaccination using lysate of

autologous whole tumour cells; or vaccination using dendritic cells that are transfected or electroporated with entire DNA or mRNA extracted from autologous tumour cells. These strategies has an advantage that all antigenic peptides of tumour cells can be processed and bound to a broad set of human leukocyte antigen (HLA) molecules, so that it is not necessary to made targeted antigens according to the HLA restriction. However, measurable specific immune responses were rarely detected following these approaches, and the efficiency of immunization was reflected merely by means of patients' clinical development.

Alternatively, defined antigenic peptides or their encoding sequences can be isolated from various tumours and used as targets for cancer immunotherapy. One of the advantages is that the antigenic peptides that meet the standard for clinical use are relatively easy to be synthesized and prepared. This would save time from conventional antigen preparation from excised tumour specimens. In addition, we are able to modify these tumour antigens according to their known structures in order to increase the immunogenecity of intrinsically poorly immunogenic tumours. More importantly, this approach greatly facilitates the quantitative and qualitative immuno-monitoring of the T-cell responses during immunotherapy because the presumed targets of the T cells are completely defined. Three T cells assays: peptide/MHC tetramer staining, intracellular cytokine flow cytometry analysis and ELISPOT assay, have emerged as the first-line methods for monitoring antigen-specific T cells induction during vaccination. These assays are relatively easy to perform, reliable, sensitive, and allow an *ex vivo* T cells analysis at single cell level. Although these assays are not, at this stage, defined as surrogate markers for *in vivo* clinical responses, they already provide information concerning the immunological potency of a given vaccine.

The efficacy of tumour vaccines is significantly affected by the immunogenecity of targeted antigens, by the route of immunization and by tumour features of susceptibility to immunological recognition. With the defined antigens, the *in vivo* characterization of the kinetics, the magnitude and the distribution of induced immune responses could be relatively easy to be traced. In comparing immune responses between strategies of vaccination under various protocols, an optimal treatment protocol can be designed to achieve more efficient clinical responses. As a result, the growing number of identified antigenic peptides could possibly make immunization be controlled more efficiently for induction of maximal immune responses.

The recent identification of human tumour antigens has already led to a better understanding of the molecular nature of tumour antigens, including mechanisms for the generation of T cell epitopes, tumour escape mechanisms and methods for evaluation of *in vivo* immune responses in patients during immunotherapy, including quantitative and qualitative immuno-monitoring. Identified tumour antigens may also be useful for the development of molecular targeting therapy and diagnostic methods. It is believed that the development of more efficient tumour vaccines is, to a great degree, dependent to the continuous identification of antigenic peptides expressed on various human tumour cells.

1.3.2. Several tumour-associated antigens have been defined

There is now substantial evidence that spontaneous immune responses against human tumours have been raised in different types of cancer, especially in melanoma and renal cell carcinoma [96, 97], but also in other types of cancer, such as non-small cell lung cancer, breast cancer and bladder carcinoma [98]. These findings implicate that there is

specific interaction of the immune system with antigenic determinants presented by tumour [99, 100]. The identification of a large number of antigenic peptides presented by HLA class I and class II molecules is likely to be important for the future design of cancer vaccines. A large set of antigenic peptide/HLA molecules complexes will alleviate HLA restriction and widen the application for eligible patients. It may also facilitate the design of a polyvalent vaccine against several antigens in order to increase the primary antitumour efficiency and decrease the risk of tumour escape by loss of antigen expression.

The first evidence for the occurrence of tumour specific T cell responses in human was seen from studies of patients with advanced malignant melanoma, in which cultured autologous melanoma cells were lysed by autologous CD8+ T cells *in vitro* in a MHC class I restricted manner. In a clinical extension of this observation, two patients with refractory metastatic melanoma were vaccinated with irradiated autologous tumour cells injected for an extended period of time. Surprisingly, complete regression of all tumour manifestation was achieved in both two patients (signature as SK-29 and MZ-2) after prolonged immunization [101, 102]. Furthermore, the clinical responses have been maintained for nearly 20 years. For the moment, melanoma is considered by many researchers as one of the relatively immunogenic human cancers and has attracted most focus.

In further effort following the observed clinical achievement after immunotherapy, a human tumour antigen was first isolated by a method developed by Boon T. and colleagues [2, 102, 103] (see also figure 1.3 below), in which melanoma-reactive autologous cytotoxic T lymphocytes generated from PBMC of a patient, MZ-2, were used to screen tumour cell genomic DNA library expression cloning. This

cancer-testis antigen was designated as MAGE-1 [2], in the context of A1 (HLA-A1). Following this successful identification, the cDNA expression cloning has become a primary technique for the isolation of tumour antigens recognized by CD8+ T cells. Later on, several other tumour-associated antigens of melanoma tumour cells were discovered by various modified methods, such as culturing irradiated tumour cells with autologous lymphocytes, a reaction known as mixed lymphocyte-tumour cell culture, and several other autologous antibody-defined tumour antigens were identified by serum IgG Ab from cancer patients, called SEREX (serological analysis of recombinant cDNA expression libraries), developed by Pfreundschuh and colleagues [104].

Murine tumour antigens were first found on methylycholanthrene (MCA)-induced fibrosarcoma in mice, Later on, similarly as human tumour antigens, several murine tumour rejection antigens were identified on methylcholanthrene-induced fibrosarcoma [21, 22], mastocytoma [105], *N*-nitroso-*N*-methylurethane-induced colon carcinoma (CT26), melanoma and spontaneous lung sarcoma and leukemia [106] (see table 1 below).

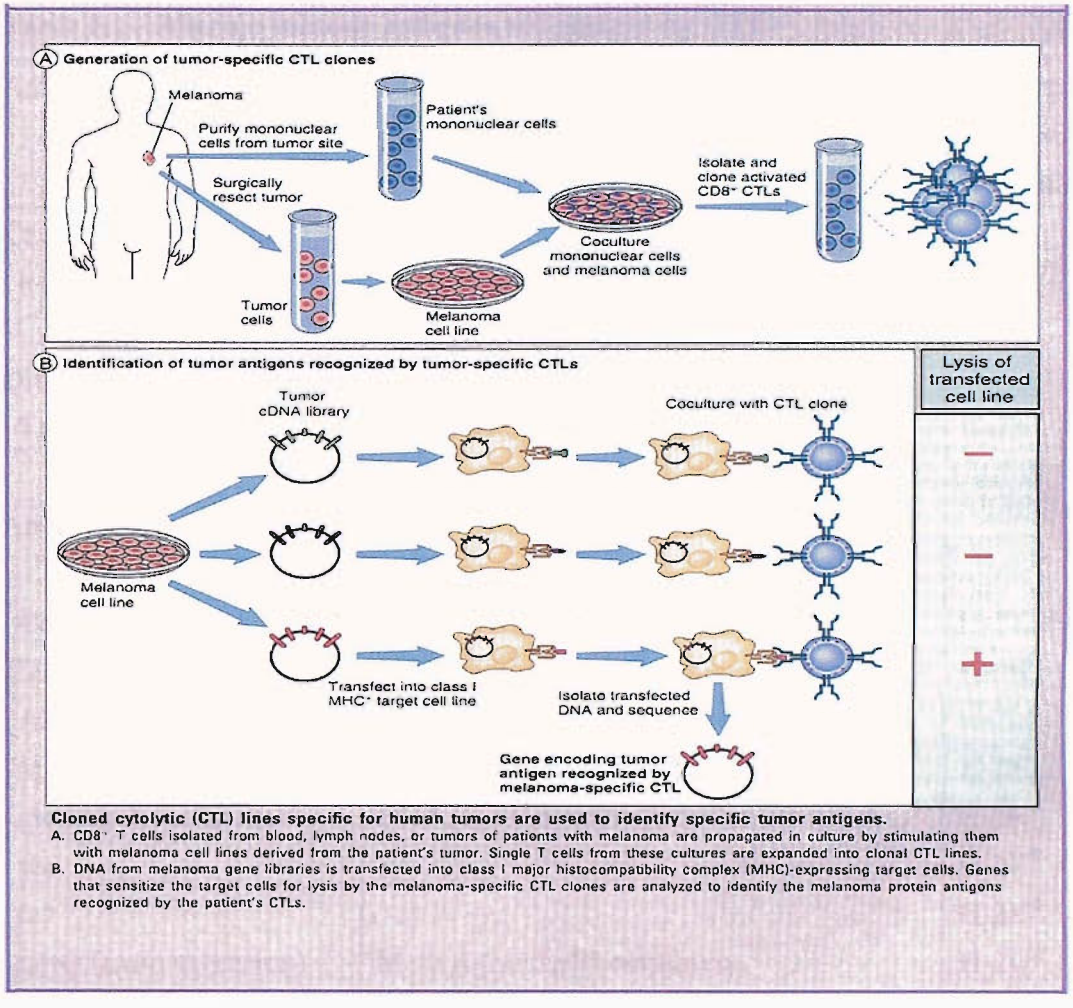


Figure 1.3. Cloned CTL lines specific for human tumours are used to identify specific tumour antigens.

A. CD8+ T cells isolated from blood, lymph nodes, or tumours of patients with melanoma are propagated in culture by stimulating them with melanoma cell lines derived from patients' tumour. Single cells from the cultures are expanded into cloned CTL lines.

B. DNA from melanoma gene libraries is transfected into class I MHC-expressing target cells. Genes that sensitize the target cells for lysis by the melanoma-specific CTL clones are analyzed to identify the melanoma protein antigens recognized by the patient's CTLs.

(Copy from ***Cellular and Molecular Immunology***, fourth edition, by **Abul K. Abbas, Andrew H. Lichtman and Jordan S. Pober,**)

Table 1. Murine tumour antigens recognized by CTL

Antigen	Tumour	Antigen presenting molecules
Cancer/Testis antigens		
P1A	P815 (methylcholanthrene-mastocytoma)	H-2L^d
Differentiation antigens		
TRP-2	B16 (melanoma)	H-2K^b
Antigens resulting from genetic alteration		
Connexin-37	Lewis (3LL)(lung sarcoma)	H-2K^b
DEAD box helicase p68	8101-RE (UV-induced sarcoma)	H-2K^b
Akt (LTR insertion)	RL♂1 (radiation induced leukemia)	H-2L^d
MAPK ERK2	CM55 (methylcholanthrene-sarcoma)	H-2K^d
methionine reductase	P815 (methylcholanthrene-mastocytoma)	H-2K^d
ras		H-2K^b
ras		H-2K^d
ramp (exon extension)	Meth A (methylcholanthrene induced-sarcoma)	H-2D^d
Viral Antigens		H-2D^k
gag IAP	LEC (spontaneous leukemia)	H-2D^b
gag	FBL-3 (Friend leukemia)	H-2L^d
AH1(gp70)	CT26 (*N*-nitroso-*N*-methylurethane induced-colon carcinoma)	

(copy from **Akiko Uenaka and Eiichi Nakayama** Murine leukaemia RL♂ and sarcoma Meth A antigens recognized by cytotoxic lymphocyte. ***Cancer Sci.*** 2003; **94 (11):** 931-936.)

The tumour antigens defined to date have been broadly classified into five groups [96] (see table 2 below). The first group of tour-associated antigens are called cancer-testis antigens, which are expressed in various tumours but not in normal tissue, except in

the immune-privileged areas of testes. These antigens include mouse P1A and human genes of MAGE, BAGE, and GAGE family [2, 103, 105, 107]. Cancer-testis antigens are thought to be tumour specific and shared among many tumours of several histological types, such as NY-ESO-1 and members of the MAGE gene family were expressed in malignant melanoma, oesophageal carcinoma and so on [8, 102]. They are, therefore, attractive targets for the development of antigen-specific immunotherapy of cancers.

The second group includes tissue differentiation antigens and idiotypic Ig molecules on lymphocytes. These antigens are called differentiation antigens because they are specific for the particular lineages or limited stages of differentiation in various cell types. The most well-known example comes from cells of the melanocyte lineage, such as gp100 and tyrosinase, which are expressed on most melanoma cells but also on normal melanocytes [108, 109, 110]. B cell-derived lymphomas can be frequently diagnosed by the detection of surface markers characteristic of the B-lymphocyte lineage, such as CD20 [56]. The idiotypic determinants of surface Ig are markers for B cell-derived lymphoma because all other B cell clones express different idiotypes.

The third group of tumour-associated antigens derives from the product of mutated oncogenes and tumour suppressor genes. These abnormal genes result from genetic alteration, such as point mutations, frame shift mutation, gene deletion, chromosomal translocations; or viral gene insertions, which frequently involve cellular proto-oncogenes or tumour suppressor genes. As a result, the protein products of these altered genes have transforming activity [4, 111, 112, 113, 114]. In these cases, most of the antigens in this group are unique to given tumours and are therefore individually specific. More importantly, because these altered antigens are not expressed in normal host cells, they do not induce self-tolerance. Examples of this group include mutated

β-catenin [114], Bcr-abl [115], CDK4 [113].

The fourth group includes viral antigens present in cancers of viral aetiologies. DNA viruses are implicated in the development of a variety of tumours in experimental animals and humans. Examples in humans include the Epstein-Barr virus (EBV), which has been implicated in the pathogenesis of Burkett's lymphoma, Hodgkin's disease, non-Hodgkin's lymphoma, B-cell lymphoma and nasopharyngeal carcinoma [116]; and human papilloma virus (HPV), which is associated with cervical cancer [117]. In most of these DNA virus-induced tumours, virus-encoded protein antigens are found in the nucleus, cytoplasm or plasma membrane of the tumour cells. As a result, these endogenously synthesized proteins can be processed, and viral peptides may be expressed on the tumour surface. Because the viral peptides are usually foreign antigens, DNA virus-induced tumours are among the most immunogenic tumours known. In contrary, the only well-defined human RNA tumour virus is human T cell lymphotropic virus-1 (HTLV-1), the etiologic agent for adult CD4+ T cell leukemia/lymphoma (ATL) [118].

The fifth group of tumour antigens are normal self proteins that are overexpressed or aberrantly expressed on many tumours; for example, HER-2/neu, a growth factor receptor, are known to be overexpressed in 30% of patients with breast and ovary cancers and a variety of other adenocarcinoma [119]. Another example is mucins that are normally high molecular weight extracellular glycoproteins containing numerous O-linked carbohydrate site chains and expressed on host epithelial cells as a physical barrier or cell-surface receptor and sensor. Cancer cells, especially adenocarcinoma, often have deregulated production of enzymes that synthesize aberrant forms or amount of mucins. The expression of distinct oligosaccharide structures, together with

different glycosylation of mucin core proteins led to potential tumour-specific epitopes that can be recognized host immune system. Several mucins have been the focus of diagnostic and therapeutic usage, including CA-125 and CA-19-9 in ovarian and breast carcinoma, and MUC-1 on breast carcinoma [120].

Table 2. Potential Sources of Tumour Antigens for Human Tumours

Category of Antigen	Example
Cancer- testis antigens	MAGE family (melanoma and breast cancer)
	BAGE
	GAGE
	CEALAGE-2
	NY-ESO-1/
	POU Homeobox Gene (OCT3, OCT4)
	SSX-1
	SCP-1
Tissue-specific differentiation antigens	Tyrosinase (melanoma)
	MART-1/Melan A (melanoma)
	gp 100 (melanoma)
	TRP-1 and TRP-2 (melanoma)
	Ig idiotypes (B-cell lymphoma) (CD20)
	TCR idiotypes (T-cell lymphoma)
	PSA (prostate cancer)
Mutated gene products	CDK4-R24C
	Beta-catenin
	CASP-8
	K-ras
	P53
	Bcr-Abl
Viral antigen products	EBV (Burkett's lymphoma, nasopharyngeal cancer)
	HPV (cervical cancer)
	HBV (Hepatocellular cancer)
Normal cellular proteins that are	HER-2/*neu*

overexpressed or aberrantly expressed in tumour cells.	CA-125 MUC-1 CA-19-9

Abbreviation: EBV. Epstein-Barr virus; HPV, human papilloma virus; HBV, hepatitis B virus; Ig, immunoglobulin; TCR, T-cell receptor

It is not entire sequences of tumour antigens that are engaged by TCR and trigger host immune responses. In general, only some short peptide sequences within tumour antigenic proteins are immunogenic. These peptide sequences, which are called "epitopes", are presented by MHC molecules according to a set of rules derived from proteasome cleavage sites, the affinity of transporters associated with antigen processing (TAP), and the anchoring pockets in the peptide-binding groove of the MHC molecules [121].

These newly defined tumour antigens have provided feasible strategies to develop therapeutic and preventive immunotherapy against various malignancies. The clinical and pre-clinical trials of immunotherapy targeting a broad set of defined tumour-associated antigens have been and are moving forward in multiple areas. For example, several therapeutic vaccines targeted various cancer testes antigens (MAGE-3) and melanoma differentiation antigens (tyrosinase, MART-1/Melan-A, gp-100) have been applied clinically for the treatment of advanced metastatic malignant melanoma [122, 123, 124, 125, 126]; peptide-based vaccine with an epitope derived from PSA was used to treat patients with prostate caner [127]; a chimeric monoclonal IgG1 antibody, Rituximab (Rituxan), targeting the CD20 surface receptor, is available commercially and is successfully used for B-cell non-Hodgkin lymphoma [128, 129]; another humanized monoclonal antibody, Trastuzumab (Herception), has been

extensively used for Her-2/neu (c-erb-B2) overexpressing advanced metastatic breast cancer [130, 131]; and various therapeutic vaccines targeting two HPV oncogenic proteins, E6 and E7, are currently being used in clinical to prevent and treat HPV (type 16 and 18)-associated cervical cancer [132]. Although, the primary results of these clinical trials varied from cases to cases depending on different setting, some promising results have been noted and many cases of tumour regression have been observed.

1.3.3. Shared tumour antigens represent the most promising targets for cancer immunotherapy.

The defined antigens include both shared and unique antigens. Literally a "unique" tumour antigen would be an antigen that was never found on another tumour. In practice, this term applies to antigens that are rarely expressed on different tumours. Conversely, "shared" antigens are frequently expressed on many, not necessarily all, tumour or all members of a subtype of tumours.

In the past, it was believed that most rejection tumour antigens recognized by host immune system are generated from normal cells by individualized tumour-specific mechanisms, such as point mutations, deletion, chromosomal translocations, or viral gene insertions involving cellular proto-oncogenes or tumour suppressor genes to form oncogenes [4, 112, 112, 113, 114]. These tumour antigens are thought to be specific to some tumour cells and individual patients. The notion came from a variety of studies in animal models and human that had suggested that most tumour rejection antigens were unique to individual tumours [112, 113, 114, 133]. There is presumably no immune tolerance to these unique antigens because they are not present in normal host cells. In addition, tumours expressing these unique mutated antigens with absolute tumour

specificity have less chance to develop loss variants, because the genes encoding these antigens are usually involved in tumour cells proliferation and survival. It has been shown that not only chemically but also ultraviolet light-induced and even spontaneous tumours express unique tumour antigens [4, 134, 135].

However, the frequency of such unique antigens identified among different types of tumours or in different individuals is relatively low. In classic immune surveillance theory, tumour cells that expressed highly immunogenic mutated unique antigens might be discovered and eliminated by specific T cells immunosurveillance before clinical diagnosis. This fact has limited the usage of such unique tumour antigens in cancer immunotherapy. In the other hand, despite the continuous selection of antigenic variants by CTL with unique tumour specificity *in vivo*, tumour cells expressing unique antigens could still escape from T cell killing. In addition, a single malignant cell that expressed multiple unique antigens has be found in many murine tumour cells [136, 137]. Such multiplicity of antigens was also discovered on human cancer cells [138].

Interestingly, mutated antigenic peptides have been more frequently identified from various tumours by tumour-reactive CD4+ T cells, suggesting that CD4+ T cells response alone may not be sufficient for the rejection of tumour [139]. Nevertheless, the potential of unique antigens have been implicated in the development of cancer immunotherapy, even though it is difficult to identify unique antigens to cancer patients.

In contrast to the unique antigens, recent evidence that various independent human tumours expressed the same T cell-defined tumour antigens has attracted interest [140,

[141]. Many of the shared antigens are derived from tissue-specific differentiation antigens, oncogenic products or cancer-testis antigens. In fact, the defined shared tumour antigens may represent potentially the most practical targets for cancer immunotherapy because they are extensively expressed on various tumour cells, and allow the design of a more favourably universal cancer vaccine for many individuals. For example, peptides derived from cancer-testes antigens are attractive targets of various cancer vaccines, since they are expressed in various types of tumour cells. A broad set of over-expressed antigens that are involved in the tumour formation, proliferation and survival are preferentially expressed on many tumour cells at relatively high level. Examples of such TAAs include Her-2/neu [58], WT-1 [142], p53 [143] and survivin [144].

Tissue-specific differentiation antigens are also useful for a broad population of patients, such as PAP, PSA and PSMA that were identified in most prostate patients [145]; MART-1, gp100 and tyrosinase are frequently detected in most melanoma patients [108, 109, 100]. As a result, a vaccine targeting these shared antigens could be relatively easy to prepare and even stocked without the necessity of the time-consuming procedures to identify unique antigens from the patient's tumour and could be extensively utilized to treat patients with various types of cancers. However, such as in the last case of melanoma, autoimmune reaction frequently occurs during immunotherapy designed to target the tissue-specific differentiation antigens. In addition, antigen loss variants may somehow develop, because these antigens are not essential for cellular proliferation and survival of tumours.

In addition, many trials suggested that a certain level of tolerance to these shared antigens might exist as they are encoded by non-mutated self-antigens; that is, these

antigens may be only poorly immunogenic. The questions regarding the shared antigens were addressed in the experiments by Brandle (1998) [146]. He reported that mice immunized by injection of L1210 leukaemia cells expressing P1A, a murine MAGE-type tumour antigens, and B7-1 (L1210.P1A.B7-1) are efficiently protected against the challenge of mastocytoma P815 tumour cells, which also express P1A. In this case, the author suggested that making generalized vaccines against shared antigens appears more practical, but one may have to use strong immunization procedures or combine with adjuvant to overcome their poor immunogenecity.

Improvement to antigenic presentation, including the addition of various strategies of adjuvant, the utilization of antigen-pulsed dendritic cells, multi-peptide vaccination, and the addition of helper antigenic peptides; or strategies to boost immune responses to these shared antigens are encouraging for future research. For example, more recently, Wolfers and colleagues (2001) [147] used tumour-derived exosome loaded dendritic cells targeting shared tumour antigens to successfully trigger T-cell-mediated anti-tumour immune responses and lead to rejection of autologous tumours and a strong inter-tumour cross-protection. Other methods to boost the immunogenecity involve strategies to overcome the self-tolerance to these shared tumour antigens.

1.3.4. Methods for identification of tumour-associated antigen

The identification of human tumour-associated antigens is an essential step in the development of efficient cancer immunotherapy. There are several documented methods (see table 3 below) have been developed for identifying tumour antigens and their coding genes in human tumours. Since the first development by Boon T and colleague (1991) [2, 102, 103], a direct genetic approach using recombinant cDNA

expression libraries has become a primary technique for the isolation of tumour-associated antigens. Transient transfectants are screened for the ability to stimulate the tumour-specific CD8+ T cells. Other methods with slight modification but similar principle were developed later on. By using these genetic approaches, a growing number of tumour-associated antigens have been identified in various murine and human tumours However, in this cDNA expression cloning system, the necessity of establishing *ex vivo* pre-characterized CTL clones with tumour restricted reactivity remains a critical hurdle of this method.

Table 3. Examples for the methods of isolation of tumour-associated antigens

Approach	Methods
Immunogenecity	cDNA expression cloning with tumour-reactive T cells
	cDNA expression cloning with patient's serum (SEREX)
Specific gene analysis expression	
Genome DNA	DNA sequencing (mutation, polymorphism)
	Comparative genomic hybridization (gene amplification)
mRNA	cDNA subtraction (RDA, PCR differential display)
	cDNA profile comparison (DNA chip/microarray, SAGE, EST database)
Protein	protein expression profile comparison (2D-EP, MS, protein chip, protein database
	Isolation and identification of HLA bound peptides using HPLC, and MS

SEREX (serological analysis of autologous tumour antigens by recombinant cDNA expression cloning), SAGE (serial analysis of gene expression), RDA (representational differential analysis), EP (electrophoresis), MS (mass spectrometry), HPLC (high-performance liquid chromography (Copy from **Yutaka Kawakami,** *et al.* Identification of human tumour antigens and its implications for diagnosis and treatment of cancer. ***Cancer Sci.*** 2004; **95:** 784-791).

Another approach was based on the fact that spontaneous occurrence of antibodies against autologous tumour antigens was found in a large number of patients bearing various tumours [54, 55]. A broad set of evidence has pointed to a hypothesis that coordinated recruitment of CD4+, CD8+ T cells and B cells responses to the same tumour antigens occurs in cancer patients [148, 149, 150]. Once immune recognition of any antigenic product is elicited; it is not restricted in merely one effector system. The spontaneously occurring antibodies found in serum of cancer patients can specifically recognized either surface or intracellular antigenic peptides of cancer cells *in vivo* and *ex vivo*. These finding have led to the development of a new antigen-hunting strategy that is called "serological identification of antigens by recombination expression cloning" (SEREX) by Pfreundschuh and colleagues [105] in 1995. SEREX is taking advantage of serum from cancer patients containing high titre of IgG antibodies to screen the recombinant cDNA expression libraries constructed from fresh autologous tumour specimen. To date, a few newly-defined tumour-associated antigens, such as SSX-1, SCP-1 NY-BR-1 and NY-ESO-1/LAGE-2 [104, 151, 152, 153, 154], were identified by this approach. Furthermore, the use of reverse T-cell immunology on some of the SEREX-defined antigens has led to the identification of several immunogenic epitopes recognized by CD8+ and CD4+ T cells from cancer patients [150, 155], such as in NY-ESO-1 [155].

Some antigens have been identified by a biochemical approach [4, 156, 157]. A mixture of endogenous peptides can be biochemically purified from tumour cells or from the MHC class I molecules of tumour cells. The purified peptides derived from tumour cells are fractionated by high-performance liquid chromatography (HPLC) and then each HPLC fraction is tested by the ability to sensitize target cells for lysis by the corresponding antigen-specific CTL. Alternatively, the elution of peptide derived

from tumour cells can be analysed by technologies of proteomics [158, 159, 160] such as tandem mass spectrometry (MS/MS) analysis, high-performance liquid chromatography elecrospray ionization mass spectrometry (HPLC ESI MS) or protein chips in comparison with proteins or peptides derived from normal host cells.

The unveiling of the sequence of human genome and improved bioinformatics tools has provided an alternative way to screen any given protein for immunogenic epitopes. Gene-expression profiling in cancer can be used to identify overexpressed or aberrantly expressed gene products that are not expressed in normal tissue. Differential gene-expression analysis at the transcription level can be performed by several methods, including serial analysis of gene expression (SAGE) [161, 162], DNA chip/microarray [163], representational differential analysis (RDA) [164], differential reverse transcriptase PCR-based display [165] and mRNA/cDNA subtraction hybridization [166]. Based on some publicly available gene-expression-profiling databases (such as Cancer Gene Anatomy Project http://cgap.nci.nih.gov/ or Stanford Genome Resource Centre http://genome-www.standford.edu) tumour antigens could be revealed by comparing the gene profile of tumour cells with a panel of normal cells.

Alternatively, if prior knowledge of the chromosomal location is available, tumour antigens can be identified by positional cloning [167, 168] or various genetic analyses along with DNA database. Allogeneic antigens such as minor histocompatibility antigens (mHa) may be identified through SNP (single nucleotide polymorphism) search obtained by random genomic DNA sequencing. Gene amplifications identified by comparative genomic hybridization (CGH) may lead to the isolation of tumour antigens.

More recently, the approach of "reverse immunology" has been applied for identification of epitopes from hypothetical tumour antigens. The candidate antigenic peptides of interest are deduced by computer programs (such as http://www.syfpeithi.de/, http://www.imtech.res.in/cgibin/propred1 and http://bimas.dcrt.nih.gov/molbio/hla_bind/) from the consensus anchor motifs for antigen-presenting MHC molecules and proteasome cleavage in the proteins known to be overexpressed or mutated in tumour cells. The predicted peptides are synthesized, loaded on antigen-presenting cells and screened by patients' immune system, including T cells or IgG antibodies [150, 155, 169, 170]. T cell recognition of parental tumours can be evaluated by tumour-reactive T cells induced *in vitro* from PBMC or TIL of patients against the candidate peptides. More recently, the catalytic subunit of human reverse transcriptase telomerase (hTERT) was characterized by epitope deduction [171]. This method is also used especially for searching CD8+ T cell epitopes in serologically defined tumour antigens or evaluating the identified antigen candidates by systemic gene analysis. .

1.3.4.1. Identification of tumour specific antigens using tumour-specific CTLs

The central element of most strategies for identifying tumour antigens is tumour-reactive T cells, especially the CD8+ T cells, which recognize antigenic epitopes presented by MHC class I complex on the surface of antigen-presenting cells (APCs) or tumour cells. As aforementioned, CD8+ cytotoxic T cells are believed to be the direct effector cellular element in host immune system responsible for the tumour surveillance. In some cases, it was found that tumour cells escaped from immune rejection *in vivo* were resistant to the original tumour-specific CTL due to the development of antigen-loss variants rather then the presence of immunosuppression

[172]. This and other evidence implies that CTL plays a critical role in tumour immunity and that antigens recognized by CTLs *in vitro* could be potentially effective tumour-rejection antigens.

Two decades ago, clones of highly specific CD8+ cytolytic T lymphocyte directed against a range of mouse tumour antigens [173, 174] were isolated and cultured *ex vivo*. One of these tumour systems is mastocytoma P815, a tumour that appeared in DBA/2 mouse after methylcholanthrene treatment. A clonal tumour cell, named P1, was derived from P815 and a panel of anti-P1 CTL clones was obtained by mixed lymphocytes/tumour cells culture (MLTC). These CTL clones were then used to select antigen-loss tumour variants that were resistant to subsets of anti-P1 CTL clones, leading to the definition of five distinct antigenic peptides expressed on P1 [105, 172].

Similarly for human tumours, mixed cellular culture of autologous tumour cells with lymphocytes derived from PBMC or TIL of tumour-bearing patients often generate tumour-specific CTLs that showed specific cytotoxicity to autologous tumour cells [175, 176]. Taking advantage of these advanced technologies, a panel of CTL clones that exerts cytolytic activities to autologous melanoma cell line, MZ2-MEL, were generated using lymphocytes from a melanoma patient MZ-2 [177]. Antigen loss variants of melanoma cells, MZ2-MEL.2.2, were obtained. A cosmid library was prepared with the DNA of a MZ2-MEL subclone and transfected into the antigen loss variant, and then was screened by appropriate CTL clone [178] for tumour-associated antigens (see figure 1.3 above). The first gene encoding for a cancer-testis antigen expressed on human malignant melanoma tumour, MAGE-1, was identified by Boon T and colleagues in 1991 [2]. The sequence of the gene found in lymphocytes of patient MZ-2 was identical to that found in MZ2-MEL melanoma cells.

Later on, a faster procedure was designed in that cDNA libraries were prepared from RNA of tumour cells, and transfected into appropriate recipient cells along with MHC genes. The episomal multiplication of the transfected plasmid in these cells ensures that large amount of the proteins containing antigens encoded by the cDNA inserts are produced within two days [179, 180]. The direct genetic approach was widely used in many research groups and has led to the identification of many other CTL-defined tumour antigens and their coding genes, such as tyrosinase [181], Melan-A/MART-1 [182] present on another melanoma cell line SK29-MEL and the members of MAGE, BAGE and GAGE family [103, 183]. To date, cloning of melanoma tumour antigens by CTL have provided a catalogue of representative melanoma antigens, including mutated peptides derived from genetic alterations in tumour cells, self-peptides derived from tissue-specific differentiation proteins, over-expressed proteins in tumour cells and cancer-testis antigens.

The hurdles of this direct genetic approach using *in vitro* growing CTL exist in three stages: the labour of isolation and *ex vivo* culture of pre-characterized tumour-specific CD8+ cytotoxic T cells; the requirement of a high throughput screening assay that provides a reliable, sensitive and fast method for the detection of CTL activities against specific antigens; and the generation of cDNA expression libraries that represent faithfully the panel of tumour-derived expression profiling.

Recently, there has been marked improvement in the technology for *in vitro* culture of CD8+ cytotoxic T cells that are preserved of their antigen-specific activities. In general, secondary signals are required in an *ex vivo* stimulation in combination with antigen signals from T cell receptors These secondary signals include those derived from interaction between costimulatory molecules on antigen-presenting cells (such

as members in B7 family) with corresponding receptors (such as CD28) on CTL, or those signal necessary for the processes of cellular survival and proliferation of the activated CTL. In this context, some cytokines, such as IL-2, IL-7 and IL-15, can be included in T cells culture medium so as to overcome the lack of costimulatory signals in *ex vivo* stimulation, and to provide anti-apoptotic effect for *ex vivo* activated CTL.

1.3.4.2. Assay for antigen-specific T cell activities

In many methods of tumour-specific antigen hunting using CD8+ T cells, an assay with high specificity and sensitivity for measurement of the CTL activity is crucial. Furthermore, as in conventional genetic approach using recombinant cDNA expression libraries, roughly at least 1-2 X 10^5 distinct recombinant plasmid cDNA need to be screened. Thus, in order to work on a repeated screening procedure through a huge pool of tumour libraries, a high throughput, easy-to-perform and reliable assay is highly recommended to measure the antigen-specific activities of CTL. In general, after recognition of cognate peptide-MHC complexes on APCs, the CD8+ cytotoxic T cells may exhibit two general effector functions: the direct target cells killing and the production of soluble factors, such as cytokines and chemokines.

Historically, the chromium (^{51}Cr)-releasing assay was used as a standard *in vitro* method to assess the direct specific killing activities of CTLs against antigen-bearing target cells [184]. However, the quantitative substance in ^{51}Cr-releasing assay is known to be radioactive. In addition, despite this assay measures the short- term (4-6 hours) cytotoxic effect of CTL, the procedure is complicated, and cumbersome. More recently, other methods that monitor the release of non-radioactive fluorescent dyes or

chromogenic molecules from lysed target cells have been introduced alternatively for measuring the antigen-specific cytotoxicity of CTL [185, 186]. Nevertheless, the latter fluorescence-releasing assays, such as LDH releasing assay, are usually labour-consuming and potentially low in sensitivity.

Alternatively, the detection of various cytokines or chemokines produced by T cells in an antigen-specific manner could be of considerable use for assessing T cell activities. Examples include the assays detect IL-2, IFN-γ, TNF-α or GM-CSF that are released in the culture supernatant. The sensitivity and efficacy of cytokine detection assay has been improved by using large-scale and small-scale enzyme-linked immunospot (ELISPOT) instead of conventional enzyme-linked immunosorbent assays (ELISA), or intracellular cytokine flow cytometry. By using ELISPOT for the detection of IFN-γ production by T cells from cDNA expression libraries, several CTL-defined epitopes on murine and human tumours were identified [106, 187, 188]. However, most of these cytokine assays are relatively expensive, complicated and laborious.

On the basis of the observation that a significant amount of TNF-β production is observed when CD8+ T cells are stimulated by antigen-presenting cells containing as little as 3% of cells presenting the specific antigen [189], an assay that is to measure the production of TNF-β by CD8+ T cells in a immune response may be valuable and sensitive in detecting the antigen-specific activity of CTLs. As described by Espevik and Nissen (1986) [190], a NK cell-resistant mouse fibrosarcoma cell, WEHI 164 clone 13, which is highly sensitive to human TNF-α and murine TNF-β when pre-treated with actinomycin-D, can be utilized to measure the amount of TNF secreted by CTL or monocytes. It has been shown in this assay using recombinant tumour necrosis factor (rTNF-β) that TNF mediated highly cytotoxicity toward WEHI cells with an

LD50 of as low as 2 X 10^{-3} ng/ml [190]. Together, these factors provide that WEHI 164 clone 13 cells can be used to examine the antigen-specific activities of CTL.

In practice, the TNF production by CTL is measured by culturing WEHI cells in the supernatant harvested from overnight CTL culture. The concentration of TNF-β in the supernatant is positively correlated with the percentage of killing of WEHI-164 clone 13 cells. The viability of WEHI cells can in turn be estimated by a standard colorimetric MTT assay, as described by Mosmann (1983) [191]. In general, the MTT assay relies on the ability of living WEHI cells to internally mobilize MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) into cells and catabolize the tetrazolium salt via mitochondria into a coloured product, formazan (see figure 1.4 below), which can also be simply read by a standard 96-well reader spectrophotometrically. The MTT approach has been valuable for the detection in various bioassays of, for example, lymphotoxin [192] and growth factors [193]. The WEHI 164 cells are well suited for the MTT assay because they produce high optic density signals. In fact, it has been shown that the cytotoxic activity measured in a 20-hour MTT assay with WEHI 164 clone 13 cells correlates closely with that measured in a standard 6-hour ^{51}Cr-releasing assay against the same target cells [190]. The combination of WEHI assay with MTT assay for quantitative assess of the antigen-specific TNF-β production by CTL is relatively easy and quick to perform and has been shown to be sensitive and reliable in identification of human tumour antigens [104, 105].

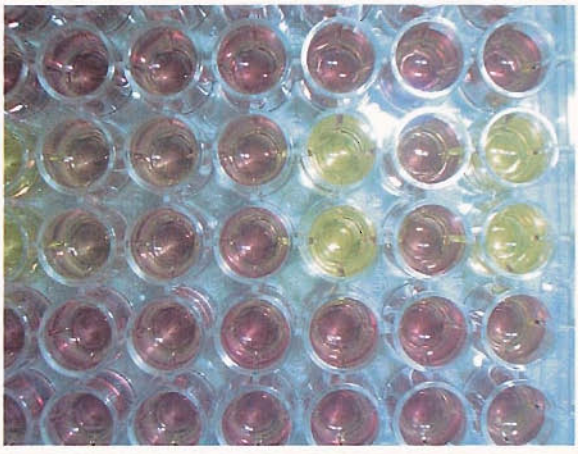


Figure 1.4. Colour change in a TNF/WEHI/MTT assay

Viability of WEHI cells from cytotoxicity by TNF-β produced by CTL in response to specific antigens is represented by purple colour (in the contrast, the yellow colour represents a higher WEHI cells killing, which in turn represents a higher level of TNF-β secreted in the supernatant by CTL.)

1.3.4.3. LacZ inducible, antigen-specific T-T hybridoma cells are alternative probe for the identification of tumour antigens

As aforementioned, most methods for the identification of unknown tumour antigens depend on the use of a large number of CTL as a detecting probe in screening recombinant DNA expression libraries or any candidate antigens from biochemically eluted protein and/or synthetic peptides. Such assays can be carried out by using short or long-term *ex vivo* culture of clonal CTL derived from PBMC or TIL of tumour-bearing host. However, the procedures involved in the maintenance, expansion, and cloning of the CTL is highly labour-intensive and time-consuming. In theory, T cells, like most other mammalian cells, can not be immortal or automatically proliferate in *ex vivo* condition. In addition, any unexpected condition could possibly

cause the CTL to die or change their properties, such as bacteria, mycoplasma or virus infection, overgrowing and so on.

In 1980, Nabholz and colleagues [194] reported an alternative strategy using somatic hybridization technology to establish a functional T-T hybridoma cell. The hybridoma cells have essentially unlimited growth potential and grow readily in standard culture medium under normal condition. Furthermore, unlike their parental T cells, the immunological activity of these hybridoma cells is not subject to cyclic fluctuations. Thus, it is relatively easy and convenient to provide hybridoma cells with extensive homogenous functions. The availability of such populations would facilitate the studies at molecular basis of the immunological activities of cytotoxic T cells. In practice, Ozaki and colleagues (1988) [195] compared in parallel the functions between T cell clones and the hybridoma cells that derived from them and reported that the functional validity (such as killing efficiency or cytokine production against antigen-bearing target) of T cell hybridoma cells were as faithful as their parental T cell clones.

Other difficulty in the work of antigen hunting lies in the inherent lack of a sensitive throughput assay for assessing the CTL cell activity. To overcome this obstacle, Karttunen and Shastri (1991) [196] have developed a "LacZ assay" to measure TCR occupancy in single T cell. The strategy is based on the identification of the nuclear factor in activated T cells (NF-AT) DNA element within the IL-2 gene as a key element involved in transcriptional regulation of IL-2 gene [197, 198]. Thus, a DNA construct including an enhancer element that contains three tandemly repeated NF-AT elements with a downstream heterogeneous *Escherichia Coli* β-galactosidase (LacZ) reporter gene (see Figure 1.5 below) was introduced into hybridoma cells. Because

the endogenous IL-2 gene and the NFAT-LacZ construct share similar requirements for NFAT mediated transcription, the occupancy of the T cell receptors by specific antigen triggers the intracellular accumulation of LacZ gene product, β-galactosidase, as well as the secretion of IL-2 in parallel manner.

To introduce a LacZ reporter gene into a hybridoma cell, Sanderson and Shastri (1995) [199] described a general approach by deriving two new LacZ inducible fusion partners, BWZ.36 and BWZ.36CD8α, by transfecting the TCR-deficient lymphoma cell line, α-β-BW5174, with the NFAT-LacZ DNA construct. Fusion of normal T cells derived from immunized mice with appropriate fusion partner allows the generation of T-T hybridoma cells in which LacZ activity is specifically induced in response to an antigen/MHC complex.

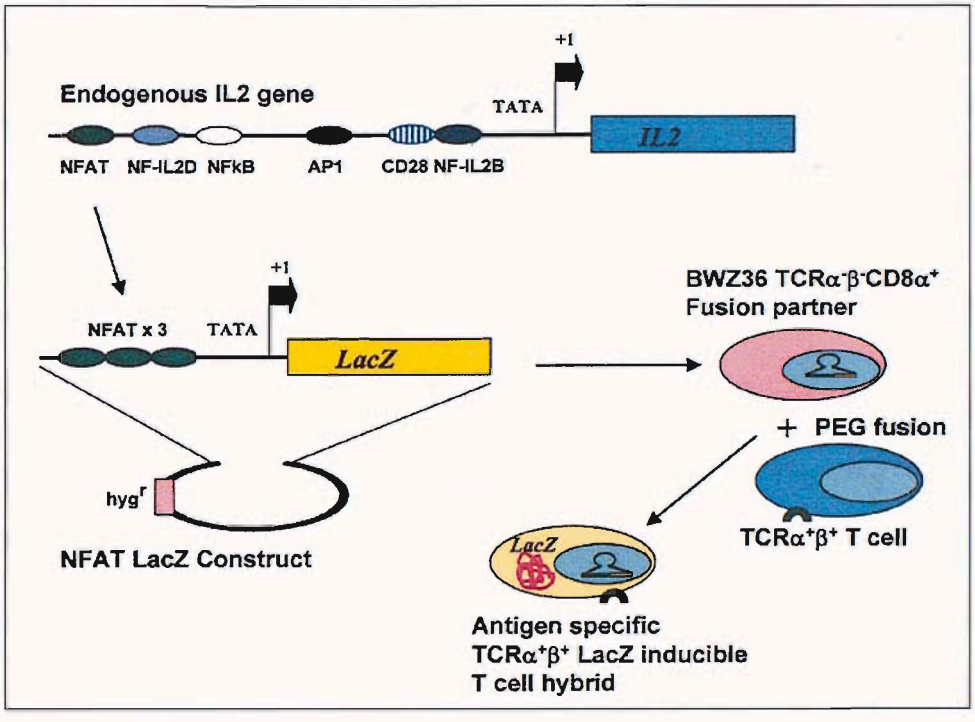


Figure 1.5. Molecular basis of LacZ inducible T cell generation.

BWZ36CD8α fusion partner was transduced with a NFAT-LacZ construct, thus after fusion with TCRαβ T cell, and LacZ activity in hybridoma cell is under the activation of TCR as for endogenous IL-2. (Copy from **Malarkannan S.,** *et al.* Generation and analysis of LacZ inducible T cell hybrids. ***Methods Mol. Biol.*** 2110; **156:** 265-272).

The TCR-mediated LacZ activities within hybridoma cells can be measured using a variety of either fluorogenic or chromogenic substrates [200]. The most common LacZ assay used in various experiments is the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG). In general, the cleavage of CPRG by enzyme β-galactosidase results in the coloured production of chlorophenol red (see figure 1.6 below), which can otherwise be quantified conventionally by measuring its absorbance using a standard 96-well plate reader. One other popular method is taking advantage of a chromogenic substance, 5-bromo-4-chloro-3-indolyl β-D-galactoside

(X-gal), to stain the formaldehyde-fixed activated LacZ-positive cells in a single cell basis. The X-gal staining could allow direct visualization under a microscopic examination of a single T-T hybridoma cell with induced intracellular β-galactosidase in intense blue colour (see figure 8), although it is tedious to quantify the number of cells under microscopy. Other substrates of β-galactosidase used in hybridoma experiments include less sensitive but cheaper chromogenic substrate, o-nitrophenyl β-D-galactopyranoside (ONPG), or the slightly sensitive but also more expensive fluorogenic substrates 4-methylumbelliferyl β-D-galactoside (MUG) and di-β-D-galactopyranoside (FDG) that can be measured using a fluorescence plate reader. The later fluorogenic FDG substrate can also be applied for the analysis of LacZ activity in viable cells by means of flow cytometry [200].

Measurement of specific T cell response with such a non-radioactive LacZ assay is simpler, faster, and more cost-effective than most other conventional assays or bio-assays. In addition, the LacZ assay allows sensitive detection of activation on a single cell basis when the number of APC is limiting. The validity of LacZ assay for identifying T cell epitopes has been proven [180, 196, 201] and several MHC class I - restricted antigens have been identified with LacZ-inducible T-T hybridoma cells [202, 203, 204].

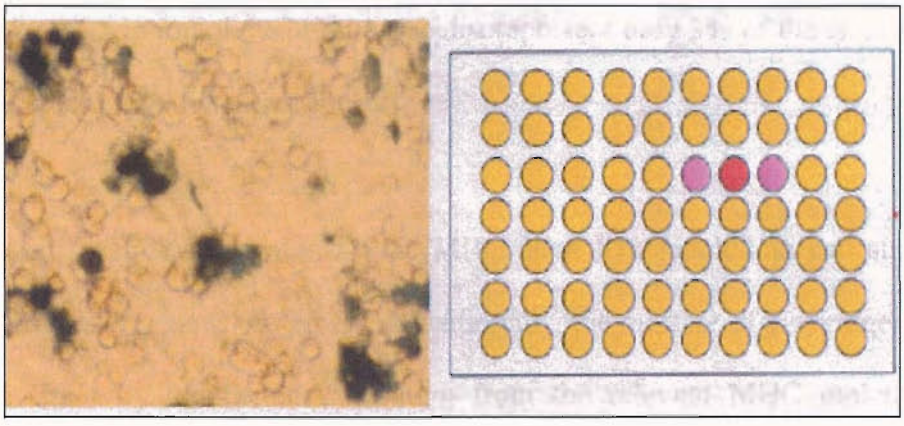


Figure 1.6. Colour changes in chromogenic X-gel stain and CPRG assay.

Examples of the substance used in measuring the enzymatic activates of induced β-galactosidase from hybridoma cells. In left panel, each blue spot represents a single LacZ-positive hybridoma cell. In right panel, the red colour in some wells represents that CPRG is catalyzed by TCR induced β-galactosidase and converted from yellow to red colour. (Copy from **Malarkannan S.,** *et al.* Generation and analysis of LacZ inducible T cell hybrids. ***Methods Mol. Biol.*** 2110; **156:** 265-272).

1.3.4.4. cDNA expression libraries represent the both expressible mRNA and functional gene product.

A molecular library is defined as a collection of various molecules that can be screened for individual species that show specific properties. Therefore, any level along the gene-mRNA-protein axis could be utilized for the generation of a library. Among these, mRNA molecules cannot be used directly in cloning and library construction, because they are very unstable and could be easily degraded. However, it is possible to synthesize complementary DNA molecules from all the mRNA in selected cells. cDNA are complementary DNA copies of mRNA. As the intervening sequences (i.e. introns) of genomic DNA have been previously removed in the process

of mRNA transcription, the cDNA molecules represent only 3% of the genomic DNA that are transcriptionally active genes.

The antigens/peptides presented by the MHC class I molecules on cell surface are derived from endogenous proteins. Conventionally, the mixture of the antigens can be prepared either by biochemically elution from the relevant MHC molecules and HPLC or by positional cloning and synthesis. However, these methods required sophisticated instrumentation, training, and the prior knowledge of the antigens. Alternatively, the cDNA expression libraries have been proven to be a feasible strategy [180, 205]. Using antigen-specific CTL, T-T hybridoma cells or serum IgG Ab as probes, the expression cloning system requires just small pools of cDNA libraries, constructed from the antigen-bearing cells, to be transfected into recipient antigen-presenting cells that lack the targeted antigens themselves. The relevant MHC molecules may be constitutively expressed in the recipient APCs or by co-transfection with the cDNA libraries. Expression of the transfected cDNA in the recipient APCs allows the generation and expression of the appropriate peptide/MHC complexes that can be detected by CTL or hybridoma cells.

Another essential element for the expression cloning system is the identification of appropriate recipient cells for antigens presentation. The most important criteria for the choice of recipient cells include: 1) whether the recipient APCs will stimulate T cells activity without transfection and result in an unacceptably high background reaction; 2) whether the antigen processing of the recipient cells is identical or similar to the antigen-bearing cells; 3) whether the recipient cells are readily transfectable and efficient in expressing the indicated protein.

To meet these criteria, several cell lines have been considered, among these, COS-7 cells [179, 180, 181, 182], L293 cells [206] and MOP cells [207] are extensively used as recipient cells in mammalian transfection-expression system. Alternatively, the transduction of retroviral cDNA libraries in autologous fibroblast cells [208] provides a method to isolate unique tumour-specific antigens without the necessity to identify the MHC-restriction.

Since these recipient antigen-presenting cells may not express the right MHC class I molecules, the relevant MHC gene must be co-transfected into the recipient cells along with the cDNA library. Therefore, the isolation of a cDNA encoding a defined tumour antigen from library-presenting cells requires the prior determination of the MHC class I molecule presenting the antigenic peptide to the CTL clone. This is readily accomplished with appropriate anti-MHC monoclonal antibodies to inhibit the tumour-specific activities of CTL [209, 210]. A second method to identify the MHC presenting molecule is to correlate recognition by the CTL clone and expression of a definite class allele. This can be performed by testing the CTL responses in the presence of several allogeneic tumour lines that share one class I allele with autologous tumour [209, 211]. These allogeneic tumour cell lines can also be generated by transfection with candidate MHC genes. If the presenting MHC class I molecule cannot be identified, an alternative method is to transfect the cDNA library into recipient cells together with two or three putative MHC class I candidate molecules.

The cDNA libraries could be constructed from cells of interest (see appendix 6) and efficiently transfected into recipient antigen-presenting cells together with gene encoding the appropriate MHC class I molecule by various protocols. These include electroporation, lipofection, calcium phosphate coprecipitation, polybrene-assisted gene transfer, and DEAE dextran-mediated transfer. Among these, the DEAE-dextran

protocol coupled to a shock treatment is simple, straightforward, and gives significant and consistent high transfection efficiencies with low toxicity [212]. DEAE-dextran is widely used for gene transfer into mammalian cells [213, 214]. Transient transfection following DEAE-dextran protocol allows the recipient cells to express the introduced gene in 24-48 hours. Furthermore, the transient expression offers several advantages over long-term stable transfection. It obviates the time-consuming procedure of selecting cells that have integrated exogenous DNA inserted. It also obviates the variability introduced by the integration of the transfected DNA, since the level of expression of each stable transfectant highly relies on the location and the number of integrated copies. The recipient cells are readily harvested 48 hours after transfection for analyzing expression of the transfected cDNA [180, 181].

1.4. Tumour escape from immunosurveillance

The process of tumour development from a single transformed cell into a mass of malignant cells involves a series of genetic changes in the progeny of transformed cells over a period of many years. The original transformed clone is gradually replaced by a variety of genetically altered clones, which accumulate and make up a heterogeneous population of malignant cells. Tumour cells are genetically unstable, and the emergence of genetic variants ensures that tumour survives in the face of host immune system. In spite of ample evidence that immune effectors can play a significant role in controlling tumour growth in the natural environment or in response to therapeutic manipulation, cancer cells can evade immune surveillance in most cases [9]. Several mechanisms have been described to be involved in the tumour escape from host immunological surveillance [9].

Some metastatic tumours have been shown to be associated with loss of the MHC class I molecular expression on the tumour surface [215] or prevention of antigenic presentation by mutations of other proteins involved in the processing of antigenic peptides (e.g., TAP) [216]. Tumours could also down-regulate tissue-specific anti-tumour responses by down-regulating the pro-inflammatory stimuli on antigen-presenting cells [217].

The increased understanding of the importance of costimulatory molecules in the regulation of T cell responses has led to a new insight of cancer immunosurveillance. Ample evidence has shown that both positive and negative costimulatory molecules are involved in T cell activation [218, 219, 220]. Among these, there is a recently documented novel mechanism by which tumour can escape from host immune system

by PD-L1. PD-L1 is a B7 family member, which negatively regulates T cell responses by engagement with a member of CD28 family, PD-1, an immunoinhibitory receptor on previously activated T cells. It has been found that PD-L1 is expressed in various tumour cell lines and tumour tissue both in human and mouse [221, 222].

A recently recognized enzyme, indoleamine 2, 3-dioxygenase (IDO), which catalyzes the initial and rate-limiting step in the catabolism of tryptophan along the kynurenine pathway [223], is suggested to play a crucial role in the tolerance of malignancy [152] using its essential role in preventing rejection of allogeneic foetuses in mouse placenta. It was shown that IDO is expressed constitutively in most human tumours derived from various histological sources; in cells exposed to interferon (γ-IFN) and in certain types of activated macrophages and dendritic cells [224, 225]. By depleting tryptophan and the production of pro-apoptotic metabolites locally, IDO seems to block the proliferation of alloreactive T lymphocytes, which are extremely sensitive to tryptophan shortage.

Other effective strategies to escape from immune surveillance include the secretion of tumour-derived immunosuppressive factors in tumour microenvironment, such as interleukin 10 (IL-10) and transforming growth factor-β (TGF-β) [226, 227], which exert either local or systemic effects on the host antitumour immune responses.

In addition, most tumour-associated antigens (TAA), unlike exogenous bacterial or viral antigen in infection, are largely self antigens or altered self antigens. As a result, vast majority of TAA are perceived as "self" by host immune system, and, in the absence of "danger signal", are frequently poorly immunogenic or simply ignored by host immune system. In this context, the induction of immunological tolerance against

TAA is presumably mediated by clonal deletion, anergy of T cells [228] or by regulatory T cell-mediated active inhibition.

1.4.1. Naturally occurring CD4+CD25+ regulatory T cells mediate peripheral tolerance to autoantigens

Two mechanisms have been put forward to account for host immune tolerance to self antigens, "central tolerance" and "peripheral tolerance". The central models has been proposed that tolerance is ensured by the absence of self-reactive T cells, which happens in the development of T lymphocytes in thymus – either by selection processes (positive and negative selection) in thymus or by functional anergy. In "positive selection", the developing CD4+ and CD8+ T cells are selected by promoting the survival, maturation and expansion of immature CD4+CD8+ (double positive) thymocytes with TCRs that are engaged in a low-affinity interaction with a self-MHC molecules on thymic epithelial cells and by permitting thymocytes whose TCRs are not self- MHC- restricted to die by apoptosis. Thereafter, CD4+CD8+ thymocytes with MHC class I-restricted TCRs become CD8+CD4-, and cells with class II-restricted TCRs become CD4+CD8-. Whereas, the "negative selection" of thymocytes works by inducing apoptosis of cells whose TCRs recognize self peptide-MHC complex on thymic antigen-presenting cells with high affinity. Thus, the process ensures the clonal deletion of self-reactive T cells occurs when the TCRs on any CD4+CD8+ thymocyte bind strongly to self-peptides presented in thymus.

However, central tolerance does not account for all tolerance since autoreactive T cells capable of causing autoimmune disease are readily found in the peripheral tissue of most normal healthy individuals. The peripheral tolerance models, on the other hand, depend on an active process to control or inhibit the activities of autoreactive

lymphocytes, such as anergy, clonal deletion and functional suppression (see figure 1.7) [229, 230]. There is ample evidence that this active peripheral tolerance is mediated by some subsets of T cells which are functionally distinct from conventional helper and cytotoxic T lymphocytes [229, 230]. Several different types of cells have been found bearing regulatory capacity, including Tr1 cells that are generated by activation in the presence of IL-10 [231], and transforming growth factor-β (TGF-β)-secreting Th3 cells found in oral tolerance [232]. More recently, a unique naturally occurring lineage of CD4+ T cells that suppress T cell effector function has attracted much attention [10].

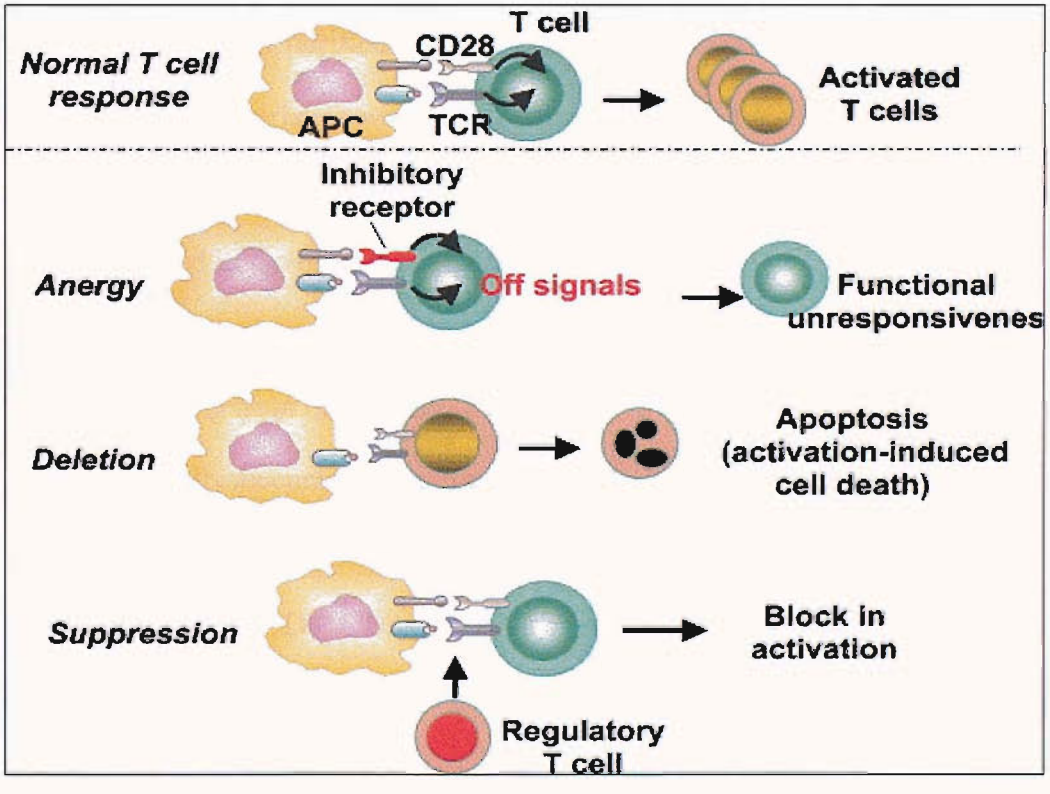


Figure 1.7. The mechanisms of peripheral tolerance

The mechanisms that maintain tolerance to autoantigen in T cells response are illustrated, and compared with normal immune responses (copy from **Abbas A. K.**, *et al.* T cell tolerance and autoimmunity. ***Autoimmune Rev.*** 2004; **3:** 471-475).

In 1969, Nishizuka and Sakakura first described the phenomenon of suppression of autoimmune disease mediated by a T cell population [233]. They found that thymectomy of mice on the third day of life (d3Tx) would result in multi-organ-specific autoimmune diseases, and process can be prevented by reconstituting these d3Tx mice with adult thymocytes or splenocytes. In 1995, CD4+CD25+ T cells were first identified by Sakaguchi and colleagues [10]. They described a subpopulation of CD4+ T cells that expressed the IL-2 receptor α chain (CD25), and, in their experiment, adoptive transfer of T cell populations depleted of the CD4+CD25+ subset into nude (athymic) mice led to a variety of autoimmune diseases that could be prevented by co-administration of purified CD4+CD25+ T cells. Later on, T cells bearing similar phenotypic characteristics were also identified in rodents and human PBMC; and *in vitro* data have shown that human CD4+CD25+ T cells exhibit similar functions to those described in the murine counterpart cells [234, 235, 236].

In differentiating from other induced regulatory T cells, these CD4+CD25+ T cells are called "naturally occurring" regulatory T cells because they are present in normal unmanipulated individuals. As summarized by Sakaguchi [237], Fontenot and Rudensky [238] in their reports, CD4+CD25+ T cells can be found in thymus of newborn mice at day 2 with functional properties of naturally occurring regulatory T cells before there are many T cells of any kind detected in spleen. And immature CD4-CD8- double negative, Thy-1.2-marked thymocytes injected into the thymuses of irradiated Thy-1.1 mice develop into CD4+CD25+ T cells in one week [239]. These factors all suggested there is a separate linage of CD4+ T cells with regulatory properties develops in the thymus.

The naturally occurring regulatory CD4+CD25+ T cells represent 5-10% of peripheral CD4+ T cells in mice and human. In animal studies, transfer of the CD4+CD25+ T cell-depleted CD4+CD25- T cells to immunocompromised hosts induced a spectrum of autoimmune diseases, including diabetes and colitis [240, 241, 242]. In contrast, co-transfer of CD4+CD25+ cells would inhibit the induction of autoimmunity [243]. The roles of CD4+CD25+ T cells in human diseases are just starting to be examined. Similar to murine autoimmune disease, CD4+CD25+ T cells from patients with multiple sclerosis [244] or Type II autoimmune polyglandular syndrome [245] were found impaired in their suppressive function. These and other studies suggested that the CD4+CD25+ T cells are crucial in the maintenance of peripheral tolerance to self antigens. More recently, it has been suggested that the roles of CD4+CD25+ T cells are not limited in the prevention of autoimmune disease but are also importantly involved in the control of actually all forms of host immune responses, including inflammation, infection, allergy, graft-versus-host diseases, transplantation and immune responses to tumour antigens [246, 247, 248].

As aforementioned, in addition to the naturally occurring CD4+CD25+ regulatory T cells, it was suggested that there are other types of regulatory T cells, which are described as "induced". A variety of *in vitro* and *in vivo* protocols have been developed to artificially generate these "induced" regulatory T cells. For example, T cells with regulatory function are generated in tolerogenic or anti-inflammatory contexts and can produce high amount of inhibitory cytokines, such as IL-10 and/or TGF-β [231, 232]. These regulatory T cells exhibit suppressive functions that are somehow dependent on the secreted cytokines. Amongst these subsets, IL-10 producing Tr1 cells have been characterized [231]. Tr1 cells appear to be distinct from

CD4+CD25+ cells in their origin and mode of action. Tr1 cells are generated from culturing naïve CD4+CD25- T cells in the presence of antigen and IL-10, and their *in vitro/in vivo* suppressive activity depends on the secretion of IL-10. Another subset of CD4+ regulatory T cells, designated as Th3 [232], has been identified in mouse models where they are characteristically generated following administration of oral antigen. Th3 regulatory cells bear suppressive functions and predominantly secrete IL-4, IL-10 and TGF-β.

1.4.2. The molecular basis of CD4+CD25+ regulatory T cells

The naturally occurring regulatory T cells are characterized by the phenotypic expression of CD4 and the IL-2R α-chain (CD25). In addition, they express little CD45RB ($CD45RB^{low}$) and a significant portion (50%) express the inhibitory costimulatory receptors - cytolytic T lymphocyte-associated antigen-4 (CTLA-4) and PD-1 [240, 241, 249]. Other phenotypic markers of these regulatory cells include glucocorticoid-induced TNFR-related protein (GITR), OX40, 4-1BB and TNFRII [246, 249]. Although the roles of these molecules in the functions of CD4+CD25+ T cells remain to be established, CTLA-4 and GITR have been implicated in the activation or inhibition of their regulatory activity respectively [247, 249]. However, most described cell surface molecules on CD4+CD25+ regulatory T cells are also expressed on other CD4+CD25- T cells upon activation. Currently, no single phenotypic marker can precisely differentiate CD4+CD25+ T cells from conventional activated CD4+ T cells. This might remain to be a main hurdle *to date* for characterization of the molecular and immunological mechanisms of these CD4+CD25+ regulatory T cells.

Although CD25 has been used to characterize the naturally occurring regulatory T cells in most experiments, CD25 is not a specific marker, since it cannot distinguish regulatory T cells from activated effector T cells., making it difficult to assess the make-up of the CD4+CD25+ T cell pool in disease state. Recently, a forkhead/winged helix transcription factor, FoxP3, was identified to be critical for the development of CD4+CD25+ T cells and their suppressive functions [250, 251]. Targeted disruption of the FoxP3 gene (Foxp3-/-) prevents CD4+CD25+ development and function, and induces lethal inflammatory disease in mice, including type-1 diabetes, allergy and inflammatory bowel disease-like enteropathy , a clinical outcome synonymous with what is believed to occur in Scurfy mice and X-linked inheritance patients (IPEX) [252, 253]. Furthermore, unlike CD25, FoxP3 is not up-regulated following activation on CD4+CD25- T cells in mice, suggesting that it is not a marker of chronic activation [176]. These studies suggest that FoxP3 has a determining role in the generation of CD4+CD25+ regulatory T cells [250, 251]. However, as FoxP3 is a nuclear protein, it is of no value as a surface marker to isolate regulatory T cells from a pool of lymphocytes *ex vivo*.

Generation of these regulatory T cells is an important function of the thymus. The antigen specificity directing selection and differentiation of the CD4+CD25+ regulatory T cells and their peripheral activation still remain unclear. There is evidence that CD4+CD25+ T cells are selected based on high affinity interaction with self-antigens in the thymus, and peripheral proliferation of regulatory T cells is highly dependent on the presence of specific antigens [254]. It has been reported that these cells have a polyclonal TCR repertoire based on diverse gene expression of various TCR$\alpha\beta$ elements, and therefore could conceivably recognize a wide spectrum of self-antigens [180]. On the other hand, it has also been shown that these cells can be expanded by

various foreign stimuli in peripheral tissue [255]. Currently, whether the TCR repertoire of CD4+CD25+ regulatory T cells is based toward only self antigens or is as diverse as that of CD4+CD25- T cells is still unknown.

The signals required for the generation and maintenance of regulatory T cells *in vivo* are not thoroughly understood. Recently, the importance of CD28 co-stimulation in thymic development and peripheral homeostasis of the regulatory T cells has been highlighted [240, 256], as illustrated by CD28-/- and B7.1/B7.2 -/- non-obese diabetic (NOD) mice which developed diabetes more rapidly than their NOD control mice. CD28 engagement might be important for their survival and self-renewing, possibly through the expression of IL-2, Bcl-2-like anti-apoptotic molecules or other cytokines [256]. In addition, although CD4+CD25+ regulatory T cells do not produce IL-2 upon TCR engagement *in vitro*, an intact IL-2 - IL-2R reaction signal is still crucial for the development and peripheral homeostasis *in vivo*, because mice deficient for IL-2, IL-2Rα or IL-2Rβ have few or no regulatory T cells and die prematurely from severe lymphoproliferative and autoimmune syndrome [257, 258].

In most of the *in vitro* experiments, it has been suggested that, although the initial activation of CD4+CD25+ regulatory T cells is antigen-specific, the suppressive effect of these cells is antigen-non-specific [11, 234, 236]. On the other hand, the regulatory cells have been shown to proliferate in an antigen-specific manner *in vivo* [259, 260, 261]. However, whether CD4+CD25+ regulatory T cells are antigen-specific during their effector phase *in vivo* remain unclear.

Joffre and colleague (2004) [262] have designed a mice model in which irradiated mice were reconstituted with *in vitro* cultured CD4+CD25- T cells from host mice and CD4+CD25+ regulatory T cells from target mice following transplantation with

chimeric bone marrow from target and third party mice which differ in their major and minor histocompatibility antigens between three strains of mice. It has been found that target bone marrow was protected, whereas third-party bone marrow was rejected. They concluded that CD4+CD25+ regulatory T cells can act *in vivo* in an alloantigen-specific manner during effector phase. However, in this experiment, both components of bone marrow were protected from rejection when higher number of CD4+CD25+ regulatory T cells was injected, suggesting disparate results. Currently, this issue remains controversial before any antigen-specific clone of CD4+CD25+ regulatory T cells could be isolated and checked their *in vivo* function.

Recent studies have shown that freshly isolated CD4+CD25+ regulatory T cells from normal naïve mice do not proliferate or produce IL-2 in response to TCR stimulation *in vitro*, as demonstrated by the lack of DNA replication and IL-2 secretion [11]. After TCR activation, they do potently suppress the activation and proliferation of other CD4+ and CD8+ T cells by inhibition of IL-2 transcription in these cells and/or by depletion of residual IL-2 from lymphoid microenvironment [12, 263]. Some experiments have documented that their suppressive function is exerted by a cell-to-cell contact manner and probably not by the secretion of certain inhibitory cytokines, such as IL-10, and TGF-β [11, 234, 236]. On the other hand, their activity *in vivo*, however, seems to have a certain dependency on the production of IL-4, IL-10 and TGF-β [264, 265, 266].

Other mechanisms reported include suppression by unknown cell surface molecules or short-acting cytokines operated during contact between regulatory T cells and effector T cells [267]. Similarly, recent evidence presented by Takeshi and colleagues (2003) [268] also supported that human CD4+CD25+ regulatory T cells suppress NKT cell function by cell-to-cell contact, not by a humoral factor or by inhibiting the

priming of antigen-presenting cells. Both the proliferation and cytokine production functions were found to be suppressed in all three subsets of Vα24+ NKT cells (Vα24CD4-CD8-, Vα24+CD4+ and Vα24+CD8+ cell).

Some studies have suggested that CD4+CD25+ regulatory T cells suppress the activation of CD8+ T cells by inhibiting the priming of dendritic cells and thus down-regulate the expressions of the some important costimulatory molecules on antigen-presenting cells, such as CD80 and CD86 [269]. However, others have suggested that the suppressive function of CD4+CD25+ regulatory T cells could be exerted in the absence of antigen-presenting cell (APC) [263, 270]. To date, the interactions between regulatory T cell, effector cells and dendritic cells still need to be further explored.

In spite of a large number of current *in* and *ex vivo* studies examining the cell biology of these regulatory cells, we have only a limited understanding of the precise molecular mechanisms that govern their development and *in vivo* activities. The current conflicting findings made it difficult to characterize the molecular basis of phenotype, activation requirements and mechanism of suppression. What is their recognition properties and affinity? How and where are these cells induced to develop? Are there other specific phenotypic markers for identification? By what mechanism do CD4+CD25+ regulatory cells prevent immunity *in vivo*? How do these cells operate in relation to other population of regulatory cells, APCs and other T cell populations? To date, no conclusive answer has been made.

1.4.3. CD4+CD25+ regulatory T cells: a common basis between tumour immunity and autoimmunity

A broad set of tumour-associated antigens has been identified by antigen-specific

CTLs, suggesting tumour immunosurveillance can occur *in vivo*. In practice, these antigens can not always efficiently activate the immune system to eliminate tumours in cancer patients. One important reason might be that many of the CTL-defined tumour antigens are self-antigens [138, 271], therefore are frequently tolerated by host immune system.

In some clinical trials of cancer immunotherapy, a number of cases using immunization by various vaccination strategies directed against tumour-associated antigens that are shared with normal host cells had obvious tumour regression observed. Meanwhile, the same patients are sometimes observed complicated with some autoimmune diseases caused by concurrent breaking of self-tolerance. For example, anti-melanoma immunity induced by vaccines directed against a variety of melanoma tissue-specific differentiation antigens sometimes was complicated with the occurrence of T cell-mediated vitiligo [272]. Similarly in many animal experiments, immunotherapy of tumour has been shown to result in severe autoimmune disease [273, 274]. These findings and others have implied that tumour immunity and autoimmunity might be linked together and the mechanisms maintaining tolerance to self-antigens may also impede the generation of effective immunity against tumours. Since CD4+CD25+ regulatory T cells emerged as the dominant T cells population governing peripheral self-tolerance to autoantigens, a hypothesis was proposed that CD4+CD25+ T cells may be involved in tumour escape from immune surveillance as they are involved in the prevention of autoimmune disease [275].

In addition, it has been addressed in some studies that increased population of the CD4+CD25+ regulatory T cells infiltrating around tumours or in tumour-draining lymph nodes was found in patients with various cancers, such as non-small cell lung

cancers, ovarian cancers [276], oesophageal cancers [277] and gastric cancer [278]. Elevated number and relative percentage of regulatory T cells in peripheral circulation of cancer patients were also documented in cases with gastric, oesophageal cancers and other epithelial tumours [277, 279]. Woo E.Y. and colleagues (2001) [276] further suggested that the increased presence of tumour-infiltrating CD4+CD25+ T regulatory cells in patients with solid tumours may contribute to a profound effect in inhibiting the antitumour functions of effector tumour-infiltrating lymphocytes.

More recently, Curiel and colleagues (2004) [280] have also demonstrated that, in patients affected with ovarian carcinoma, human CD4+CD25+FOXP3+ regulatory T cells preferentially move and accumulate in tumour and malignant ascites, but rarely enter draining lymph nodes in later cancer stages. The tumour infiltrating regulatory T cells could suppress tumour-specific T cell immunity and contribute to tumour growth *in vivo*, and therefore, the increased number of tumour-infiltrating regulatory T cells is associated with a high death hazard and reduced survival. The hypothetic close relationship between effector and regulatory T cells has also been demonstrated by an *in situ* staining technique and multicolour confocal microscopic analysis that the CD4+CD25+FoxP3+ T cells were in close contact with CD8+ T cells in tumour microenvironment. More importantly, it was found that tumour cells and microenvironmental macrophages can produce a chemokine, CCL22, which mediates the trafficking of regulatory T cells into the tumour microenvironment. This specific recruitment of regulatory T cells represents a mechanism by which tumours may foster immune privilege.

Hiroyoshi and colleague (2003) [281] demonstrated their experiment, in which vaccination in BALB/c mice with a pool of SEREX-defined tumour-associated

antigens derived from MCA-induced sarcoma cell lines could surprisingly heighten susceptibility to tumour challenges. It was found that CD4+CD25+ regulatory T cells in vaccinated mice were activated and suppressed the tumour immune response. The majority of SEREX-defined tumour antigens were shown no structural abnormality and therefore were considered to be wild-type autoantigens. In this experiment, the immuno-regulation of regulatory T cells was known to function through their inhibition to the immune activity of NKT cells.

In another study, passive transfer of the CD4+CD25+ regulatory T cells derived from fibrosarcoma-bearing mice was shown to impair the generation of tumour immunity in the recipient [248]. Similar observations that CD4+CD25+ regulatory T cells rendered animals unresponsiveness to tumour challenge were also noted in a variety of murine tumours [282, 283]. These and other findings have led to a hypothesis and a variety of animal experiments in which tumour immunity is challenged in hosts that are selectively depleted of CD25+ regulatory T cells by *in vivo* administration of an anti-CD25 monoclonal antibody.

Simuzu and colleague (1999) [13] have demonstrated that in some murine models of tumour immunotherapy, in which tumour rejection is usually unsuccessful because the targeted tumour-associated antigens are frequently poorly immunogenic, tumour immunity can be further enhanced by *in vivo* depletion of CD4+CD25+ regulatory T cells. In another case, depletion of regulatory T cells before vaccination with bone marrow-derived dendritic cells (BM-DC) loaded by stressed poorly-immunogenic melanoma tumour cells, B16-F10, considerably increased the ability of vaccinated host to survive from the following tumour challenge and allowed the development of long-lasting tumour immunity [284]. More recently, Hiroshi Nagai and colleague

documented that *in vivo* elimination of CD25+ regulatory T cells leads to tumour rejection of B16F10 melanoma when combined with interleukin-12 gene transfer [285]. Similarly, in a broad range of other studies, depletion of CD4+CD25+ T cells *in vivo* by anti-CD25 mAb could provoke tumour immunity in multiple strains of mice and induce rejection of a variety of immunogenic tumours, such as leukemia, sarcoma and malignant melanoma [14, 15, 96, 97].

The exact mechanisms by which the *in vivo* depletion of CD25+ regulatory T cells by administration of anti-CD25 mAb could enhance antitumour immunity are largely unknown. It has been suggested that the enhanced tumour immunity is mediated primarily by tumour-specific CD8+ cytotoxic T cells. However, other cellular elements of host immune system, such as CD4+ helper T cells and NK cells, might possibly also involve in this approach. Surprisingly, in the studies reported, the selective *in vivo* depletion of CD25+ regulatory T cells that has enhanced tumour immunity or induced efficient rejection of tumours did not provoke evident organ-specific autoimmune diseases. As a result, this approach, alone or in combination with other vaccination strategies, has been suggested to hold great potential in the future development of cancer immunotherapy.

1.4.4. Depletion of CD25+ regulatory cells uncovers immune responses to shared tumour rejection antigens

Earlier experiments in our laboratory, Golgher [16] using murine colorectal tumour CT26, has demonstrated tumour rejection in BALB/c mice that were selectively depleted of CD25+ regulatory T cells by intraperitoneal injection of a monoclonal antibody against CD25 (PC61) before challenge with subcutaneous injection of CT26

tumour cells (figure 1.8). The observed tumour immunity in this mice model is long-lasting and not only restricted to CT26 tumour, but also effective against the challenges of another colon tumour, C26, and tumours of other different histological types, such as two B cell lymphoma cells, BCL1 and A20, and renal cell carcinoma cells, RENCA (figure 1.9). Surprisingly, there was no pathological evidence of autoimmune diseases found in any immunized mice that received two sequential intraperitoneal injections of anti-CD25 mAb.

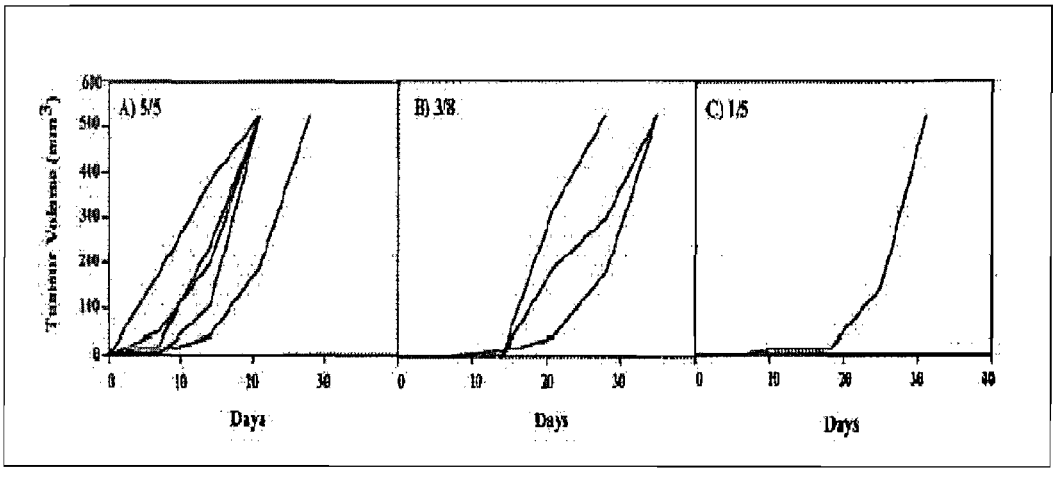


Figure 1.8. Tumour growth and long-term immunity in anti-CD25 mAb-treated mice.

Mice were injected i.p. with 1mg of either isotype control antibodies (A) or 1mg of CD25-specific mAb (B) 1and 3 days prior to subcutaneous inoculation of 5 X 10^4 CT26 cells. Each line represents an individual mouse. Approximately 10 weeks after injection of CT26 (group B). BALB/c mice were re-challenged with 10^5 CT26 cells (C). The numbers in the boxed indicate the number of tumour-bearing mice per total number of mice injected in each group (Copy from **Golgher, D.,** *et al.* Deletion of CD25+ regulatory cells uncovers immune responses to shared murine tumour rejection antigens. ***Eur. J. Immunol.*** 2002; **32:** 3267-3275).

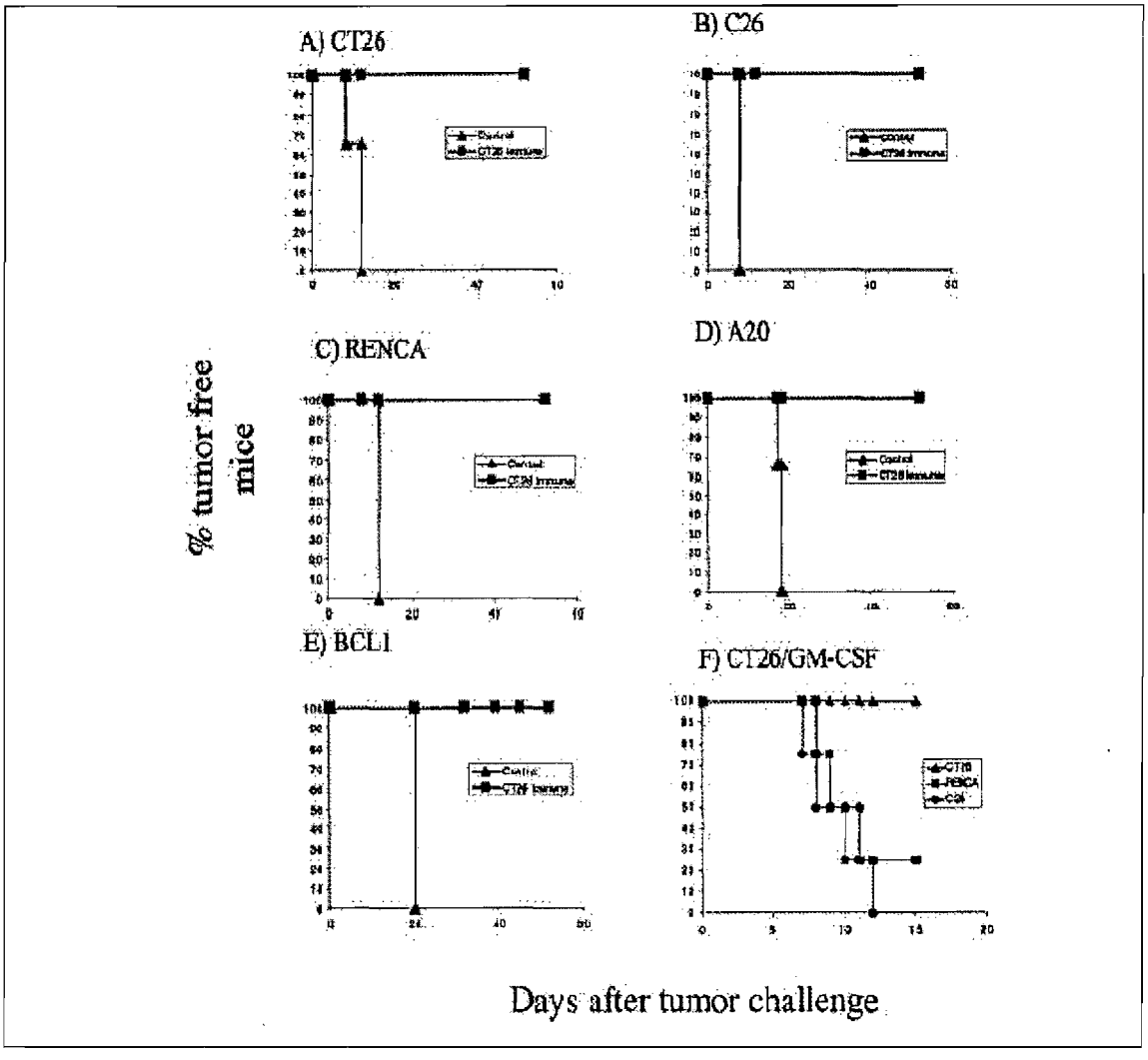


Figure 1.9. CT26-specific immune responses induced in the absence of CD25+ regulatory T cells are against shared tumour rejection antigens.

Mice injected with anti-CD25 mAb and that had rejected an inoculum of CT26 cells were re-challenged approximately 45 days later with 1 x 10^6 (A) CT26 (n=5), (B) C26 (n=5), (C) RENCA (n=5), (D) A20 (n=4) or (E) BCL1 (n=5). The percentage of tumour-bearing mice in each group is shown along with the percentage of tumour-bearing control naïve BALB/c mice (n=5). (F) Mice were vaccinated with irradiated CT26/GM-CSF (1 X 10^6 cells) and challenged 45 days later with 1 X 10^5 CT26 (n=3), C26 (n=4) or RENCA (n=4). Similar results were obtained in two independent experiments (Copy from **Golgher, D.,** *et al.* Deletion of CD25+ regulatory cells uncovers immune responses to shared murine tumour rejection antigens. ***Eur. J. Immunol.*** 2002; **32:** 3267-3275).

In another experiment, in which different subpopulations of T cells from survival immunized mice were adoptively transferred to SCID mice respectively; it was shown that both CD4+ and CD8+ T cells alone were able to mediate the challenge of CT26 tumour in recipient SCID mice. Similar results were also obtained by another forward experiment in which mice were challenged with CT26 tumour after *in vivo* depletion of CD4+ and/or CD8+ T cells by monoclonal antibody in combination with depletion of CD25+ regulatory T cells. On the other hand, both the CD4+ and CD8+ T cell subpopulations provoked in the immunized mice were required to work together for full protection against the challenges of other tumours, C26 and A20 tumours. Alone, each T cell subset confered partial protection against unrelated tumours.

Previously, Dranoff and colleagues (1993) [286] demonstrated a well-defined animal model, in which tumour immunity against CT26 tumour cells was generated using different strategy of vaccination (signature as CT26/GM-CSF model). In their experiment BALB/c mice were injected with irradiated CT26 tumour cells genetically engineered to secrete a murine cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF). Vaccinated mice were shown to reject the challenge of parental CT26 tumour. Both short-term and long-term CD8+ T cells lines were isolated from immunized mice and were shown to be able to kill parental CT26 tumour cells *in vitro*. Following adoptive transfer, these tumour-specific CD8+ T cell can cure other mice of established CT26 tumour [286]. Later in 1996, a non-mutated immunodominant peptide, AH1 ($gp70_{423-431}$), derived from the *envelope protein* (gp70) of an endogenous ectotropic murine leukemia provirus (MuLV), *env-1*, was identified as the reactive antigenic epitope in this CT26/GM-CSF system [287].

The *env* gene products of MuLV, including AH1, are known to be expressed in a

variety of H-2^d murine tumour cells, such as CT26 colon adenocarcinoma, B16 melanoma and TS/A mouse mammary adenocarcinoma [288, 289], whereas they are silent in the normal tissues of BALB/c mice (H-2^d). In addition, the gp70 gene products are not expressed in thymus. This could explain why central tolerance is not observed to these endogenous gene products. It is not clear whether the expression of the MuLV *env* products is critically involved in the initiation of transformation or tumourigenesis, or is the result of genetic deregulation associated with the process of tumour transformation. However, the selective expression of this *env* antigen in multiple non-viral-induced tumours provides further evidence of a set of unique shared tumour antigens that could be utilized as targets for anti-tumour immunity.

In comparing our model with CT26/GM-CSF model, it has been shown that the tumour immunity generated in our model is distinct to that generated following vaccination of mice with CT26/GM-CSF. First of all, it was CD8+ T cells that mediate the regression of CT26 tumour in mice vaccinated with CT26/GM-CSF. However, both CD8+ and CD4+ T cells were found to be capable of mediating rejection of CT26 in our model. Secondly, the tumour immunity induced in CT26/GM-CSF model is only CT26-specific, whereas the immunity arisen after deletion of CD25+ regulatory T cells has been shown to render cross-protection against various tumours of different histological origins. Finally, as aforementioned, it has been reported that reactive immunodominant antigenic epitope in CT26/GM-CSF model is AH1, a MHC class I H-$2L^d$ restricted peptide derived from *envelope protein* (gp70) of an endogenous ectotropic murine leukaemia virus [211]. However, we have shown that *gp70* is unlikely to be the bioactive antigen in our model by taking advantage of another experiment in which "cross-protective" mice in our model can not resist infection by a recombinant vaccinia virus expressing gp70 (rVVgp70),

whereas mice vaccinated with CT26/GM-CSF were able to resist the infection by the same rVVgp70 virus.

The results of our experiments have been further supported by another research group, Noelia Casares and colleagues (2003) [290]. In addition to those mentioned above, they also reported that the antitumour function mediated by CD4+ T cells in the absence of CD25+ regulatory T cells relies on their IFN-γ production, which exerts a potent anti-angiogenesis activity. They concluded that depletion of CD25+ regulatory T cells *in vivo* in combination with immunizations using either CTL or CD4+ helper cell reactive antigenic peptides would favour the induction of antitumour memory T cell responses.

Our results have implied that depletion of CD25+ regulatory T cells *in vivo* preferentially favours the stimulation of both CD8+ and CD4+ T cells to reject the following tumour challenges and long-term immunity. In addition, some unknown shared antigens that are expressed on a range of tumours derived from unrelated histological origins were uncovered through the deletion of CD25+ regulatory cells before immunization with CT25 tumour. Characterization of these shared antigens would be worthy for further exploring the mechanisms of antigenic recognition by T cells, the precise role of CD4+CD25+ regulatory T cells in tumour immunity and the interaction between effector and regulatory T cells at tumour site. Furthermore, the molecular identification of these shared antigens could provide possibilities for the development of cancer vaccines that could be relatively universal against a broad range of different tumours.

In order to further dissect the mechanisms by which tumour could escape from

immunosurveillance and the roles of CD4+CD25+ T cells in tumour immunity, we wish to identify the shared tumour antigens that are recognized by T cells and responsible for the observed tumour rejection in this animal model. Such antigens would subsequently facilitate in unveiling the strategies to break through the suppressed tumour immunity and the development of successful and relatively universal cancer immunotherapy against a set of malignancies. .

Although the need for CD4+ helper T cells in regulating CD8+ T cells-mediated anti-tumour activity has been reported [291, 292], the effector cells responsible in most observed tumour immunity are CD8+ T cells that recognize the antigens expressed by MHC class I molecules on tumour cells. Our initial experiments are directed toward the identification of the CD8+ T cell-reactive shared tumour antigens.

To facilitate the approach in the identification of the unknown CD8+ T cell stimulating shared tumour antigens, we attempted to isolate tumour-specific CD8+ T cells from the immunized mice, generate specific CTL clones and culture to expand these T cells *ex vivo*. The *ex vivo* culture of CTL clones were used as probes to subsequently screen the CT26 tumour cDNA libraries by a expression cloning system. Meanwhile, we tried to generate LacZ inducible hybridoma cells by fusion tumour-specific CD8+ T cells with a fusion partner, BWZ.36.CD8α. for an alternative strategy in the antigen hunting.

2.1. Mice

BALB/c mice (H-2d) were bred locally under specific pathogen-free condition in University of Southampton. Female mice, 6-8 weeks of age, were used in immunization. During experimental procedures, all mice were housed in conventional facilities with standard control.

2.2. Immunization of mice

Mice were intraperitoneally injected of anti-CD25 monoclonal antibody (PC61, 1 mg/per mouse) twice on Day-3 and day-1. On day+0, mice were subcutaneously inoculated with live CT26 tumour cells ($1x10^5$ cells/ per mouse). On day+7 to day+10, the immunized mice are sacrificed; spleens and flank lymph nodes were harvested and PBMC were prepared by standard procedure.

2.3. Cell lines

CT26 (ATCC) is a colon epithelial tumour derived by intra-rectal injection of *N*-nitroso- *N*-methylurethane in BALB/c mice [293, 294], A20 (ATCC) is a BALB/c-derived B cell lymphoma cell line [295]; pi-BCL1 was a new variant of the BCL1 syngeneic mouse B-cell lymphoma established in BALB/c mouse strain [296]. The immunophenotypic expressions and the growth characteristics of the pi-BCL1 and original BCL1 appeared very similar. However, pi-BCL1 can be easily grown and propagated in liquid culture. RENCA was a mouse renal carcinoma cell line derived

from BALB/c mouse strain [297]. CT26 cells and RENCA cells were a generous gift from Professor Drew Pardoll (Johns Hopkins University, Baltimore, MD). A20 cells and pi-BCL1 cells were generous gifts from Dr. Martin Glennie (University of Southampton, GB). MCFL cell is derived from syngeneic fibroblast cell line; K41 is wild-type mouse embryonic fibroblast cell line [298]. All these cells were maintained in complete RPMI medium (Gibco-BRL) supplemented with 10%FCS, 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 1mM sodium pyruvate, non-essential amino acid (NEAA, Gibco-BRL) and 50μM 2-mercaptoethanol.

The fusion partner cells, BWZ.36.CD8α [200], derive from the original thymoma cell line, TCRα-β-BW5147, that was stably transfected with a NFAT-LacZ construct as well as CD8α gene. The cells were maintained in ICTM medium supplemented with 360μg/ml Hygromycin-B and 1mg/ml G418.

WEHI 164 clone 13 cells were a generous gift from Dr. Pierre van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium) and were maintained with RPMI medium in non-tissue culture flasks. WEHI cell line is a TNF-β highly sensitive mouse fibrosarcoma cell line after pre-treatment with actinomycin-D [190, 299].

COS-7 cells are derived from the kidney of an African green monkey, transfected with replication defective SV40. MOP cells are derived from mouse (strain NIH/swiss) fibroblast cell (NIH/3T3 cell line), which is transformed by replication defective polyoma virus. L cells are derived from murine (strain C3H/An) fibroblast cell line (H-2k). COS-7 cell and MOP cell were purchased from CRUK and maintained in DMEM supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 10% foetal calve serum (FCS). L cells were maintained in complete RPMI culture medium.

Hybridoma cells secreting anti-CD25 monoclonal antibody (PC61, rat IgG1) were grown in ICTM medium supplemented with 360μg/ml Hygromycin-B and 1mg/ml G418. The PC61 mAb was purified by precipitation in saturated ammonium sulphate.

2.4. Antibodies

Monoclonal antibodies against mouse MHC class I molecules, HB-27 (28-14-8S, H-2L^d), HB-79(34-1-2S, H2-K^d/D^d) were kindly provided by Dr. Martin Glennie (University of Southampton, GB); HB-159 (SF1-1.1, anti-H-2K^d), and HB-102 (34-5-8S anti-H-2D^d) were purchased from BD Pharmingen. These anti-MHC class I monoclonal antibodies were used in blocking experiments to determine the haplotype of the reactive peptide/MHC complex. Anti-CD3e (500A2), anti-CD8a (Ly-2, clone 53-6.7) and anti-CD4 (L3T4) monoclonal antibodies were purchased from Pharmingen and used in FACS analysis. The anti-CD8a (Ly-2, clone 53-6.7) was also used in the CD8+ T cells blocking experiment.

R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD44 (IM7), anti-mouse CD45RC (DNL-1.9), and anti-mouse CD45RA (14.8) were purchased from BD Pharmingen. Rat monoclonal anti-mouse CCR7 (IgG2a) is a generous gift from Professor Jőrg Swirner (Department of Immunology, Georg-August-University Gőttingen, Germany). This antibody was used in combination with secondary antibody, R-PE-conjugated F(ab')s fragment Donkey anti-Rat IgG (H+L) (Jackson ImmunoResearch Lab), in FACS analysis.

Monoclonal antibodies against different TCR variable β chains (TCRV$_\beta$) (Vβ2, Vβ3, Vβ4, Vβ6, Vβ7, Vβ8.1/8.2, Vβ8.3, Vβ9, Vβ10b, Vβ13, Vβ14) were also a generous

gift from Dr. Martin Glennie (University of Southampton, GB) and used in TCRV$_\beta$ gene usage determination for clonality assessment [300].

2.5. Isolation and culture of antigen-specific T cells

2.5.1. T cell culture and generation of antigen-specific T cell line

The method used by Khanna and colleagues (1999) [79] to *in vitro* culture and expand EBV-specific CTLs was modified and applied in our T cell culture. In brief, single cell suspensions in complete medium were prepared from spleens and plated in 24-well flat bottom plates, at a concentration of $4x10^6$ cells per well. $5x10^5$ cells/well irradiated (50Gy) CT26 tumour cells and 2 $X10^6$ cells/well irradiated (25Gy) syngeneic spleen cells were added as stimulator and feeder cells. These T cell lines were re-stimulated with 1-2 x 10^5 irradiated CT26 and $2x10^6$ syngeneic spleen cells regularly every 7-10 days. Culture medium was changed every 2-3 days. IL-2 (in a final concentration of 20U/ml) and IL-15 (in a final concentration of 10ng/ml) were included in the culture medium the second day after the addition of CT26 cells/ spleen cells. However, in the beginning of T cells culture, these cytokine were started after the first 4 weeks.

Viable T cells were further expanded and tested for their antigen-specificity against CT26, A20 tumour cells by cytotoxicity assay by chromium-releasing assay or WEHI/MTT assay (see below). In general, 5000 cells of A20, CT26, RENCA or BCL1 tumour cells were used as target cells and were plated on a 96-well microwell, syngeneic fibroblast cells, MCFL, were used as a negative control. Indicated number of T cells from each well of master plate was added for reaction. T cells with positive

reaction were transfer to wells of another 24-well plate and further cultured for cloning.

2.5.2. Cloning of antigen-specific T cell by limiting dilution

The tumour reactive T cells were counted and diluted serially to make 3-5 cells/ml in complete medium and then were plated in a 96-well flat bottom plate, 100μl per well (this would be at a cellular concentration of 0.3-0.5 cell/well). $2X10^5$ irradiated syngeneic splenocytes and $2x10^4$ irradiated CT26 tumour cells were included added as well as IL-2 (20IU/ml) and IL-15 (10ng/ml). After 10 to 14 days, the growing cells were checked under microscope and were expanded into another 24-well plate with additional irradiated CT26 tumour cells and syngeneic spleen cells. After another 2-3 weeks of further culture, the growing T cells were tested for the antigen-specificity by cytotoxicity assay.

2.5.3. CD4+ T cells were depleted by MACS

T cells culture were depleted of CD4+ T cell subpopulation from single cell suspensions prepared from spleen cells of immunized mice or from the culture of T cell line. For depleting the CD4+ T cell subset from the T cells, these cells were labelled by anti-CD4+ monoclonal antibody (L3T4, Miltenyi Biotec, Bergisch Gladbach, Germany) conjugated with MACS beads and were separated using a MACS VS+ separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instructions. The efficiency of CD4+ T cells depletion (>90%) was confirmed by FACS analysis. The CD8+enriched T cells (the eluted portion) were washed once and re-suspended in complete culture medium.

2.6. Assays for antigen-specific CTL activity

2.6.1. ^{51}Chromium -release assay

A standard 4-hour ^{51}Cr-release assay [98] was used to assess cytotoxic activity of T effector cells. Target cells are incubated with 20μCi/ml of sodium chromate ($Na_2{}^{51}CrO_4$) (Amersham Biosciences, Bucks, UK) for 1 hour at 37°C. Excess chromium was removed by washing three times with medium. The ^{51}Cr-labled target cells were plated in 96-well U-shape culture plates (Life Technologies), at a concentration of 5000 cells per well. Samples were prepared in triplicate for each reaction. CTL cells are added in indicated serial concentration in 50μl with a total final volume of 100ul per well. Plates were then centrifuged at 700 rpm for 3 minutes and incubated for 4-hour at 37°C. After that, cells were pelleted at 1500 rpm for 5 minutes and 50μ of supernatant is collected and counted in Packard Topcount Microplate Scintillation Counter (Long Island Scientific Equipment Corp, NY. USA). The percent specific lysis is calculated using equation as below:

% specific lysis =experimental release – spontaneous release (O.D.) / maximum release – spontaneous release (O.D.) X 100

Maximum release was determined by lysis of labelled sample using 2% Nonidet P40 (NP-40). Background was measured as the amount of spontaneous chromium release from target cells without the presence of effector cells.

2.6.2. TNF/WEHI assay

Target cells were plated on 96-well culture plates, duplicated in each reaction. 5000 cells of CTL were added in each well to a final volume of 100µl/well. After incubation at 37°C for 20-24 hours, 50µl of the supernatant was withdrawn and transferred to a new microplate containing 3 X 10^4 WEHI-164 clone 13 cells in 50µl of complete RPMI medium supplemented with 2µg/ml of actinomycin D and 40mM LiCl.

After another 20 hours of incubation at 37°C, the percentage of viable cell was measured by a MTT colorimetric assay. 50µl of MTT (SIGMA) (2.5mg/ml in phosphate-buffered saline) was added in each well and the plates were incubated for 2 hours at 37°C. The coloured product Formazen crystals are dissolved by adding 100µ of a lysis solution (prepared by mixing 1 volume of N, N-dimethylformamide with 2 volumes of 30% SDS, and PH adjusted to 4.7 by adding 2.5% of 80% acetic acid and 2.5% 0.1M HCL).

The plates were returned to incubator overnight. Survival of WEHI cells was measured by reading the absorbance (optical density O.D.) on a Dynatech MR5000 microplate reader (Dynatech, Billinghurst, Sussex, UK) at wavelength of 570nm and at wavelength of 650nm as reference wavelength

The CTL-mediated cytotoxicity was measured by the following equation that represented as percentage of killing of WEHI cells by CTL supernatant:

% dead cells = 100 – ((D.D. in wells with CTLs and target cells – O.D. in well of WEHI cell-total lysis) / (O.D. in wells with WEHI cells only-total survival – O.D. in

wells of WEHI cells-total lysis) X 100

Total lysis of WEHI cells was determined by the addition of 50μl 2% NP-40 into WEHI cell culture and total survival was measure by culturing WEHI cells only in complete culture medium.

2.7. Generation of antigen-specific CD8+ T-T hybridoma cells

Antigen-specific CD8+ T-T hybridoma cells were generated by fusion of reactive CD8+ T cells from spleens of immunized mice, T cell lines or CTL clones using chemical reagent- polyethylene glycerol (PEG).

2.7.1. Specific Culture media for hybridoma cells

ICTM medium was made from IMDM medium (Gibco) supplemented with 10% FCS, 100U/ml penicillin, 100ug/ml streptomycin, 2mM L-glutamine and 10% tumour cocktail medium (IMDM medium supplemented with 10% (w/v) dextrose, essential and non-essential amino acid, 10mM sodium pyruvate, 10% (w/v) sodium bicarbonate and 50μM 2-mercaptoethanol).

50% PEG solution (polyethylene glycol) was purchased from SIGMA.

The HAT selective medium was made by dissolving 50x HAT powder (SIGMA) in 250ml ICTM (2xHAT).

HT medium was made by dissolving 50x HT powder (SIGMA) in 500ml ICTM (1xHT).

2.7.2. Somatic cell hybridization- fusion by PEG

CD4+ T cell-depleted PBMC cells prepared from spleens of immunized mice or CD8+ T cells from CTL clone were *in vitro* restimulated with irradiated CT 26 tumour cells and syngeneic splenocytes in the presence of IL-2 three to five days earlier before fusion. The fusion partners, BWZ.36/CD8α cells, were split by 1: 2 or 1:3 one day before fusion. On the day of fusion, BWZ.36/CD8α cells were harvested and checked by methylene blue exclusion test to make sure the viability of cells above 90%.

Just before fusion procedure, the 50% PEG solution, IMDM, ICTM and a 250-ml beaker of water were warmed to 37°C. All procedures of fusion were kept at 37°C by continuously manipulating and swirling the conical tube within a 250ml beaker with 37°C water bath.

Equal amount of CD4+ depleted T cells and BWZ.36CD8α cells (usually about 1-10 X 10^6 cells each) were prepared in pre-warmed IMDM medium and mixed in a 50ml conical tube. The cells were pelleted and all the supernatant was removed. The cell pellet was loosened by gentle tapping the bottom of the tube. 1ml of warm PEG was added slowly over 1 minute along the side of the tube. After 1 minute, 10ml of pre-warmed IMDM was then added over 2-3 minutes along the side of the tube. The tube was topped up to 50 ml IMDM and left in the 37°C water bath for another 5 minutes.

After spinning down, the supernatant was removed and the cells were washed once by

adding slowly 50ml of warm IMDM without disturbing the pellet. Then the cell pellet was gently loosened and re-suspended in pre-warmed ICTM in a concentration of $2x10^5$ normal T cells per ml. These cells were plated out at 100µl/well in 96-well flat bottom plates (this amount is equivalent to a cellular concentration of $2x10^4$cells/well). The plates were cultured at 37°C in a humidified 5% CO_2 incubator. On the second day after fusion, 100ul of 2 x HAT medium was added to each well. Thereafter, every 5 days, 100µl of the supernatant medium was removed from cell culture and replaced with 100µl of 1 x HAT medium supplemented with 360µg/ml Hygromycin-B and 1mg/ml G418.

Following this protocol, most of the cells should die within 2-3 days, and colonies of growing hybridoma cells should be visible under microscope around 8-10 days later. The growing hybridoma cells could be screened for their antigen-specificity around 10-14 days after fusion.

The tumour-specificity of the viable T-T hybridoma cells were measured by the LacZ assay (see below). In general, 50µl of the growing hybridoma cells (around 1 X 10^5 of hybridoma cells were used in each reaction) from each well of the original culture plates (master plate) was harvested to each well of another 96-well U-bottom plate (assay plate). Then a panel of serial dilution of target cells (A20, CT26, RENCA or BCL1 tumour cells) from 1 X 10^5 cells/well were added to a final volume of 200µl/well. A murine embryonic fibroblast cell line, K41, or a syngeneic fibroblast cell line, MCFL, was also used as a negative control.

The putatively positive hybridoma cells were recovered from master plates and transferred to wells of a 24-well culture plate in 1 x HT culture medium supplemented

with hygromycin-B and G418.

2.7.3. Sub-cloning of hybridoma cell

Putatively positive hybridoma cells were sub-cloned by standard limiting dilutions as soon as possible to avoid the overgrowth of non-specific cells. These procedures were repeated whenever the performance of cells appeared suboptimal. Briefly, hybridoma cells were serially diluted in HT medium and plated over 96-well flat bottom plates at a concentration of 0.5cell/well. Syngeneic splenocytes were added as feeder cells at a concentration of 2 X 10^5 cells per well (in a final volume of 200μl/well). Growing hybridoma cells were tested about 7-10 days later for their antigen-specificity by CPRG assay. Positive hybridoma cells were further expanded and frozen down for latter use.

2.8. CPRG assay for TCR-controlled LacZ activities in hybridoma cells

Target cells are plated on a 96-well plate, duplicated in each reaction. 1-2 X 10^5 cells of hybridoma cells were added in 1 X HT medium to a final volume of 200μl. The plate was cultured at 37°C, 5% CO2 overnight. On the next day, the microwells were washed once by 200μl of PBS. Then 100μl of CPRG solution (Roche; made by dissolving chlorophenol red β-D galactopyranoside in PBS containing 1mM $MgCl_2$ and 0.125% NP-40 as manufacturers' instruction) was added to each well. The plates were left in room temperature overnight. After that, the amount of induced β-galactosidase was measured by reading the absorbance (optic density O.D.) on a Dynatech MR5000 microplate reader (Dynatech, Billinghurst, Sussex, UK) at wavelength of 570 nm and at wavelength of 640nm as reference,

2.9. Blocking of CTL activity by monoclonal antibodies (anti-MHC class I and anti-CD8a mAb)

In experiment to determine the restricted MHC class I molecules expressing the reactive antigens on target cells, the monoclonal antibodies against indicated MHC class I molecules (H-2-L^d, H-2-K^d and H-2-D^d) were included in target cell culture medium at a concentration of 20-50μg/ml and pre-culture for 1 hour at room temperature before the addition of CTL cells or hybridoma cells. Excess antibodies were removed along with supernatant after centrifugation without washing the cells. Isotype-matched antibodies were always used at similar concentration as a negative control.

In another experiment, anti-CD8a monoclonal antibody was added in CTL culture at a concentration of 20-30μg/ml and culture for 1 hour before assay to test the CD8-dependence of CTL or hybridoma cells.

2.10. Fluorescence-activated Cell Sorting Analysis and Tetramer Staining

Cells were stained by specific monoclonal antibodies conjugated with indicated immunofluorescence in PBS containing 2% FCS and 0.01% sodium azide according to a standard procedure. Stained cells were counted by BD FACScalibur flowcytometry (Becton Dickinson) and analyzed using BD FACStation data management system.

The AH1/H-2-L^d tetramer was a generous gift from Dr. Martin Glennie (University of

Southampton, GB) and used to stain the TCR of CTL for characterization of the population of AH1/H-2-L^d–specific CD8+ T cells according to the manufacturer's instructions. Briefly, T cells were prepared as for standard FACS procedures and stained by indicated tetramer together with an anti-CD8a monoclonal antibody. Stained cell were analysed by flow cytometry as above. A P815A/H-2-L^d tetramer was used as a control.

2.11. Enzyme-linked immunospot (ELISPOT) assay

The ELISPOT assay used to quantify antigen-specific, IFN-γ-releasing effector cells were performed as described everywhere. Briefly, nitrocellulose-bottomed 96-well plates (Multiscreen MAHA; Millipore,) were coated with capture anti-IFN-γ antibody (clone R4-6A2, BD Pharmingen) overnight at 4°C. Target cells (A20, CT26 tumour cells) and T cells were added in duplicates and incubated overnight at 37°C. Biotinylated secondary antibody (clone XMG1-2-biotin, BD Pharmingen) was added, and cells were cultured at RT for 2 hour. Then streptavidine-alkaline phosphatase and alkaline phosphatase substrate (BCIP/NBT) were added. Reactive spots were counted in blinded manner.

2.12. Cytokine Secretion by CTL Cells (ELISA assay)

T cells from CTL clone 2 were cultured in complete culture medium supplemented with 20U/ml IL-2 and 10ng/ml IL-15 at a concentration of 1 X 10^6 cells/well in a 24-well plate and restimulated in the presence of either 1 X 10^6 irradiated syngeneic spleen cells with 5 X 10^5 irradiated CT26 tumour cells or Dynabeads-coated anti-CD3e (145-2C11/biotin, Pharmingen) /anti-CD28 (37.51/biotin, Pharmingen).

48-72 hours later, supernatant of each culture was harvested and analyzed by cytokine-specific ELISA assay using the appropriate antibody pairs (R4-6A2 and XMG1-2/biotin for IFN-γ, TRFK-5 and TRFK-4/biotin for IL-5, JES5-2A5 and SXC-1/biotin for IL-10). Standard curves were generated using recombinant cytokines; the limit of detection for all cytokines was 0.1ng/ml.

2.13. Peptide-pulsed immune sensitization assay.

Peptide AH1 (SPSYVYHOF) is a CD8+ T cell-reactive epitope derived from gp70, an *envelope (env)* product of endogenous murine leukemia virus (MuLV) [287]. It is known to be mouse MHC class I H-2-L^d restricted. AH1 was used to pulse syngeneic MCFL cells to test the antigenic specificity of CTL cell line by a standard method, A peptide (GILGFVFTL) derived from influenza matrix protein was used as a negative control.

Briefly, 5000-10000 of recipient cells were cultured with indicated peptides (10-20μM) in serum-free medium at 37°C for 1 hour. After that, recipient cells were washed once and CTL were added for a TNF/WEHI assay or ^{51}Cr-release assay.

2.14. *In vitro* proliferation (CFSE) and cell survival assay

Cell division was evaluated using the method of Lyons AB (1999) [301]. CD8+ T cells were harvested and incubated with 5μM of Carboxyl-fluorescence diacetate succinimidyl ester (CFSE, molecular Probes, Eugene, OR) for 10 minutes at room temperature before culture with either irradiated spleen cells with irradiated CT26 cells or Dynabead-coated ant-CD3/anti-CD28. Cells were removed at different times and CFSE fluorescence intensity measured by flow cytometry. Cell survival was

measured by methylene blue exclusion assay at different times and represented by percentage of cells death.

2.15. Tumour challenge animal experiment

For tumour challenge experiments, SCID mice or BALB/c mice received 3-5 X 10^6 *in vitro*-activated CD8+ T cells by i.v. injection in the lateral tail vein. On the following days, mice were challenged with 5 X 10^5 CT26 tumour cells injected s.c. over the flank. Survival of mice was determined as the time lapsed before sacrifice was judged necessary to remain within the humane end-point specified in PPL 30-1891 (in brief, 1st sign of pain /distress; lessening of body temperature; tumour $\geq$ 10% of body weight; ulceration).

Purified CD8+ T cells were harvested from vaccinated BALB/c mice or from *in vitro* CTL culture that was restimulated by irradiated CT26 tumour cells/syngeneic spleen cells or Dynabead coated with anti-CD3/anti-CD28 for 5 days before use.

2.16. Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA was purified from T cell of CTL clone using Dynabeads mRNA DIRECT™ kit (DYNAL UK LTD) according to manufacturer's instruction. The mRNA products were quantified by RiboGreen RNA Quantification Kit (Molecular Probes). 1ng of mRNA was used in first-strand cDNA synthesis.

First-strand cDNA was synthesized from mRNA of cell lines with oligo$(dT)_{15}$ primer (Invitrogen) and M-MLV reverse transcriptase (RNase H -, point Mutant) (Invitrogen)

according to manufacturers' instructions.

All PCR were performed under the condition below: 94°C for 3 minutes, followed by 25 cycles of 94°C, 60°C and 74°C for 1 minute each, and finally 74°C for 5 minutes. In PCR examination of TCRVß genes of T cells, primers (CBR: 5'-AAG AAG CCC CTG GCC AAG CAC-3', TCRVβ8.3: 5'-TCA TAT GGT GCT GGC AAC CTT-3') were used. In PCR examination of inserts in plasmid vector (see appendix 1 and 2), primers (T7 promoter: 5'-TAA TAC GAC TCA CTA TAG GG-3', SP6 promoter: 5'-GAT TTA GGT GAC ACT ATA G-3', BGH reverse: 5'-TAG AAG GCA CAG TCG AGG-3') were used. For reference, we performed PCR with mouse β-actin.

2.17. DNA plasmids.

Two plasmids, pIRES (Invitrogen), containing inserts of DNA encoding for mouse MHC class I molecules H-2Dd and H-2Kd were gifts from Dr. Martin Glennie (University of Southampton, GB). pEGFP-N1, which encodes a red-shifted variant of wild-type GFP, was purchased from BD Biosciences Clontech and used in substitute of cDNA library for the titration of the transfection efficiency of recipient cells.

2.18. Verification of transfection efficiency

Recipient cells were cultured in wells of 6-well plate in a concentration of 2-3 X 10^5cells/well. On the next day, these cells were incubated with indicated concentration of 0.5ml transfection medium containing 1μg of plasmid DNA (pEGFP-N1 or pIRES/H-2-Dd). After varied period, the transfection medium was flicked; 0.5ml of PBS supplemented with 10% DMSO was added and cells were incubated at RT for 2

minutes. Thereafter, culture medium was changed with complete culture medium. After 48 hours in 37°C incubator, the expression of GFP or H-2-Dd was verified by flow cytometry.

Cos-7 cells are derived from the kidney of an African green monkey, transfected with a variant of SV40 that had been rendered replication defective by a 3 bp deletion in the SV40 *ori* region. The cells constitutively express the SV40 large T antigens (SV40-LT) that bind to SV40 *ori* [302] allowing the host DNA polymerase to start a bi-directional DNA synthesis, therefore, support the replication of any transfected plasmids that contain the SV40 *ori*. A large number of mammalian expression vectors that contain the SV40 *ori* are available and therefore are amplified to a high copy number in transfected cells, allowing the production of 1000-fold level of mRNA and proteins after 48 hours after transfection.

2.19. Screen of CT26 cDNA library

2.19.1. Titration of CT 26 cDNA library

The CT26 cDNA library was kindly prepared by Dr Firouzeh Korangy (Medizinische Hochschule Hannover, Hannover, Germany) for our experiment. First-strand cDNA was reverse transcribed from purified mRNA of CT26 tumour cells with a size range from 400bp to 5kb. After making second-strand cDNA, the subsequent cDNA molecules were clone by inserting into EcoR1 site of an expression vector, pcDNA3 (see appendix 1, Map of pcDNA3) (Invitrogen), in a non-directional manner. The efficiency of insertion was about 85-90%. The cDNA plasmids were then

electroporated into XL1-Blue cells (Strategene) and these bacteria were store at -80°C in glycerol stock after amplification.

The insertion size of the cDNA library has been previously examined and confirmed (see figure 2.1). In brief, bacteria of 1μl of the library were plated on a 10-cm agar plate containing ampicillin (100ng/ml). After overnight culture at 37°C, 20 colonies were picked randomly from the pools of grown colonies and expanded in agar broth containing ampicillin (100ng/ml). Plasmids were extracted from bacteria by a QIAprep spin miniprep kit (QIAGEN) and cDNA were purified by digesting with a restrictive endonuclease enzyme (EcoR1) according to the manufacturer's instruction. The size of cDNA was determined by electrophoresis on a 1% agar mini-gel containing ethidium bromide and differentiated under UV illumination.

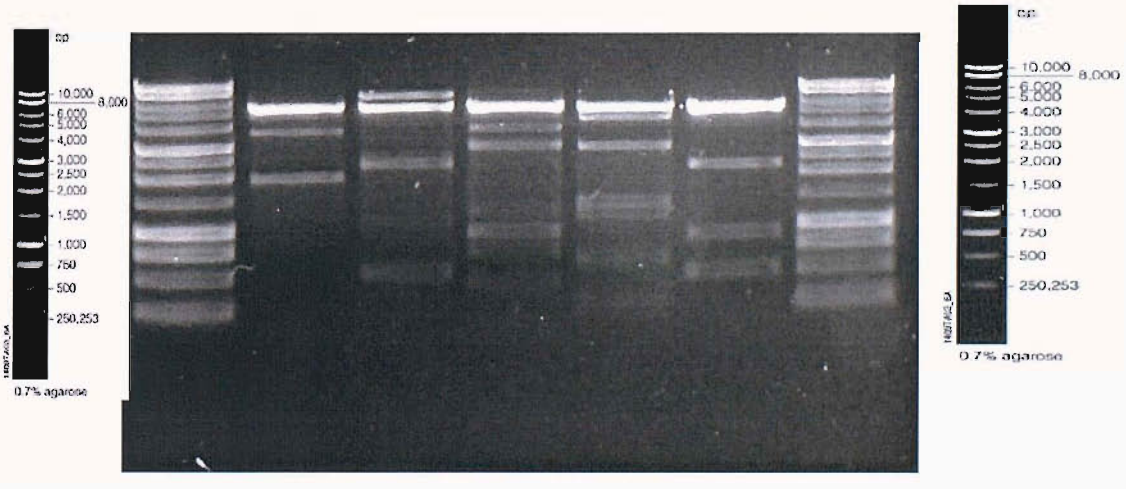


Figure 2.1. Complexity of CT26 tumour cDNA library.

The range of sizes of CT26 cDNA library was examined. Plasmids of randomly selected bacterial colonies were extracted; the inserted cDNA molecules were recovered by endonuclease cleavage (EcoR1) and were separated by electrophoresis on a 1% agar mini-plate.

2.19.2. Preparation of plasmid containing cDNA libraries from pool of bacteria

First, the titre of the CT26 cDNA library was determined by limited dilution. Briefly, 10μl of bacteria was taken directly from the frozen sample and 10 X serial dilutions were made (range from 10^3 to 10^8 X dilution). Then 50μl of every dilution fraction was plated on a 10-cm agarose culture plate containing ampicillin (100ng/ml). After overnight incubation at 37°C, the visible bacterial colonies were counted from each agarose plate. The titre of the cDNA library was deducted. It was estimated to be 3-4 X 10^5 colonies per μl of bacterial library (cpu).

According to the counted cpu, indicated amount of bacteria from the frozen pool of whole CT26 cDNA libraries was plated on a 22 x 22 cm agar plate containing ampicillin (100ng/ml), thus to gain a density of colonies nearly 100 colonies/cm^2. The agar plate was cut in pieces by sterile scalpels, so as the cDNA library was then divided into smaller pools with about 100 colonies in each piece. Normally, at least a batch of 1-2 X 10^5 colonies of bacterial should be screened to represent the entire cDNA library.

Each pool of 100 colonies was put in a 50ml conical tube containing 7ml of LB broth supplemented with ampicillin (100μg/ml) and amplified at 37°C overnight under vigorous shaking. The next day, 0.5ml of each pool was frozen down in glycerol and kept at -80°C as a master sample. Containing plasmid DNA was extracted from each pool of bacteria by QIAprep 96 Turbo Miniprep Kit (QIAGEN) in a 96-well format according to the manufacturer's instructions. The DNA plasmid was dissolved in water containing 20ng/ml RNase and stored in -20°C for later use.

2.19.3. Transient transfection of COS-7 cells with cDNA libraries

On the day before transfection, COS-7 cells were seeded in 96-well flat-bottom plates, 10000-20000 cells/well containing 200µl DMEM medium supplemented with 10% FCS. Thus, COS-7 cells would grow to a nearly confluent density. Recipient cells were transfected with 100ng of each plasmid DNA sample of libraries together with 100ng of plasmid DNA containing gene encoding for indicated H-2d molecules.

The transfected plasmids were dissolved in DMEM medium supplemented with 200µg/ml of DEAE-dextran (SIGMA), 200µM of chloroquine (SIGMA) and 10% of heat-inactivated NuSerum (BD, biosciences) in a final volume of 35µl/well. After incubation for 90 minutes at 37°C, microwells were gently flicked of DMEM medium. 50µl of PBS containing 10% DMSO are added and microwells were incubated for another 2 minutes at room temperature. Then, PBS/DMSO was removed and 200µl of fresh DMEM supplemented with 10% FCS were added.

The plates were returned to incubator for 48 hours at 37°C. After that, the transfected COS-7 cells were ready to be test for their expressed antigens by either CTL or hybridoma cells.

2.19.4. Recovery of the identified tumour-associated antigens

When any positive sample was suspected, the validity of the result should be confirmed by comparing in parallel a panel of assays with plasmid DNA of interest transfected into COS-7 cells that were co-transfected with cDNA of restricted H-2^d and irrelevant H-2^d to rule out the possibility of false positive.

Then the truly positive fraction of bacterial (around 100 colonies) was recovered from glycerol frozen stock, culture in LB agar supplemented with 100ng/ml of ampicillin, and subsequently fractionated into smaller pool (15-25 as one fraction). The same screening procedures were repeated until a single positive colony of bacteria containing cDNA plasmid of interest was identified.

The DNA plasmid of interest was extracted from the positive colony of bacteria. The size of the insert cDNA wanted was checked by restriction enzyme, EcoR1 (Promega), cleavage. Alternatively, the cDNA insert can be confirmed by PCR using indicated primers.

2.20. Recloning of cDNA of interest into new vector

The cDNA inserts of interest were prepared by digestion from cloning site of original plasmid using restriction enzymes (HindIII + XhoI) (Promega). The vectors (pcDNA3.1/V5-His) (see appendix 2) (Invitrogen) were prepared similarly. Then the inserts were ligated into cloning site of new vectors by T4 DNA ligase (Promega) according to the manufacturer's instructions. The ligated plasmid was transformed into competent E. coli (TOP10, ONE SHOT, Invitrogen), which were then cultured overnight on LB agar supplemented with 100ng/ml ampicillin. Viable bacteria was harvested and checked for the presence of insert by restriction enzymes digestion or by PCR annealing.

2.21. Sequencing of cDNA and searching for genes of homology

DNA sequence analysis was performed by DNA Sequencing Facility, Department of

Biochemistry, University of Oxford (http://www.bioch.ox.ac.uk/~dnaseq/). In brief, double-stranded DNA plasmids containing insert cDNA of interest were sent for DNA sequencing using 5' primer TAATACGACTCACTATAGGG (T7 promoter) and 3' primer TAGAAGGCACAGTCGAGG (BGH reverse), ABI BigDye terminators kit, Applied Biosystems 9700 thermal cyclers and Applied Biosystem 3730 X 1 DNA Analyzer. The computer search for sequence homology was performed by the program BLAST with GenBank databases (http://www.ncbi.nlm.nih.gov/blast/ MAR-10-2005).

2.22. Epitope deduction from MHC class I molecules binding motif and peptide sensitizing assay.

Candidates of CTL-reactive epitope within the isolated antigen were deduced by computer software [303, 304] (http://www.imtech.res.in/cgibin/propred1/ March-14-2005 and http://bimas.dcrt.nih.gov/molbio/hla_bind/ March-14-2005). The synthetic peptides (nonamers) were purchase from GL Brioche (Shanghai, China) Ltd. 10-20μM of each nonamer was used to pulse target cells (MCFL cells) in serum-free medium at 37°C for 1 hour before CTL sensitization test by TNF/WEHI assay or ^{51}Chromium-release assay.

3. Cross Reactive CTL from CD25+ Regulatory T cell-depleted Mice

3.1. Introduction

In BALB/c mice immunized with live CT26 tumour cells in the absence of CD25+ T regulatory T cells, both CD8+ and CD4+ T cells were shown to be capable of mediating rejection of CT26 tumour cells and cross protection from the challenge of A20, BCL1 and RENCA tumour cells. In order to identify the CD8+ T cell-reactive antigens in this animal system using a genetic approach, CD8+ T cells that have cross-reactivity were isolated from immunized Treg-depleted mice and cultured *in vitro*.

To generate a CD8+ cytotoxic T cell line responsible for the cross protection against CT26 and other tumours in our animal model, a modified method used by Khanna and colleagues (1999) [79] was applied in the culture of mouse T cells. In brief, a single cell suspension was prepared from spleens and lymph nodes of mice immunized with CT26 tumour cells in the absence of CD25+ regulatory T cells (see *materials and methods* above). All T cells were *in vitro* re-stimulated by irradiated CT26 tumour cells and irradiated syngeneic spleen cells as feeder cells regularly every 7-10 days. IL-2 (at a final concentration of 20 IU/ml) and IL-15 (at a final concentration of 10ng/ml) were included in culture medium starting from 4 weeks later to ensure the deletion of potential alloreactive T cells [79].

IL-15 was originally identified as a cytokine with IL-2-like activity [305, 306]. Similarities

of the functional properties between IL-15 and IL-2 come from the fact that these two cytokines employ the same β- and γ-chains in their receptors. The specificities of their receptors are provided by a unique α-chain [307]. However, there is evidence suggesting that IL-2 and IL-15 has different roles in regulating T cell turnover [308]. It was reported that, IL-15 could downregulate the negative regulator or the death signals of the immune system through deactivating the Nuclear Factor of Activated T cells (NF-AT) in T cells, thus promote T cells division and growth. In addition, IL-15 has been proven to be essential for *in vitro* expansion and survival of CD8+ T cells [309, 310]. As a result, IL-15 was included in combination with IL-2 in our T cell culture.

Two factors were thought to be important in our T cell culture. First, the procedure to separate viable T cells from dead cells by Ficoll gradient centrifugation was avoided in order to prevent cellular loss [78]. Second, the irradiated CT26 tumour cells used to stimulate the T cells have been titrated to around 1-2 X 10^5 cells/well on a 24-well plate (with an Effector-to-Tumour E:T ratio > 10) to avoid possible growth-inhibition exerted by tumour. It was known that there may be some tumour-derived soluble factors or surface molecules expressed by some tumour cells would hamper the function or proliferation of T cells, such as IL-10 [311], TGF-β [312] or B7-H1 [222]. In our previous works, CT26 tumour cells inhibit T cell activation in co-culture experiments, although no IL-10 or TGF-β secretion in the supernatant of culture was identified.

Although standard 4-hour ^{51}chromium-relaese assay has long been used for the examination of the antigen-specific cytotoxicity of CD8+ T cells, it is radioactive and labour-intensive and not suitable for antigen hunting, which requires high throughput assay. Alternatively, TNF/WEHI assay were verified as a suitable bioassay for the antigen-specific activities of CTL and has used in many laboratories for identification

of tumour-associated antigens through tumour cDNA libraries [104, 105].

For alternative trial, antigen-specific hybridoma cells were generated by chemical fusion between antigen-specific CD8+ T cells and fusion partner, BWZ36.CD8a cells. In many experiment, hybridoma cells have been shown convenient and sensitive in substitute of CTL for the assay of TCR specificity. The TCR activity of hybridoma cells would drive the expression of a downstream LacZ gene, of which the β-galactosidase product in turn is relatively easy to be analyzed by a CPRG assay.

3.2. Results

3.2.1. Adoptive transfer of CD8+ T cells from immunized mice

To verify the role of CD8+ T cells in BALB/c mice immunized with CT26 tumour in the absence of CD25+ T regulatory cells, purified CD8+ T cells were harvested from immunized mice and were transferred to SCID mice. On the next day, SCID mice were challenged with live CT26 tumour cells. As shown in figure 3.1, SCID mice that were adoptively transferred with CD8+ T cells from immunized mice were able to reject the challenge of CT26 tumour cells, whereas SCID mice that were transferred with naïve CD8+ T cells from normal BALB/c mice died within 30 days.

Since the presence of T cells in recipient SCID mice was derived from donor only, the immunization rendered in recipient SCID mice were CD8+ T cells from the immunized BALB/c mice. In addition, the purified CD8+ T cells were titrated as few as 4 $x10^5$ cells to be able to render recipient SCID mice immunity against the challenge of CT26 tumour cells (see figure 3.2).

In Golgher experiment [16], both CD8+ and CD4+ T cells were required together for complete cross-protection from the challenge of C26 and A20 tumour cells. However, CD8+ T cells alone were able to rendered partial cross- protection in immunized mice. Therefore, in the next stage, antigen-specific CD8+ T cell lines were generated from immunized mice.

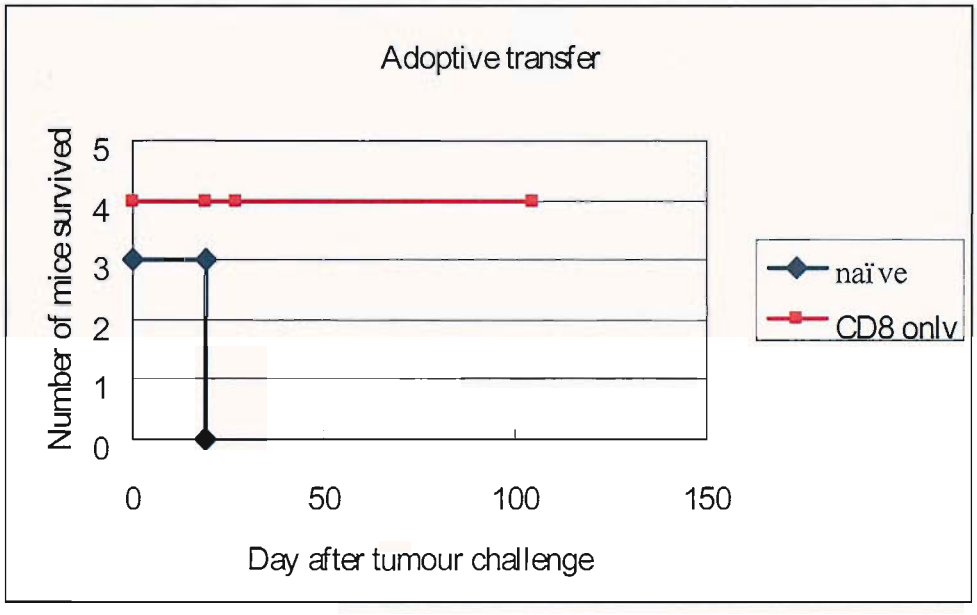


Figure 3.1. Adoptive transfer of CD8+ T cells from immunized mice to SCID mice

4 X 10^6 of purified CD8+ T cells from immunized BALB/c mice (immunized with CT26 tumour cells after depletion of CD25+ regulatory T cells by mAb, PC61. See *Materials and Methods*) were transferred to SCID mice via tail vein (■, n=4). On the next day, SCID mice were challenge with 10^5 of CT26 tumour cells by subcutaneous injection. Naïve CD8+ T cells from normal BALB/c mice were used as a control group (♦, n=3)

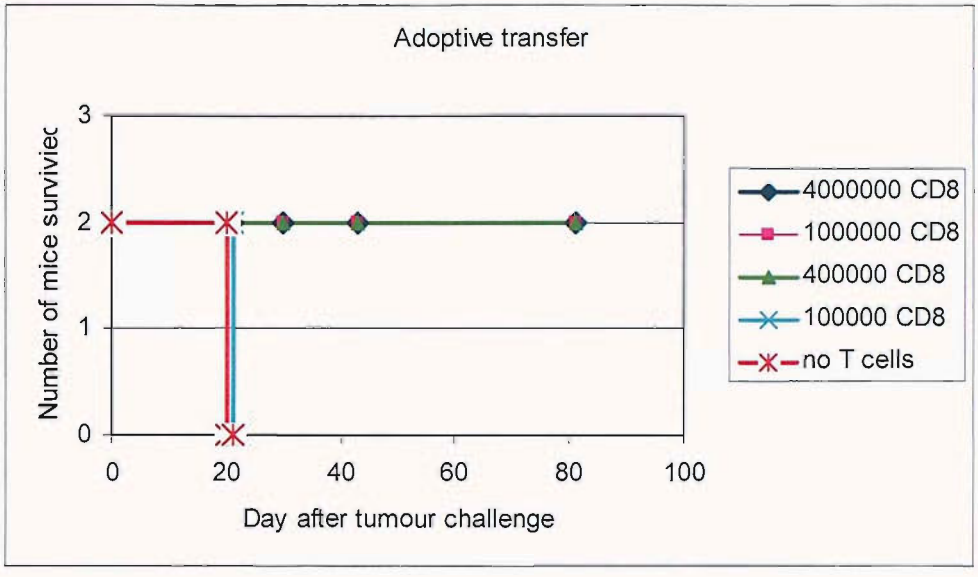


Figure 3.2. Titration of CD8+ T cells effective in adoptive transfer experiment

A panel of purified CD8+ T cells from immunized BALB/c mice, ranged from 1 X 10^5 to 4 x 10^6, were transferred to SCID mice via tail vein. On the next day, SCID mice were challenge with 10^5 of CT26 tumour cells by subcutaneous injection. (n=2 in each groups)

3.2.2. Generation of two antigen-specific CD8+ T cell lines

A single cell suspension was prepared from spleen cells of immunized mice and cultured *in vitro* as mentioned in material and methods. In the first 3-5 weeks of in vitro culture, the number of total viable cell decreased rapidly. This fast reduction of cell number might be owing to death of non-specific T cells and non-T cells. After that time, the number of viable cells that were presumably tumour-specific increased gradually (figure 3.3).

Two CD8+ T cell lines (T cell line 1 and T cell line 2) were successfully grown form spleen cells of immunized mice and showed specificity to both CT26 and A20 tumour

cells. Among theses cells, T cell line 1 has shown stable growth rate in *ex vivo* culture for more than 6 months. Proliferation of the CTL cells depends on the regular *in vitro* re-stimulation with irradiated CT26 tumour cells and syngeneic spleen cells in the presence of IL-2 and IL-15.

The prepared single T cell suspension must be depleted of CD4+ T cell by MACS beads conjugated anti-CD4+ monoclonal antibody and MACS separation column. There was only one factor different in setting-up of the two CD8+T cell lines. CTL line 1 was depleted of CD4+ T cells two months after commencing the culture, whereas CTL line 2 was depleted at the beginning of culture. These T cells were characterized by FACS analysis and were shown that more than 90% of the cells were CD8+ and CD3+ in both cell lines (figure 3.4).

The tumour-specific cytotoxic activities of the two CTL cell lines were confirmed two months later before further expansion. In our previous work [16], CD8+ T cells from the immunized BALB/c mice were shown to be able to reject the challenge of CT26 tumour cells and partially to cross-protect from the transplantation of A20, BCL1 and RENCA tumour cells. Thus, in our initial works, these cell lines were tested for their specific reactivities against both CT26 and A20 tumour cells. In 4-hour ^{51}Cr-release assays (figure 3.5), CTL from both of the two T cell lines were shown with significant specific cytotoxicity against CT26 and A20 tumour cells as compared to a syngeneic fibroblast cell line, MCFL cells. In another experiment, the number of T cells with tumour-specific IFN-γ production against CT26 and A20 tumour cells were also demonstrated by ELISPOT assays (figure 3.6).

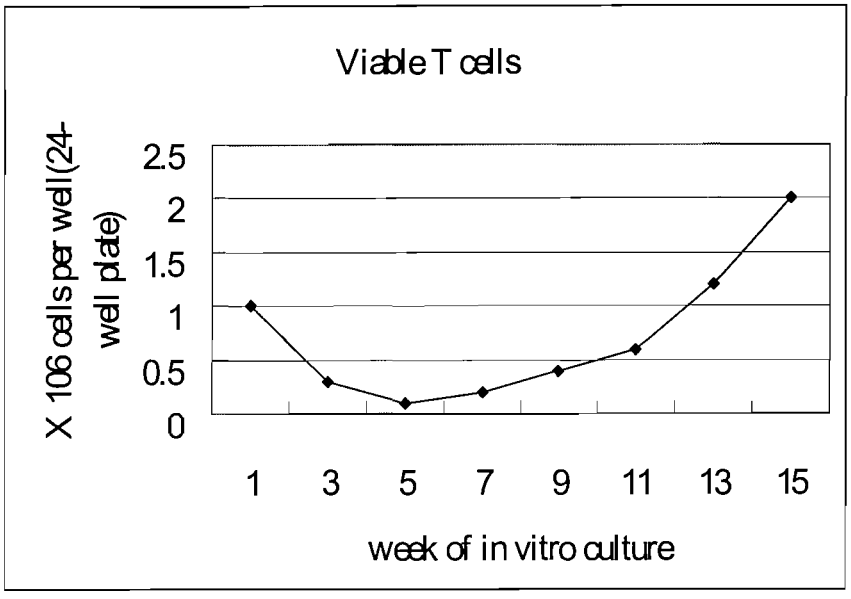


Figure 3.3. Number of viable *in vitro* T cells culture

At different time points, T cells were harvested and counted. Numbers were represent as X 10^6 per well of 24-well plate.

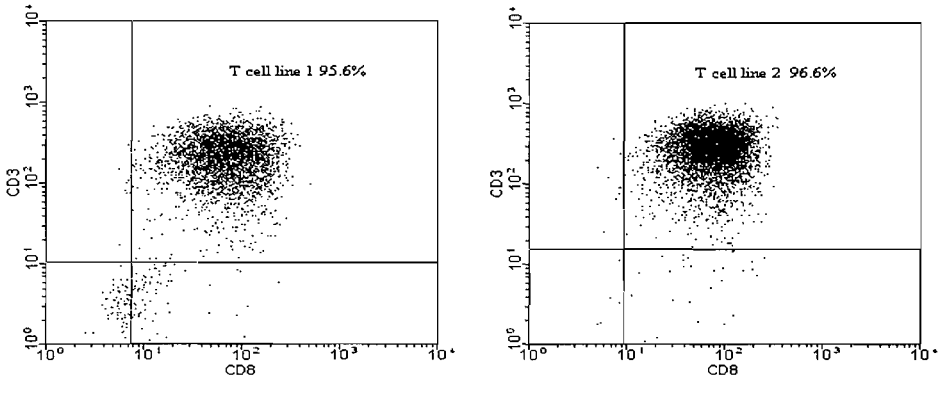
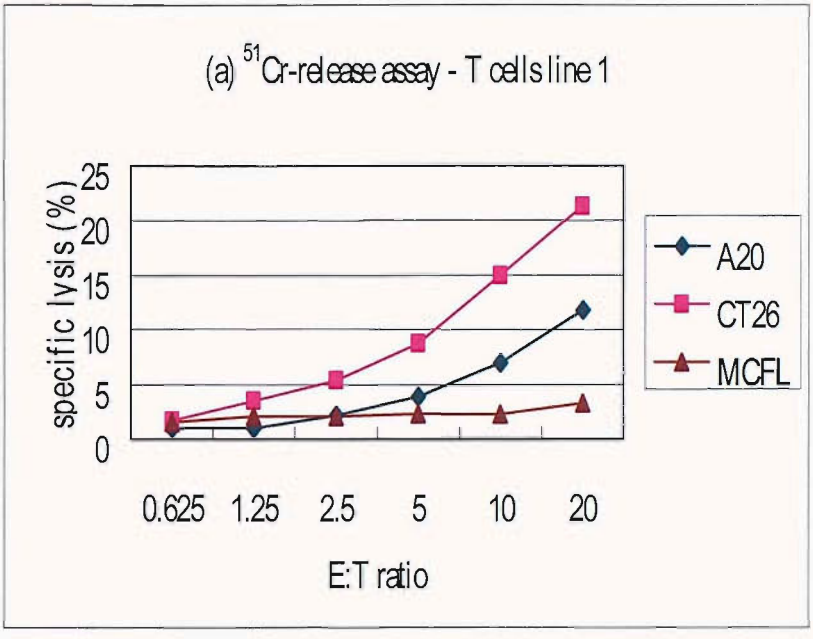


Figure 3.4. Phenotypic expression on CTL line 1 and CTL line 2.

Cells were stained with anti-CD3ϵ mAb-biotin and anti-CD8a mAb-FITC followed by streptavidine-PE (indirect immunofluorescent staining). Background staining was determined by an irrelevant IgG2 mAb-FITC plus anti-CD3ϵ mAb-biotin/ streptavidine-PE (not shown). FACS profile presents dots obtained by analysis of 10^4 events.



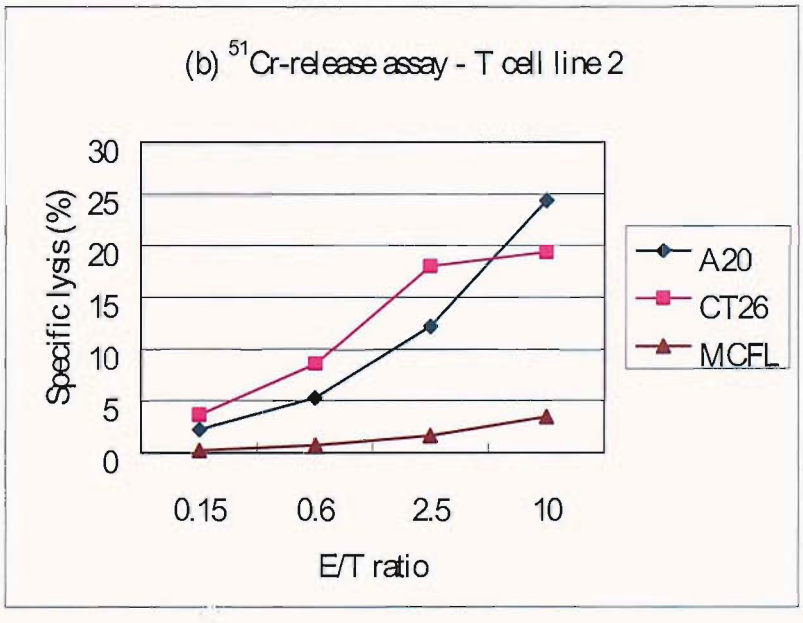


Figure 3.5. Chromium-release assay: cytotoxicity of T cell line 1 and line 2 against CT26 and A20 tumour cells.

The CTL line *1* (a) and CTL line *2* (b) were tested for 4-h ^{51} Cr-release assays against A20 cells (♦), CT26 cells (■) and syngeneic MCFL cell (▲) at various E:T ratios. Target cells were added in 5000 cell/well. Each point represents an average of triplicates. ^{51}Cr-release in wells containing A20, CT26 plus T cells from CTL line *1* and line *2* was significant ($P<0.05$) higher than in control well (MCFL plus CTL line *1* /*2* cells).

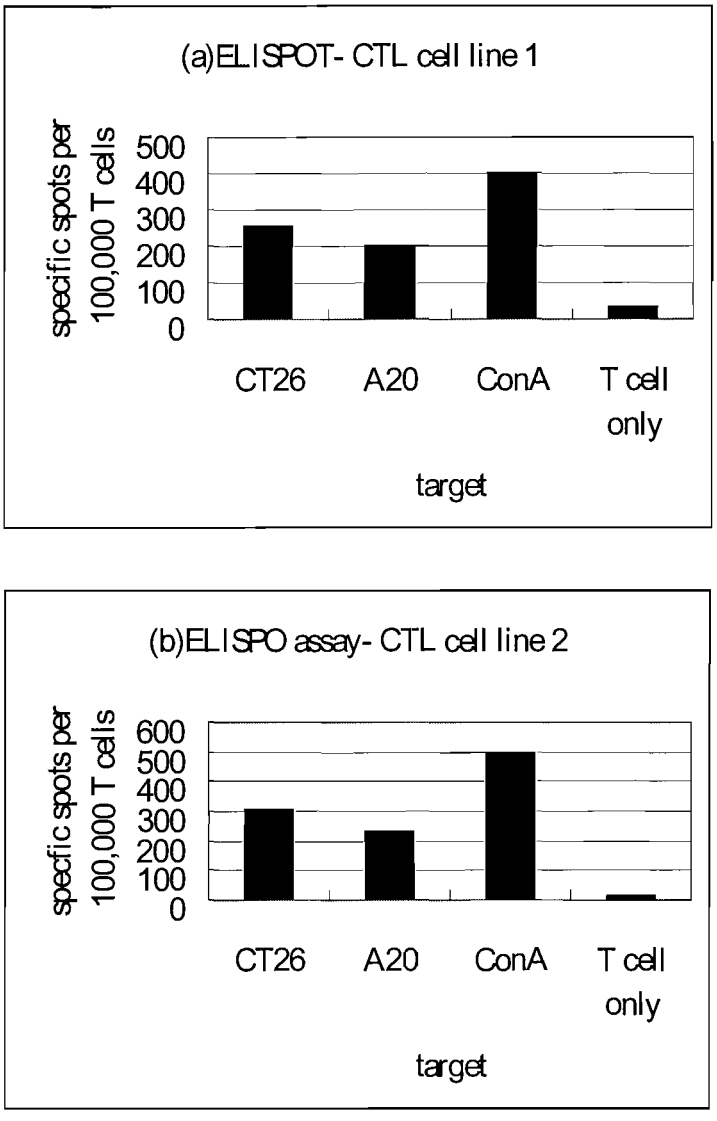


Figure 3.6. ELISPOT assay: recognition of CT26 and A20 tumour cells by T cell line 1 and 2.

In a standard ELISPOT assay, 1 X 10^5 cell/well of CTL cells from both CTL cell line 1 (a) and cell line 2 (b) were added in duplicates, together with CT26 and A20 tumour cells. The specific INF-γ release was counted as described in *Materials and Methods.*

3.2.3 TNF/WEHI assay is parallel to standard chromium-releasing Cytotoxicity assay for the CTL activity

The ^{51}chromium-release cytotoxicity assay is not a suitable assay for a high-throughput screen of CTL activities. ELISPOT for detection of the antigen-specific IFN-γ-releasing T cells is very sensitive, but relatively expensive. In contrast, TNF/WEHI assay in combination with a MTT assay has been shown to be a reliable, high throughput and cost-effective assay for antigen-specific CTL activity.

As shown in figure 3.7, the positive linear relationship between the percentage of killing of WEHI cell and the concentration of TNF (pg/ml) in culture supernatant was demonstrated using human recombinant TNF-β (hrTNF-β) (R & D System, MN) with known concentrations. High level of TNF-mediated cytotoxicity towards WEHI 164 cells was observed. In our experiment, it was shown that the killing effect of WEHI 164 clone 13 cells started even when the rTNF-β concentration in the supernatant was as low as 0.001pg/ml.

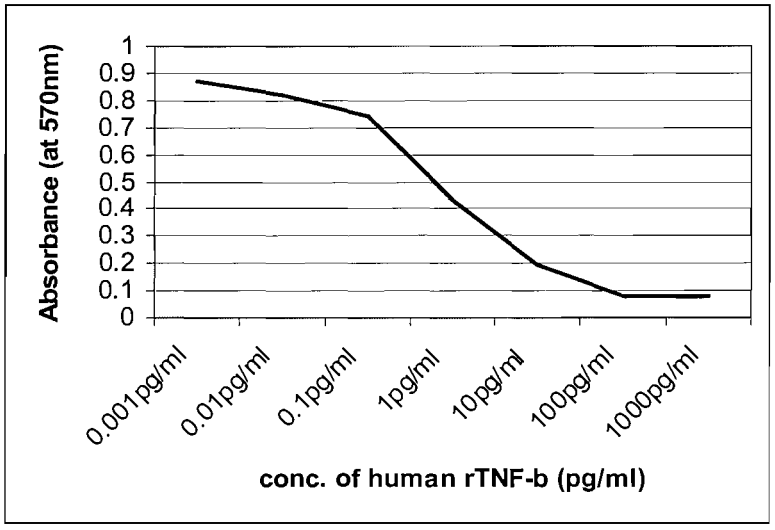
The amount of TNF production by CTL against specific tumour cells was measured by culturing WEHI cells in supernatant from the cellular reaction medium and represented by the percentage of killing of WEHI 164 clone 13 cells. We next moved to examine the validity of this assay in our T cells system by comparing the percentage of killing of WEHI cells cultured with dilutions of the supernatant TNF from T cell cultures. As shown in figure 3.8, WEHI cells killing was titrated with dilution of supernatant at all E:T ratios tested. This indicated that the response-range of the WEHI assay was suitable for use with undiluted supernatants, and that undiluted supernatant even for high E:T ratio was not sufficient to saturate the WEHI

response. In addition, the killing of WEHI cells was corrected with the amount of TNF production in the cellular supernatant, the latter was in turn correlated with the amount of antigens presented to CTL.

Since there was no detectable TNF secreted by CT26 and A20 tumour cells (data not shown), the TNF present in the supernatant of CTL-tumour cells culture was derived from CD8+ T cells only and the secretion was shown to be antigen- and tumour-specific in a dose-dependent manner. As shown in figure 3.9, CD8+ T cells from both CTL lines were known to specifically react to A20 and CT26 tumour cells and produce TNF that was represented by the killing of WEHI cells. Results of the TNF/WEHI assays for CTL line 1 and 2 were compared with their profiles in ^{51}Cr-release cytotoxicity assays (figure 3.5 above). The assay for antigen-specific TNF secretion was as sensitive as the assay for their direct cytotoxic effect towards CT26 and A20 tumour cells.

The percentage of WEHI cell killing and the amount of TNF secretion against different target cells could be differentiated at a lower effector-to-target ratio. This could be another benefit for the TNF/WEHI assay, because fewer CTL are required. The tumour-specific TNF production of CTL was CD8-dependent, as seen by the fact that the TNF secretion of the CTL line 1 against CT26 tumour cells could be completely blocked by an anti-CD8a monoclonal antibody (figure 3.10).

(a)



(b)

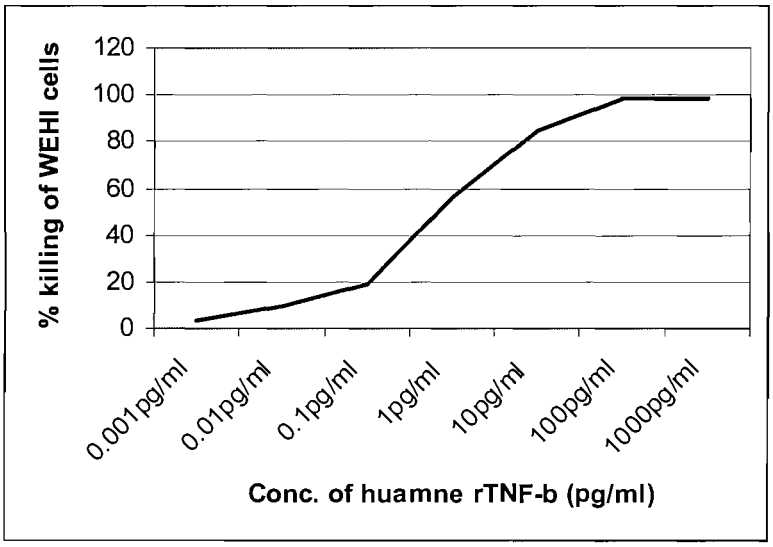


Figure 3.7. Verification of TNF/WEHI assay: killing of WEHI 164 clone 13 cells by humane recombinant TNF-β (hrTNF-β).

5000 cells/well of WEHI 164 clone 13 cells were cultured in RPMI complete medium with indicated concentrations of human rTNF-β. The viability of WEHI cells was examined by MTT assay and converted into the killing percentage of WEHI cells (see Materials and Methods). Each point represents an average of triplicate. Results were presented by absorbance at 570nm (**a**) or by percentage of killing of WEHI cells (**b**) against various concentrations of human rTNF-β

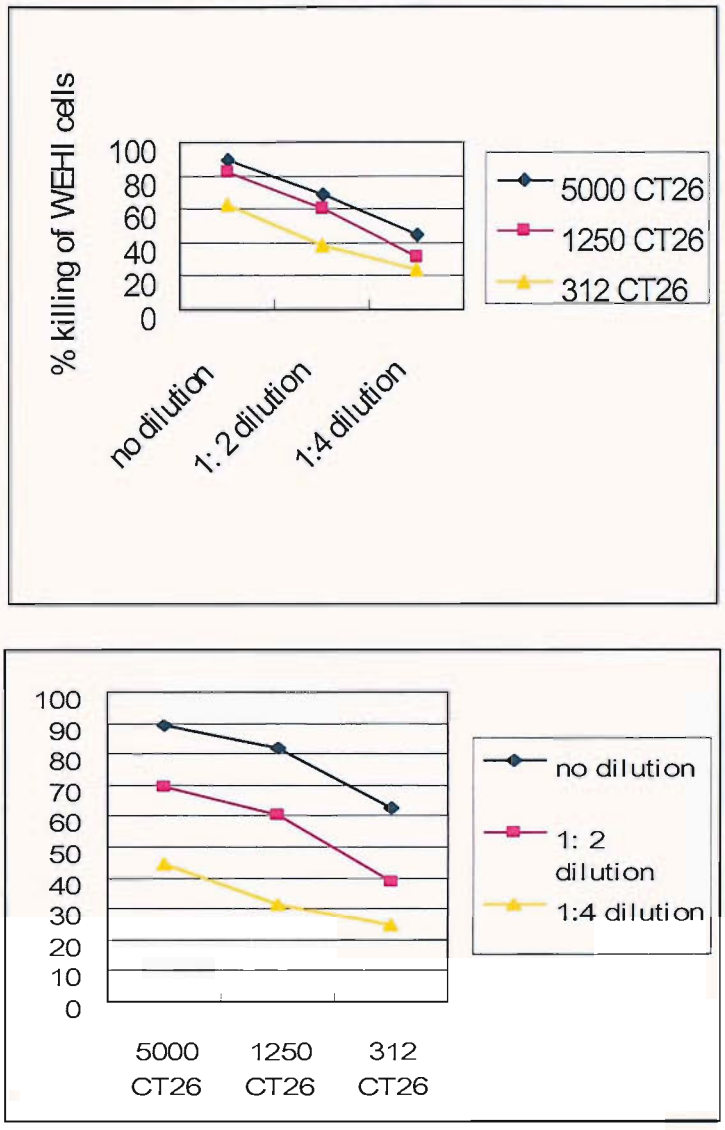
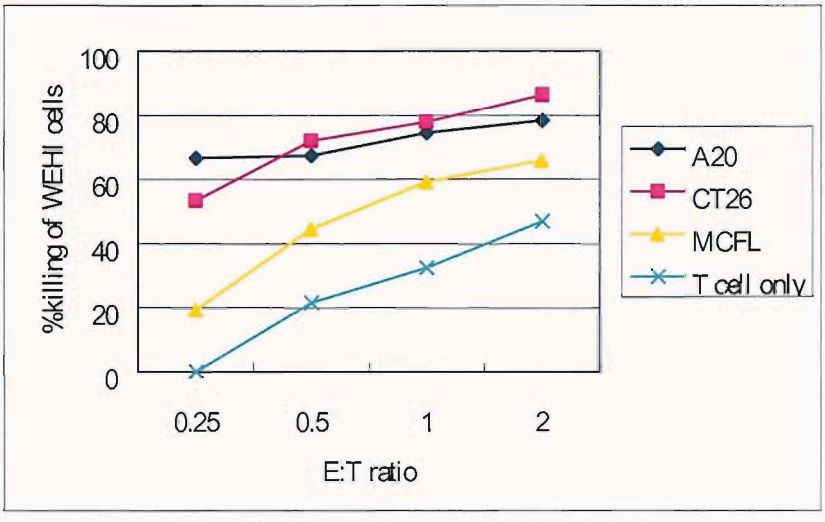


Figure 3.8. TNF/WEHI assay: titration of TNF production of CTL line *1* against CT26 tumour cells

5000 T cell from T cell line 1 was add in reaction with various number of CT26 cells. The TNF amount of T cells was examined by a TNF/WEHI assay. The supernatant harvested co-culture medium was left undiluted, 1:2 diluted or 1:4 diluted before the addition of WEHI cells. Each point represents an average of duplicate (see *Materials and Methods*). Upper panel: percentage killing of WEHI cells was titrated by serial dilution of co-culture supernatant. Lower panel: percentage killing of WEHI cells was titrated by number of CT26 tumour cells in co-culture with T cells.

a) TNF/WEHI assay of CTL line *1*



b) TNF/WEHI assay of CTL line *2*

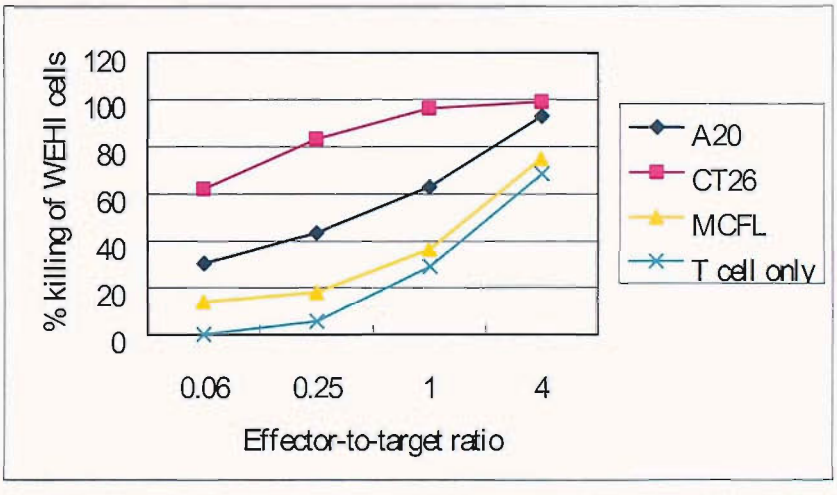


Figure 3.9. TNF production of CTL line *1* against A20 and CT26 cells.

The CTL line *1* (**a**) and CTL line *2* (**b**) were tested for the antigen-specific production of TNF against A20 cells (♦), CT26 cells (■) and syngeneic MCFL cells (▲) at various E:T ratios. CTL cell only (**X**) was tested as a TNF background of T cells. Target cells were added in 5000 cells/well. Each point represents an average of duplicate. The amount of TNF production was measured by its cytotoxic effect to WEHI cells (see *Materials and Methods*).

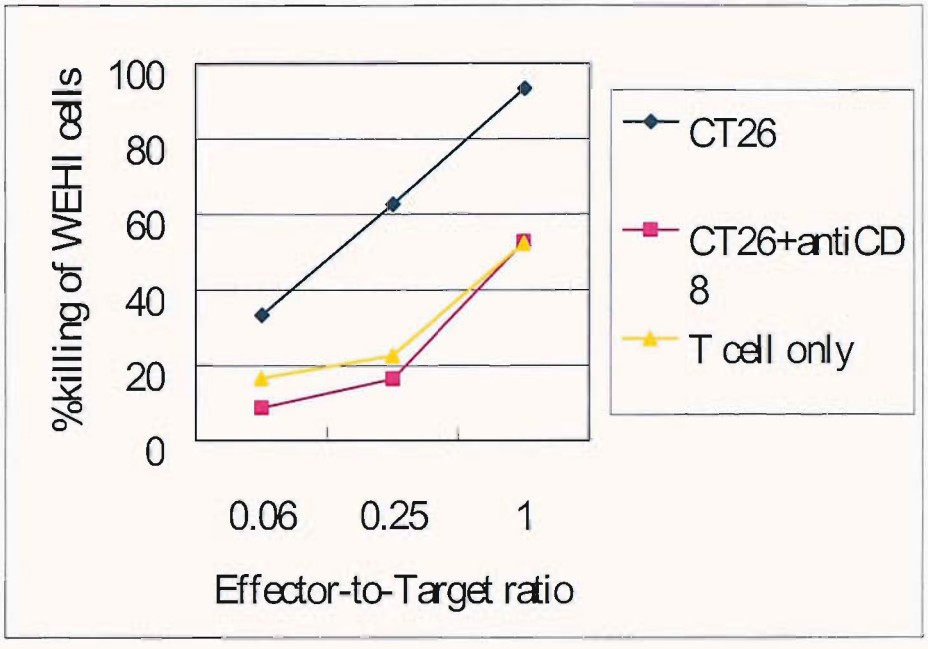


Figure 3.10. TNF production of CTL line 1 against CT26 tumour cells could be blocked by anti-CD8a monoclonal antibody.

TNF production of the CTL line *1* cells against CT26 tumour cells was measured without (♦) or with (■) anti-CD8a monoclonal antibody (clone 53-6.7) at various E:T ratios. Supernatant from culture of CTL cells only (▲) was tested as a TNF background of T cells. Target cells were added in 5000 cells/well. Antibody was added at a concentration of 20μg/ml and culture for 1 h before reaction. Each point represents an average of duplicate. The amount of TNF production was measured by its cytotoxic effect to WEHI cells (see *Materials and Methods*).

3.2.4. Several cross-reactive CTL clones were subcloned from of 2 T cell lines

The H-2 restriction for the two T cells lines were determined using blocking antibodies. Thus, indicated mAbs against different H-2^d molecules were included in the CTL-tumour cells reaction. In an initial work, for CTL cell line 1, it was found that the TNF secretion of CTL against CT26 can be blocked to different degrees by two antibodies, HB-27 (anti-H-2-L^d) and HB-79 (anti-H-2-K^d/D^d) (figure 3.11). One reasonable explanation was that there could be more than one clone among this CTL cell line that were responsive to different antigen/H-2^d complexes on antigen presenting cells.

The TNF production of T cell line 1 can be completely blocked by anti-H-2-K^d/D^d, and partially by anti-H-2-L^d. It might suggest that H-2-L^d restricted CD8+ T cell population in T cell line 1 was a minority. Thus, in response to CT26 tumour cells, the amount of TNF production by CD8+ T cells with H-2-L^d restriction may not be sufficient to reach the detection threshold of WEHI/TNF assay.

As s result, limiting dilution was applied for the isolation and culture of single T cell clone from T cells line 1. Consequently, three clones of CTL (clone 3, clone 6 and clone 9) were successfully selected as potentially tumour-specific CD8+ cytotoxic T cells. Taking advantage of TNF/WEHI assays and ^{51}Chromium-release assay, these three clones were shown to produce TNF in response to, and were cytotoxic towards both CT26 and A20 tumour cells (figure 3.12 and figure 3.13).

The H-2 restrictions of these clones were again determined using mAb blocking MHC class I molecules in ^{51}Cr-release assays. It was shown that the cytotoxicity towards

CT26 tumour cells of T cells from CTL clone 3 (figure 3.14) and clone 6 could be blocked by an anti-H-2-D^d monoclonal antibody, whereas reaction of CTL clone 9 can be blocked by an anti-H-2-K^d monoclonal antibody (figure 3.15).

Since the tumour-specific reaction profiles of both H-2-D^d restricted CTL clone 3 and clone 6 were similar in either TNF/WEHI assays or ^{51}Cr-releasing assays (figure 3.12 and figure 3.13), we next assessed their clonality by TCR variable β gene usage assay using a panel of 13 anti-TCRV_β monoclonal antibodies to stain their TCR as detailed in *Materials and Methods*. It was shown that both CTL clone 3 and clone 6 can be stained by an anti-TCR$V\beta$8.3 monoclonal antibody (figure 3.16); suggesting that clone 3 and clone 6 might be the same clone of CTL.

On the other hand, the TNF production of T cell line 2 can be only blocked by anti-H-2K^d/$D^{d.}$ (figure 3.17). After limiting dilution, only one clone of CTL was isolated from CTL cell line 2, designated as CTL clone 2. To determine the H-2 restriction using blocking mAb in a ^{51}Chromium-release assay, the cytotoxicity of CTL clone 2 could only be blocked by mAb against H-2-D^d, indicating the recognition of antigen by CTL clone 2 is H-2-D^d restricted (figure 3.18).

To differentiate CTL clone 3 and clone 2, TCR genes usage assay by anti-TCRV_β mAb binding and FACS analysis was performed. It was shown that, although both CTL can be bound by anti-TCRβ8.3 mAb, T cells from CTL clone 2 can be bound by mAb anti-TCRα8.3 (figure 3.16 above), whereas T cells from CTL clone 3 can not be bound by anti-TCRα8.3. Thus, CTL clone 3 and CTL clone 2 are two different clones.

To verify the cross-reactivity of these different CTL clones, TNF/WEHI assay was

performed against several H-2^d tumour cells line, including CT26, A20, BCL1 and RENCA. As shown in figure 3.19, T cells from CTL clone 2 was shown to produce TNF in responses to CT26, A20 tumour cells and a murine lymphoma cell line, pi-BCL1; CTL clone 3 was shown to product TNF in responses to CT26, A20, pi-BCL1 and RENCA cell (figure 3.20.); and CTL clone 9 was shown to be specific to CT26, A20 and pi-BCL1 (figure 3.21).

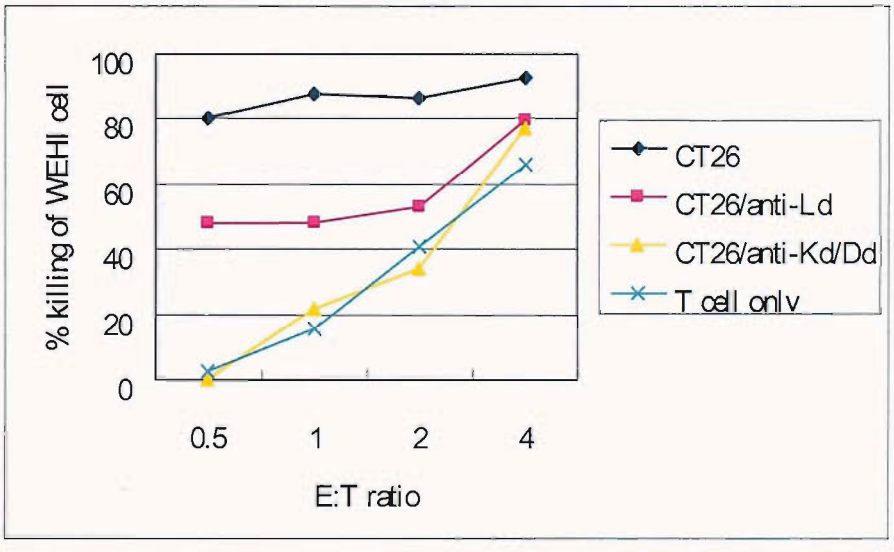


Figure 3.11. TNF production of CTL line 1 against CT26 cells could be blocked by anti-H-2-L^d and anti-H2-K^d/D^d monoclonal antibodies.

T cells from CTL line *1* were culture with CT26 cells (♦), or CT26 cells in the presence of anti-H2-Ld (■) and anti-H-2Kd/Dd (▲) monoclonal antibodies in a different E:T ratios. TNF production by CTL in the supernatant was examined by TNF/WEHI assay Supernatant from culture of T cells only (**X**) was also examined as the TNF background of CTL. Each point represents an average of duplicate (see *Materials and Methods*).

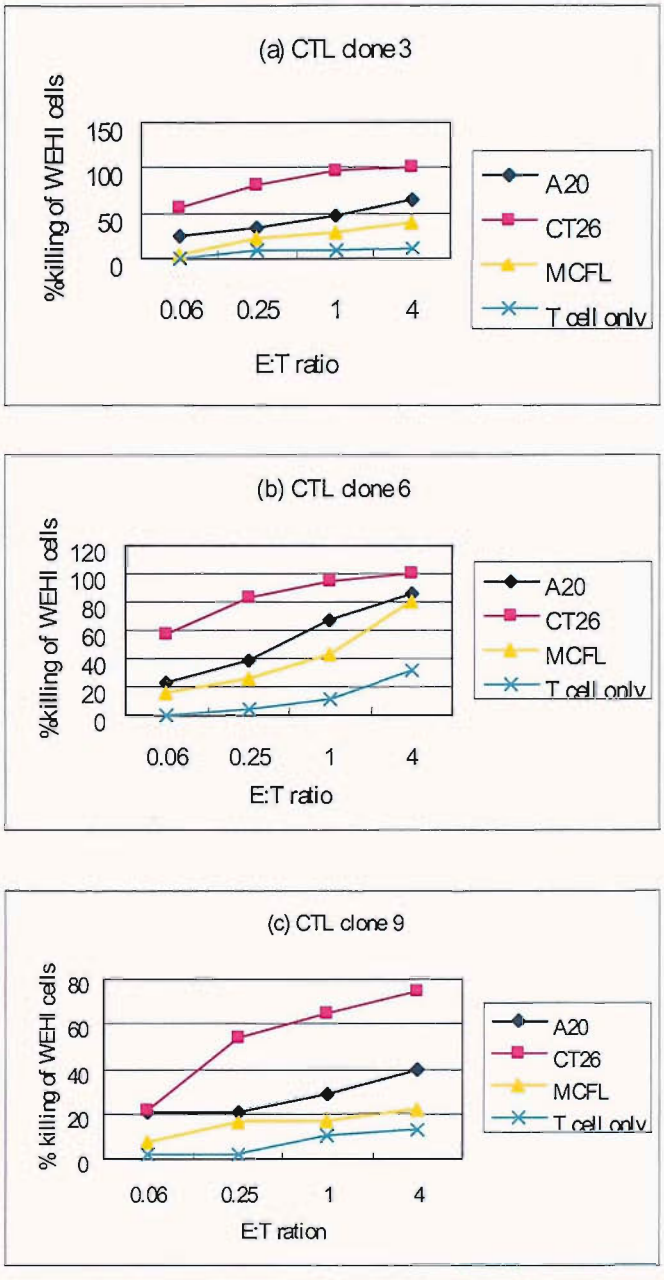


Figure 3.12. TNF production against A20 and CT26 cells of CTL clone 3, clone 6 and clone 9.

The CTL clone 3 (**a**), 6 (**b**) and 9 (**c**) were tested for the antigen-specific production of TNF against A20 cells (♦), CT26 cells (■) and syngeneic MCFL cell (▲) at various E:T ratios. Supernatant from CTL cells only (**X**) was tested as a TNF background of T cells. Target cells were added in 5000 cell/well. Each point represents an average of duplicate. The amount of TNF production was measured by its cytotoxic effect to WEHI cells (see *Materials and Methods*).

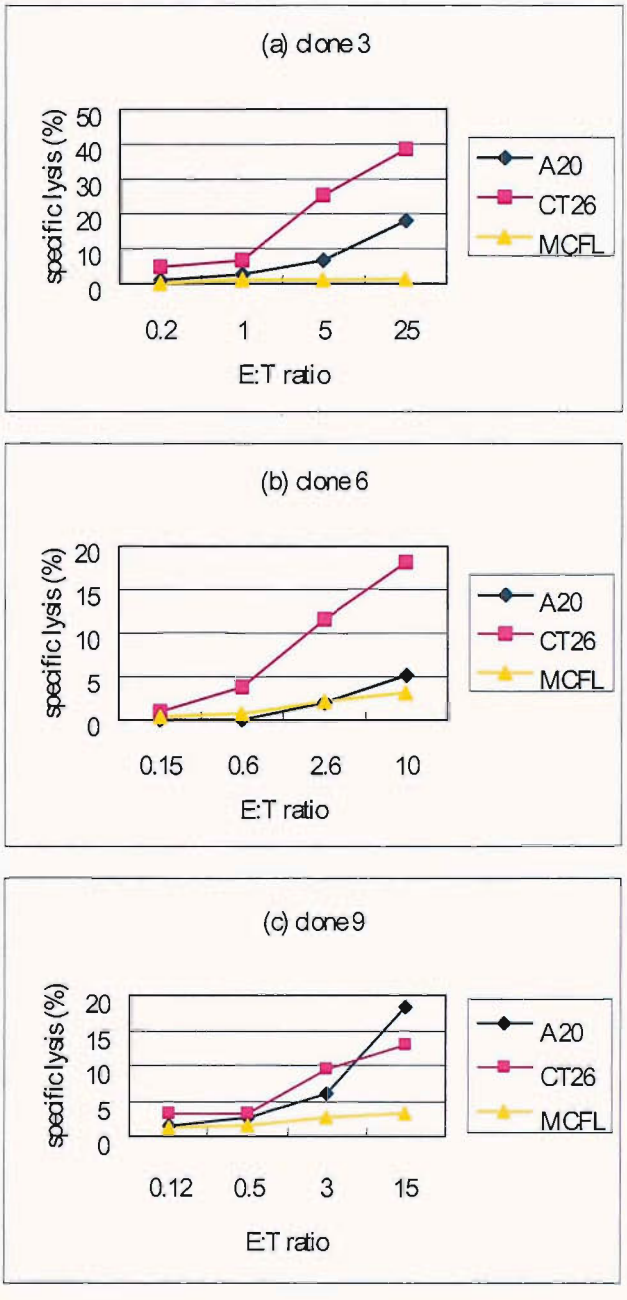


Figure 3.13. Cytotoxicity of CTL clones 3, 6 and 9 against A20 and CT26 tumour cells.

The CTL clone 3 (**a**), CTL clone 6 (**b**) and CTL clone 9 (**c**) were tested for 4-h ^{51} Cr-releasing assays against A20 cells (♦), CT26 cells (■) and syngeneic MCFL cells (▲) at various E:T ratios. Target cells were added in 5000 cell/well. Each point represents an average of triplicate (see *Materials and Methods*).

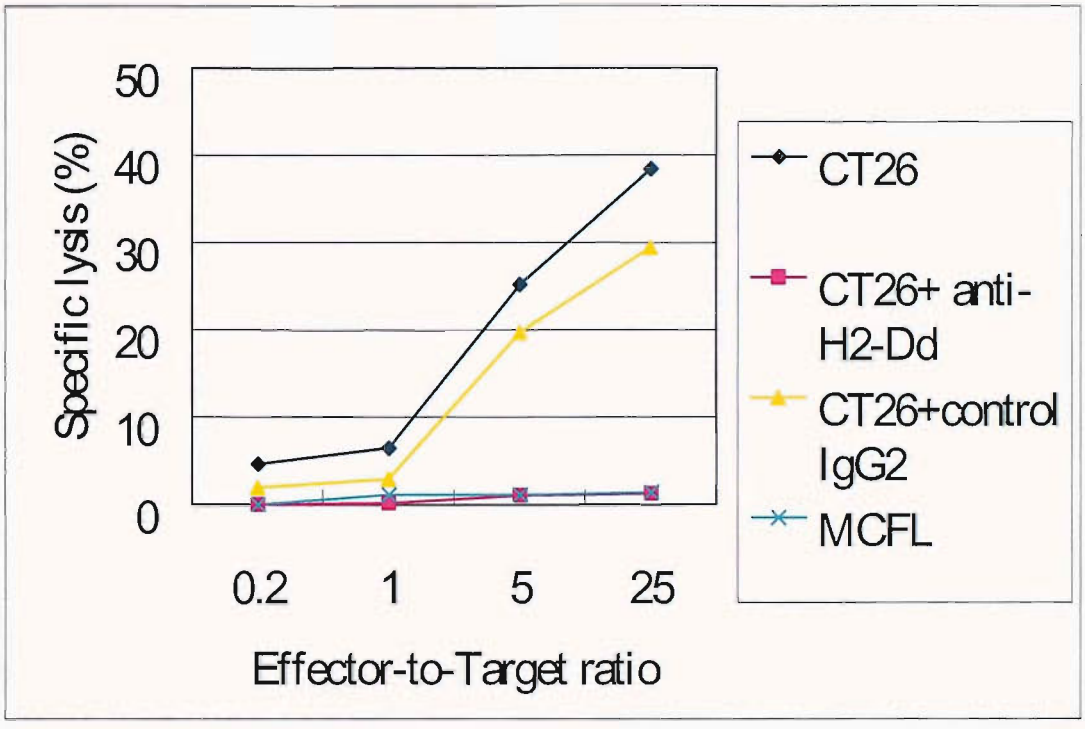


Figure 3.14. Cytotoxic activity of CTL clone 3 could be blocked by anti-H2-D^{d} monoclonal antibody.

The CTL clone *3* was tested for 4-h ^{51} Cr-releasing assays against CT26 cells (♦), CT26 cells in the presence of anti-H-2D^{d} monoclonal antibody (clone 34-5-8S) (■) or control IgG2 Antibody (▲) and syngeneic MCFL cells (**X**) at various E:T ratios. Target cells were added in 5000 cells/well. Antibody was added in a concentration of 30μg/ml. and cultured for 1 h before reaction. Each point represents an average of triplicate (see *Materials and Methods*).

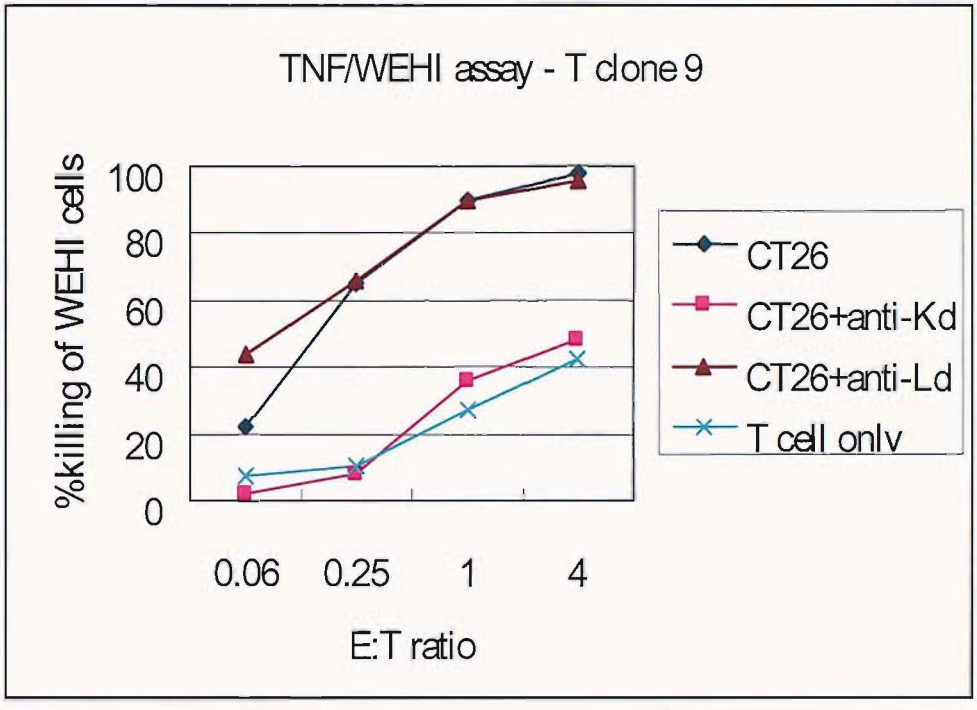
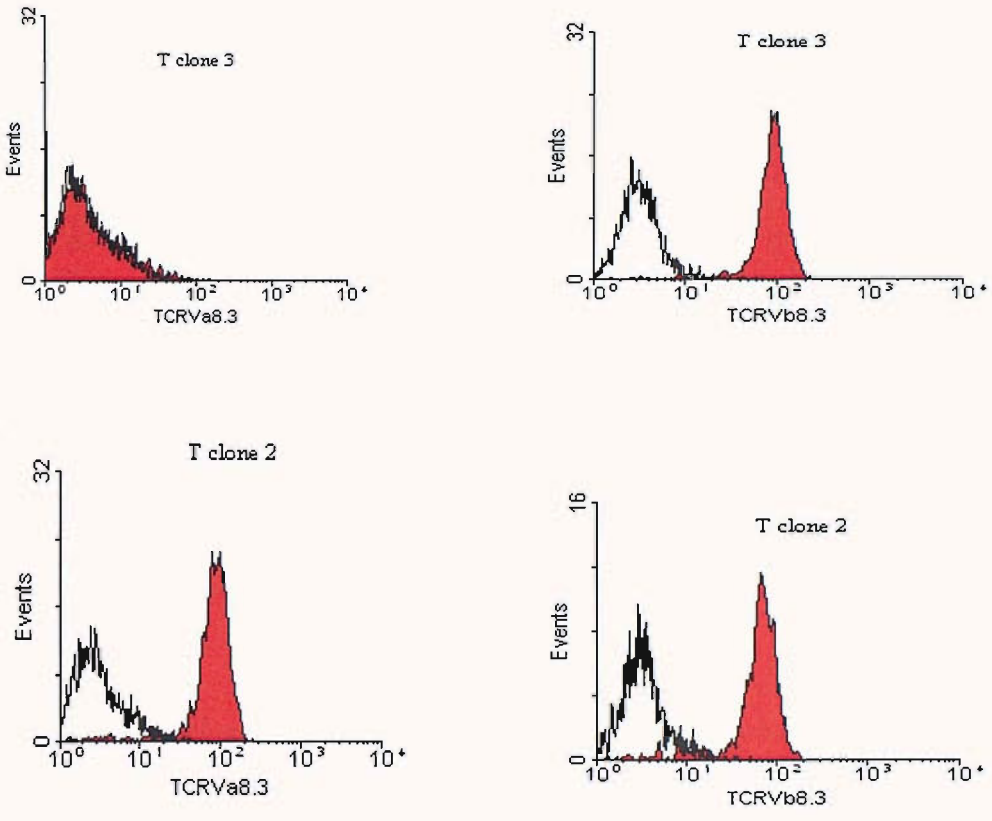


Figure 3.15. TNF production of CTL clone 9 against CT26 tumour cells could be blocked by anti-H-2-K^d monoclonal antibody.

CTL clone *9* was tested for TNF production against CT26 cells (♦), CT26 cells with anti-H-2K^d (■) or anti-H-2L^d (▲). Supernatant from culture T cells only was measured as TNF background of CTL (**X**). 5000 target cells were added in each well. Antibodies were added in a concentration of 20μg/ml and cultured for 1 h before reaction. The amount of TNF production was measured by the apoptotic effect to WEHI cells. Each point represents an average of duplicate (see *Materials and Methods*).



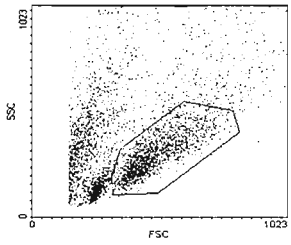


Figure 3.16. TCR clonality of CTL clone 3 and clone 2

Cells were stained with a panel of FITC conjugated mAb against various TCRVβ and TCRVα8.3. Cell stained with an irrelevant IgG2 mAb-FITC were set as a negative control. FACS profile presents dots obtained by analysis of 10^4 events.

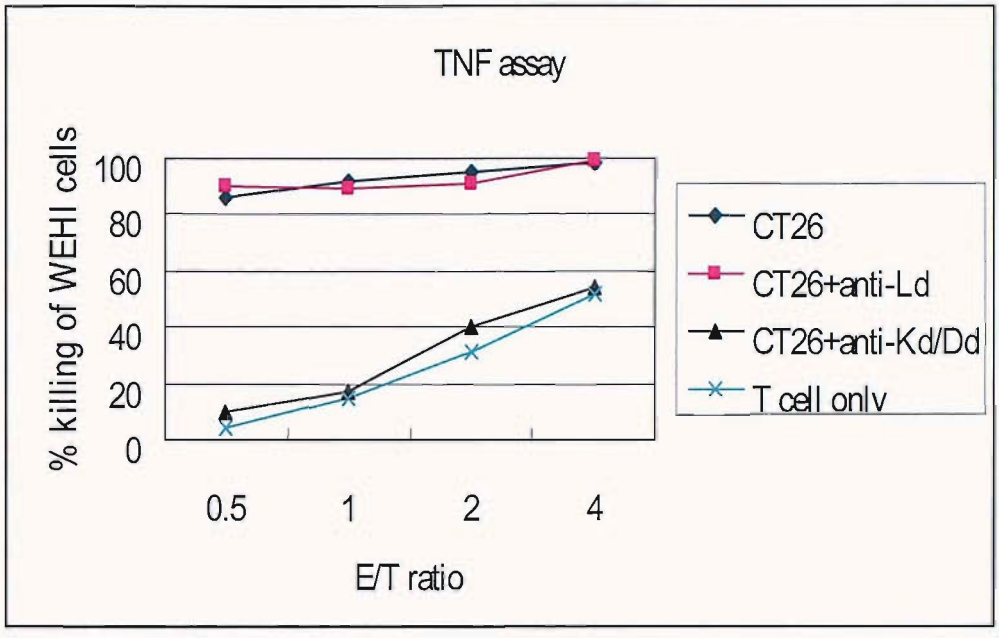


Figure 3.17. TNF production of CTL line 2 against CT26 cells could be blocked by anti-H2-K^d/D^d monoclonal antibodies.

T cells from CTL line *1* were culture with CT26 cells (♦), or CT26 cells in the presence of anti-H2-Ld (■) and anti-H-2Kd/Dd (▲) monoclonal antibodies in a different E:T ratios. TNF production by CTL in the supernatant was examined by TNF/WEHI assay Supernatant from culture of T cells only (**X**) was also examined as the TNF background of CTL. Each point represents an average of duplicate (see *Materials and Methods*).

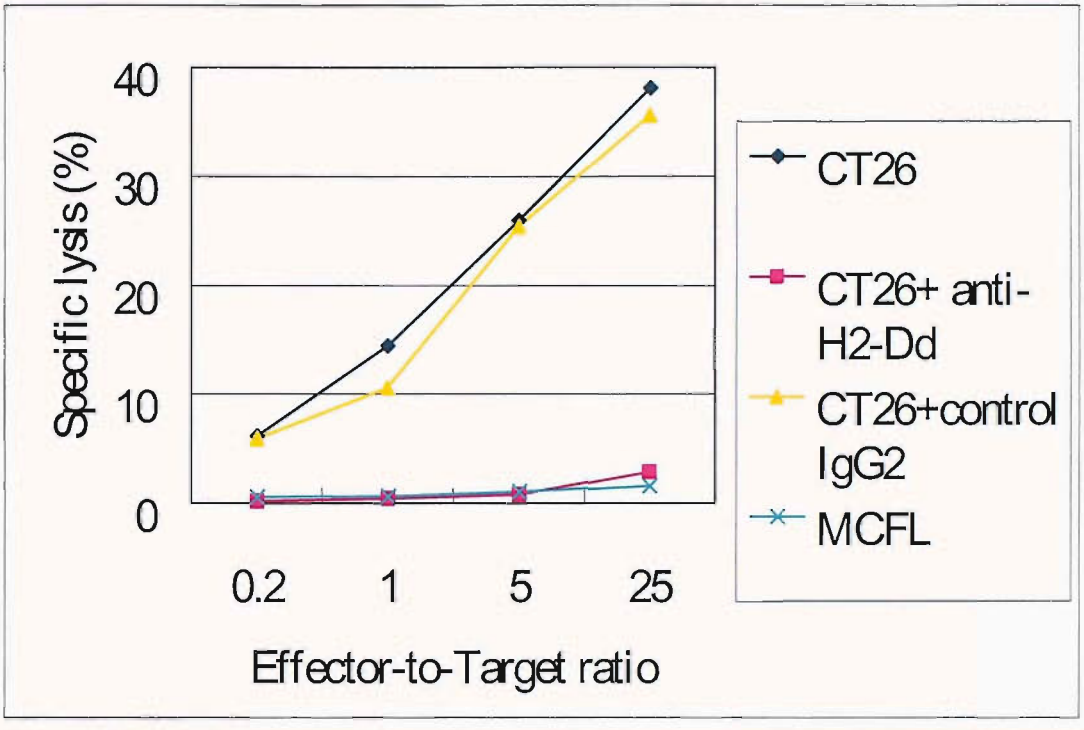


Figure 3.18. Cytotoxicity of CTL clone 2 could be blocked by anti-H2-D^{d} monoclonal antibody.

The CTL clone *2* was tested for 4-h ^{51} Cr-releasing assays against CT26 cells (♦), CT26 cells in the presence of anti-H-2Dd monoclonal antibody (34-5-8S) (■) or control IgG2 antibody (▲) and syngeneic MCFL cells (**X**) at various E:T ratios. Target cells were added in 5000 cell/well. Antibody was added in target cells a concentration of 30μg/ml and cultured for 1 h before reaction. Each point represents an average of triplicate (see *Materials and Methods*).

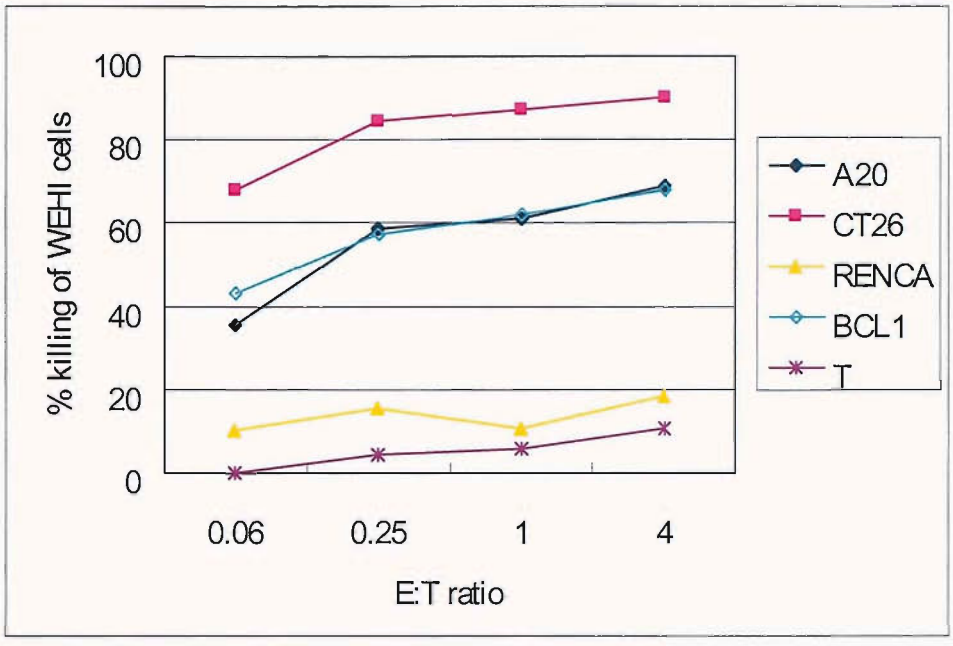


Figure 3.19. TNF/WEHI assay of CTL clone *2* against H-2^d tumour cell lines.

The CTL clone *2* was tested for the antigen-specific production of TNF against A20 cells (♦), CT26 cells (■), RENCA cells (▲) and pi-BCL1 cells (◊) at various E:T ratios. Supernatant from T cells only (**X**) was tested as the TNF background of CTL cells. Target cells were added in 5000 cells/well. The amount of TNF produced was measured by its cytotoxic effect to WEHI cells. Each point represents an average of triplicate (see *Materials and Methods*).

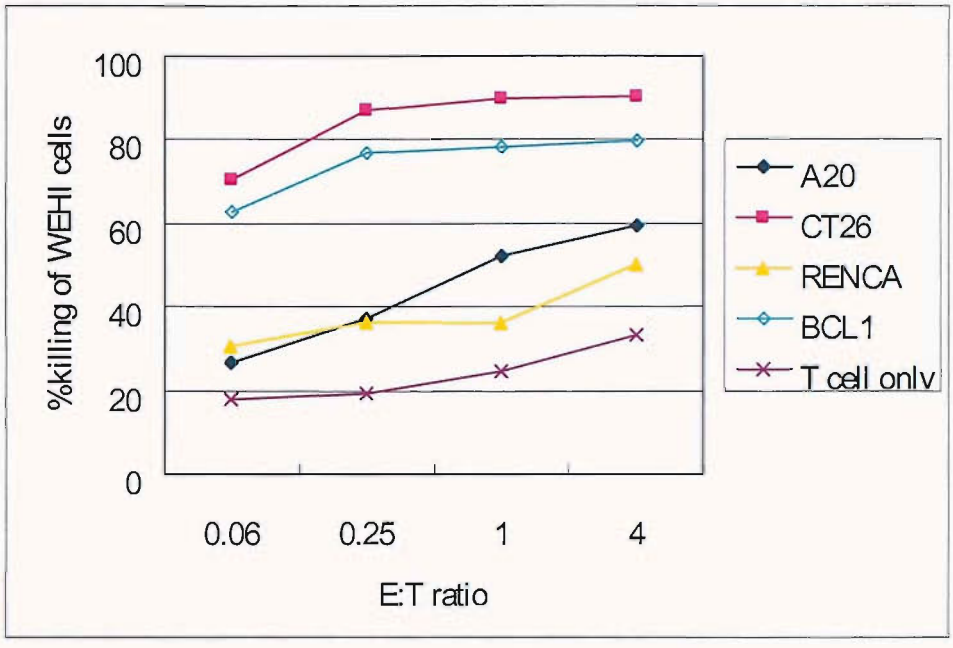


Figure 3.20. TNF/WEHI assay CTL of clone *3* against H-2^d tumour cell lines.

The CTL clone *3* was tested for the antigen-specific production of TNF against A20 cells (♦), CT26 cells (■), RENCA cells (▲) and pi-BCL1 cells (◊) at various E:T ratios. Supernatant from T cells only (**X**) was tested as a TNF background of CTL cells. Target cells were added in 5000 cells/well. The amount of TNF production was measured by its cytotoxic effect to WEHI cells. Each point represents an average of triplicate (see *Materials and Methods*).

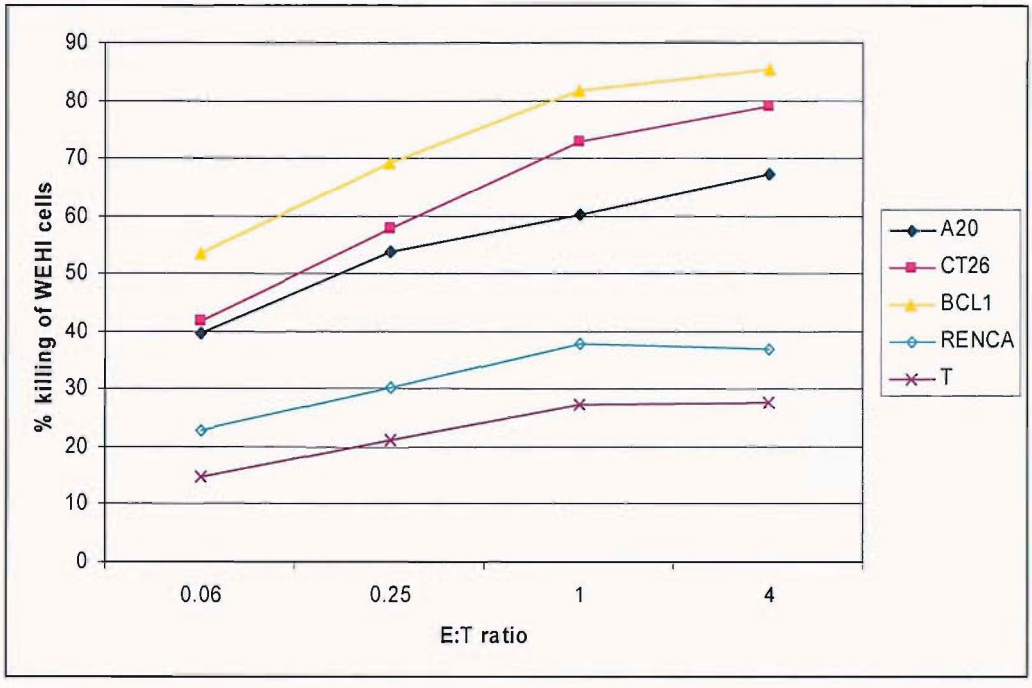


Figure 3.21. TNF/WEHI assay of CTL clone 9 against H-2^d tumour cell lines.

The CTL clone *9* was tested for the antigen-specific production of TNF against A20 cells (♦), CT26 cells (■), pi-BCL1 cells (▲) and RENCA cells (◊) at various E:T ratios. Supernatant from T cells only (**X**) was tested as a TNF background of CTL cells. Target cells were added in 5000 cells/well. The amount of TNF production was measured by its cytotoxic effect to WEHI cells. Each point represents an average of triplicate (see *Materials and Methods*).

3.2.5. Outgrowth of AH1-specific CTL population from long-term culture of T cells line 1

A key feature of the protective immune responses elicited to CT26 tumour cells in the absence of CD25+ regulatory T cells is the lack of a detectable CTL response to the immunodominant epitopes from gp70 (AH1) *in vivo* [16]. It is not known whether this is due to the lack of priming of AH1-specific CTL in these mice or whether there is another mechanism rendering the AH1 response subdominant.

After continuous *in vitro* culture for more than 6 months, T cells from CTL line 1 were found different from the original cells in some properties; for example, in a ^{51}Cr-releasing assay, the cytotoxicity towards CT26 tumour cells was much more prominent than that towards A20 cells (figure 3.22.) as compared with the same assay in early culture (figure 3.5 above). In addition, when we repeated the H-2^{d} antibodies blocking experiments 6 months later, it was found that the cell populations in this long-term CTL cell line were different from their original cell line since their CTL activities could only be blocked nearly completely by an anti-H-2-L^{d} monoclonal antibody (figure 3.23). This was different from what was found in early culture of T cell line 1, in which the TNF production against CT26 tumour cells can be completely blocked by mAb against anti-H-2-K^{d}/D^{d} and partially by anti-H-2-L^{d} (figure 3.11). As a result, we considered that the predominant cell population in long-term T cell line 1 has changed.

Since the well-known antigenic peptide, AH1 (SPSYVYHOF) [287] was defined as an H-2-L^{d}-restricted CTL epitope derived from *env* protein of *gp*70 and expressed extensively on CT26 and other tumour cells. We wished to examine if the

predominant T cell population in this long-term CTL cell line was altered and specific to AH1 expressed by H-2-L^d on CT26 cells. We used an AH1/H-2-L^d tetramer to stain the TCR of T cells from long-term CTL line 1 and analysed by flow cytometry (see *Materials and Methods*). As expected, more than 80% of CD8+ T cells from the long-term CTL cell line 1 were shown to be stained by AH1/H-2-L^d tetramer (as shown in figure 3.24). In the early subcloning of CTL from T cell line 1, we could not isolate cloned T cells that were H-2-L^d restricted.

We verified this observation *in vitro* by showing that T cells from early passage of T cell line 1 were not reactive to AH1-pulsed MCFL cells in a TNF/WEHI assay (figure. 3.25a.). Furthermore, it has been demonstrated in previous experiment by Golgher that gp-70 may not be the targeted antigen in our model because CD25+ regulatory T cell-depleted vaccinated mice were not able to resist infection by a recombinant vaccinia virus expressing gp70 (rVVgp70) [16].

We re-tested long-term T cell line 1 for AH1 reactivity and were able to detect AH1-activity (figure. 3.25b.). The specificity of T cells from long-term CTL line 1 towards the antigenic peptide, AH1, was also supported by a ^{51} Chromium-release assay using BCL1 pulsed with AH1 (figure. 7.26).

The *in vitro* condition in the culture of T cell line 1 was almost the same along the period. Obviously, the initial cell population in T cell line 1 were predominantly specific to antigens expressed by H-2-K^d/D^d molecules on CT26 tumour cells. After 6 months, the predominant population of T cells in long-term cell line 1 were reactive to AH1/H-2-L^d complex on target cells. Obviously, AH1-specific CD8+ T cells were outgrown in the long-term T cell line 1.

The AH1-specific CTL was actually presented in the initial cell culture but with very small percentage (see figure 3.5 above). One possibility could be that AH1-specific T cells need the presence CD4+ T cells in initial activation; however they might have growth superiority in the absence of CD4+ helper T cells. On depleting CD25+ regulatory cells in our model, both tumour-specific CD4+ T cells and multiple clones of CD8+ T cells were activated in vaccinated mice. Whereas, after *in vitro* culture for a long time, when CD4+ helper T cells were depleted, these AH1/H-2L^{d}-specific CD8+ T cells may grow and proliferate faster than other CTL and finally outgrow in the long-term T cell line. However, this assumption needs to be further dissected in the future.

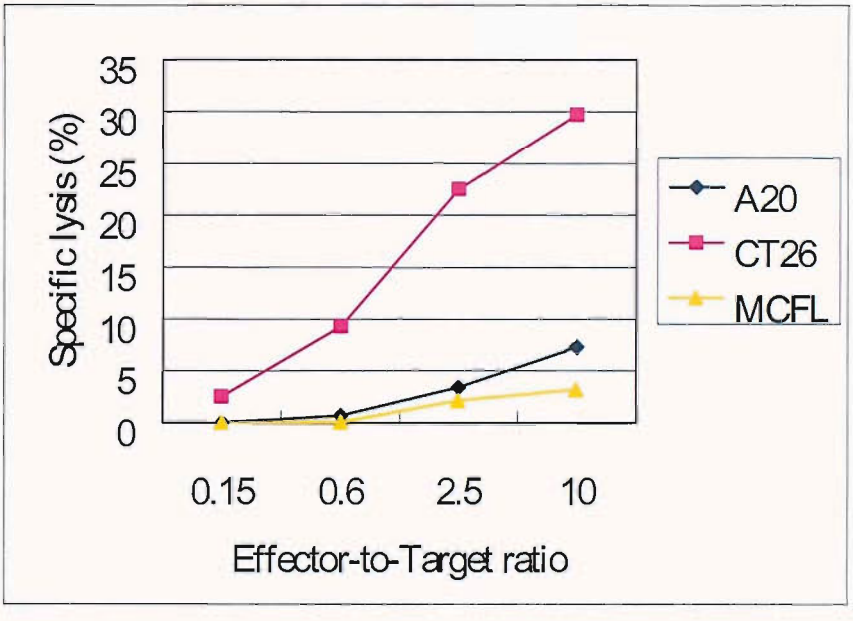


Figure 3.22. Cytotoxicity of long-term T cells line 1 towards A20 and CT26 tumour cells.

After culture for more than 6 months, the T cell line 1 was tested for 4-h ^{51} Cr-release assays against A20 cells (♦), CT26 cells (■) and syngeneic MCFL cell (▲) at various E:T ratios. Target cells were added in 5000 cell/well. Each point represents an average of triplicate (see *Materials and Methods).*

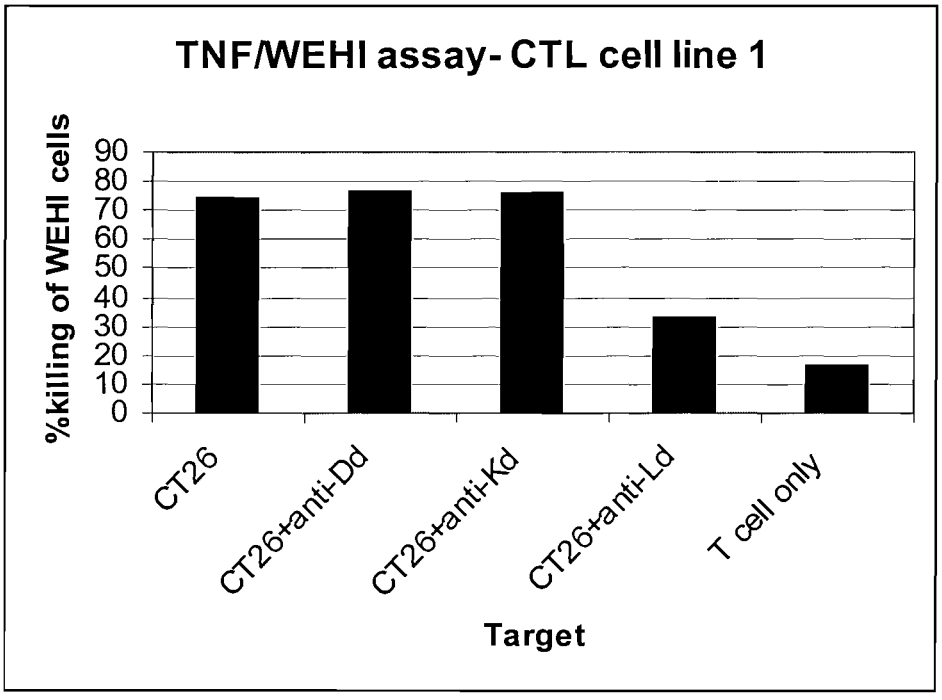


Figure 3.23. TNF production of T cells from long-term T cell line 1 towards CT26 tumour cells can be blocked by anti-H-2-L^d mAb.

T cells from long-term T cell line 1 was tested for TNF production against CT26 cells alone, CT26 cells with anti-H-2-D^d, anti-H-2-K^d, or anti-H-2-L^d in TNF/WEHI assay. Supernatant from T cell culture only was measured as TNF background. 5000 CT26 tumour cells were added in each well. Antibodies were added in a concentration of 20μg/ml and cultured for 1 hour before the addition of T cells. The amount of TNF production was measured by percentage of killing of WEHI cells. Each point represents an average of duplicates.

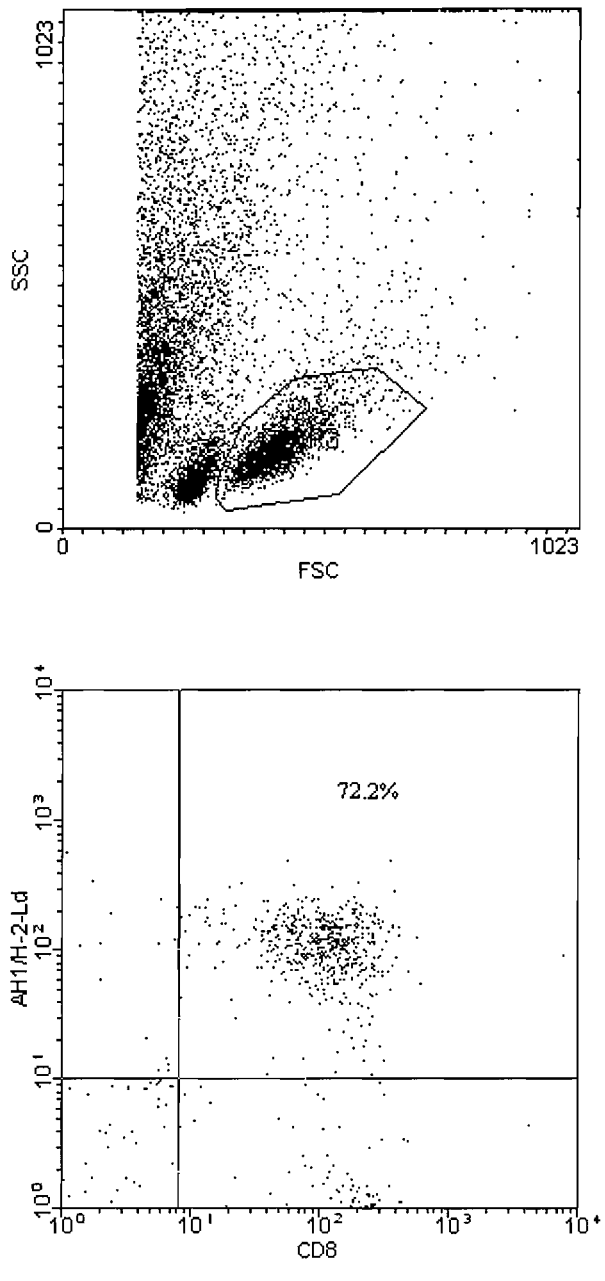
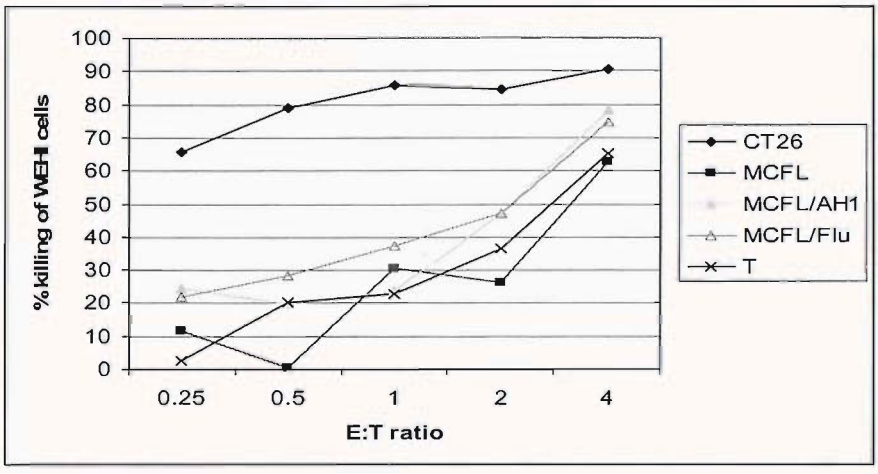


Figure 3.24. AH1/H-2-L^d Tetramer staining and flow cytometric analysis of long-term T cell line 1

Cells from long-term (>6 months) T cell line 1 were stained with anti-CD8a mAb-FITC and AH1/ H-2L^d tetramer-RPE and analyzed by flow cytometry. Cells stained with P815/H-2-L^d tetramer-RPE were used as a negative control. FACS profile presents dots obtained by analysis of 5 X 10^3 events.

(a)



(b)

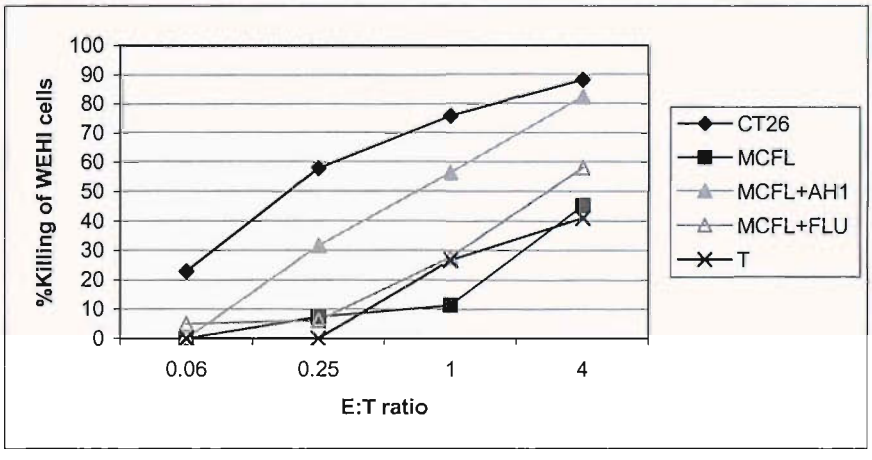


Figure 3.25. TNF production of T cell line 1 against syngeneic MCFL cells pulsed with AH1.

MCFL cells were culture with 10μM of peptide AH1 (▲), or irrelevant peptide (Flu) derived from influenza matrix protein (△) in serum-free complete medium for 1 h at 37°C. Then these cells was washed once and cultured with CTL from T cell line 1 in the beginning of culture **(a)** or CTL after 6 months of culture **(b)**. The production of TNF of CTL was examined by **TNF/WEHI assay**. CT26 tumour cells (♦) and MCFL cells (■) were used as positive and negative control. Supernatant from T cells only **(X)** was also examined as the TNF background of CTL. Each point represents an average of duplicate (see *Materials and Methods*).

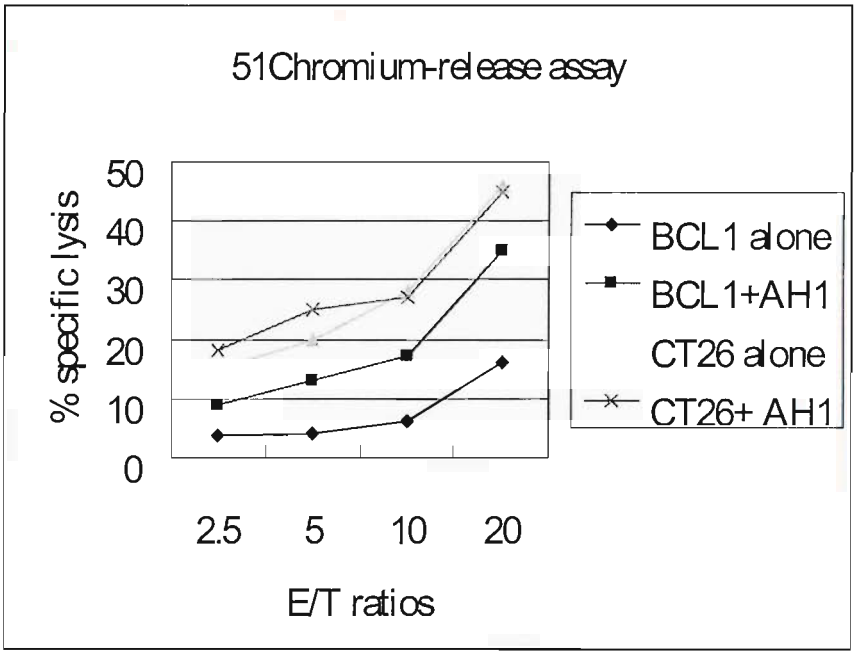


Figure 3.26. ^{51}Cr-release assay of T cells from long-term T cell line 1 against target cells pulsed with AH1.

5000 cells/well of target cells (CT26 tumour cells alone (▲), CT26 pulsed with AH1(x), BCL1 tumour cells alone (♦), and BCL1 pulsed with AH1(■)) were culture with 10μM of peptides AH1 in serum-free medium for 1 hour at 37°C. After that, the target cells were labelled with chromium (^{51}Cr) and T cells from long-term T cell line 1 were then added in indicated E/T ratios. Each point represents an average of triplicates.

3.2.6. Loss of specificity in CTL cell from long-term culture of cloned T cells

The major impediment in identification of tumour-associated antigens using CD8+ T cells is the need for large numbers of tumour-specific T cells. To become fully functional, the CD8+ T cells from *in vitro* CTL clones need to be activated by either peptides with spleen cells or anti-CD3/anti-CD28 monoclonal antibodies in the presence of endogenous cytokines.

We have established several tumour-specific CTL clones in *ex vivo* culture. However, we were interested in examining the persistence of antitumour effector functions in our cloned CTL that were grown *in vitro* prolonged culture. There was a limited amount of information in the literature concerning the stability of CTL clone grown in long-term culture. Jeffrey and colleagues [313] reported that prolonged culture of vaccine-primed lymphocytes resulted in decreased antitumour killing and a change in cytokine secretion. On the other hand, Kwong and colleagues [314] have demonstrated phenotypic stability in a long-term human CTL line directed against an immunodominant epitope of carcinoembryonic antigen in terms of either lytic properties, cytokine production and the expression of adhesion molecules.

Change in TNF production toward CT26 tumour cells was noted first for T cells from long-term CTL clone 2 that were cultured *in vitro* with regular re-stimulation for more than 18 months. As represented by TNF/WEHI assays in figure 3.27, the antigen-specific TNF production of long-term cloned CTL towards CT26 tumour cells decreased more than half in comparison with the profile performed about one year ago. Similarly, the decreased specific-cytotoxicity towards CT26 tumour cells was demonstrated by ^{51}Chromium-release assay in long-term CTL clone 2 (see figure 3.28,

in comparison with figure 3.18 that was performed about 18 months ago).

The phenotypic expressions on our long-term CTL cells were found to be somehow different from those on short-term T cells. As shown in figure 3.29, CTL in earlier culture was analyzed to be CD45RA ^{low} and CD45 RC ^{low}. Whereas, the long term CTL clone 2 have turned to be CD45RA ^{high} and CD45 RC ^{high}. However, the significance of these changes and their influences to cellular cytotoxic functions remains unclear.

In addition, although the expressions of CD8 and CD3 on long-term CTL remain similar, the expression of their TCR variable $\alpha\beta$ gene was different. The expression of TCRVα8.3 was shown downregulated (figure 3.29) on our long-term CTL cells, whereas their TCRVβ8.3 expression remained unchanged (figure 3.30). Presumably, the decreased expression of TCRVα8.3 gene on long-term CTL cells has led to the diminished cytotoxicity towards CT26 tumour cells.

The antigen-specificity of CTL was changed after long-term in vitro culture in term of TNF production and cytotoxicity towards CT26 tumour cells. However, the mechanisms that cause this change remain unknown. Furthermore, it was also not clear that the decreased expression of TCR variable α chain was owing to expression downregulation under long-term culture or due to predominant outgrowth of non-specific T cells in long-term CTL that might be not truly a purified clone originally.

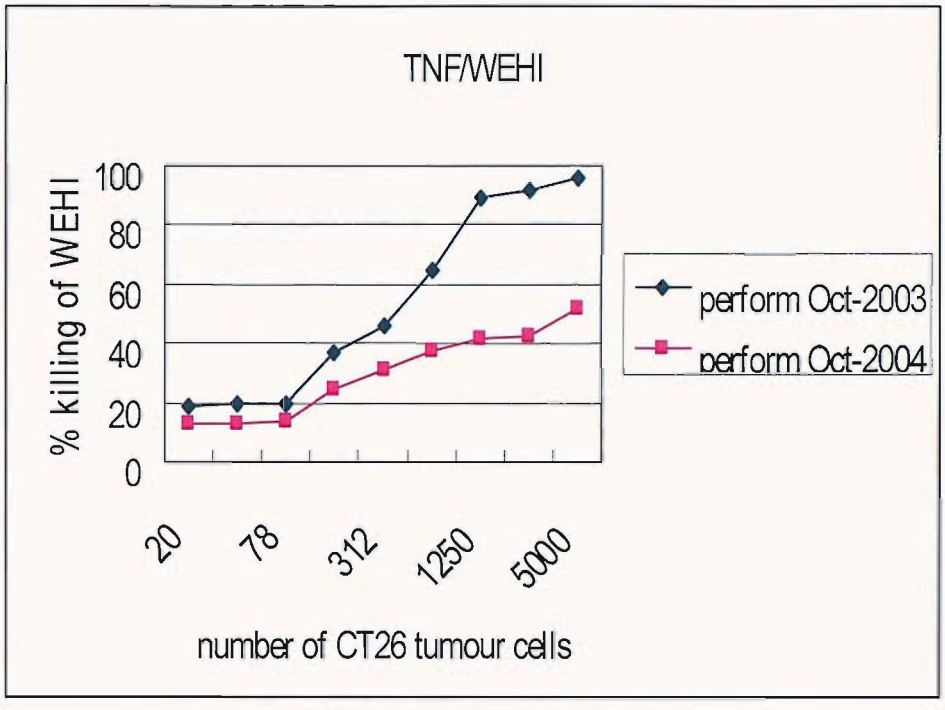


Figure 3.27. Decreased TNF production towards CT26 tumour cells in T cells from long-term CTL clone 2

A summary of two independent TNF/WEHI assays of T cells from CTL clone 2 performed in different time point, Oct. 2003 (♦) and Oct. 2004 (■) in the period of continuous *in vitro* culture. WEHI cells sensitivity to TNF was confirmed on each occasion using standard dilations and remained unchanged.

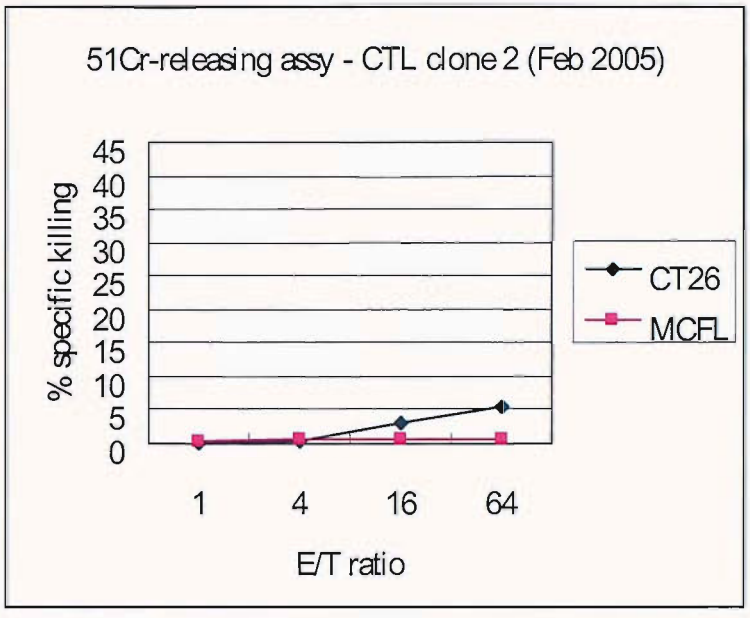


Figure 3.28. ^{51}Cr-release assay of long-term CTL clone 2 toward CT26 tumour cells

Standard cytotoxicity assay towards CT26 tumour cells (♦) for T cells from CTL clone 2 that were continuously culture in vitro for more than 18 months. MCFL cells (■) were used as a negative control.

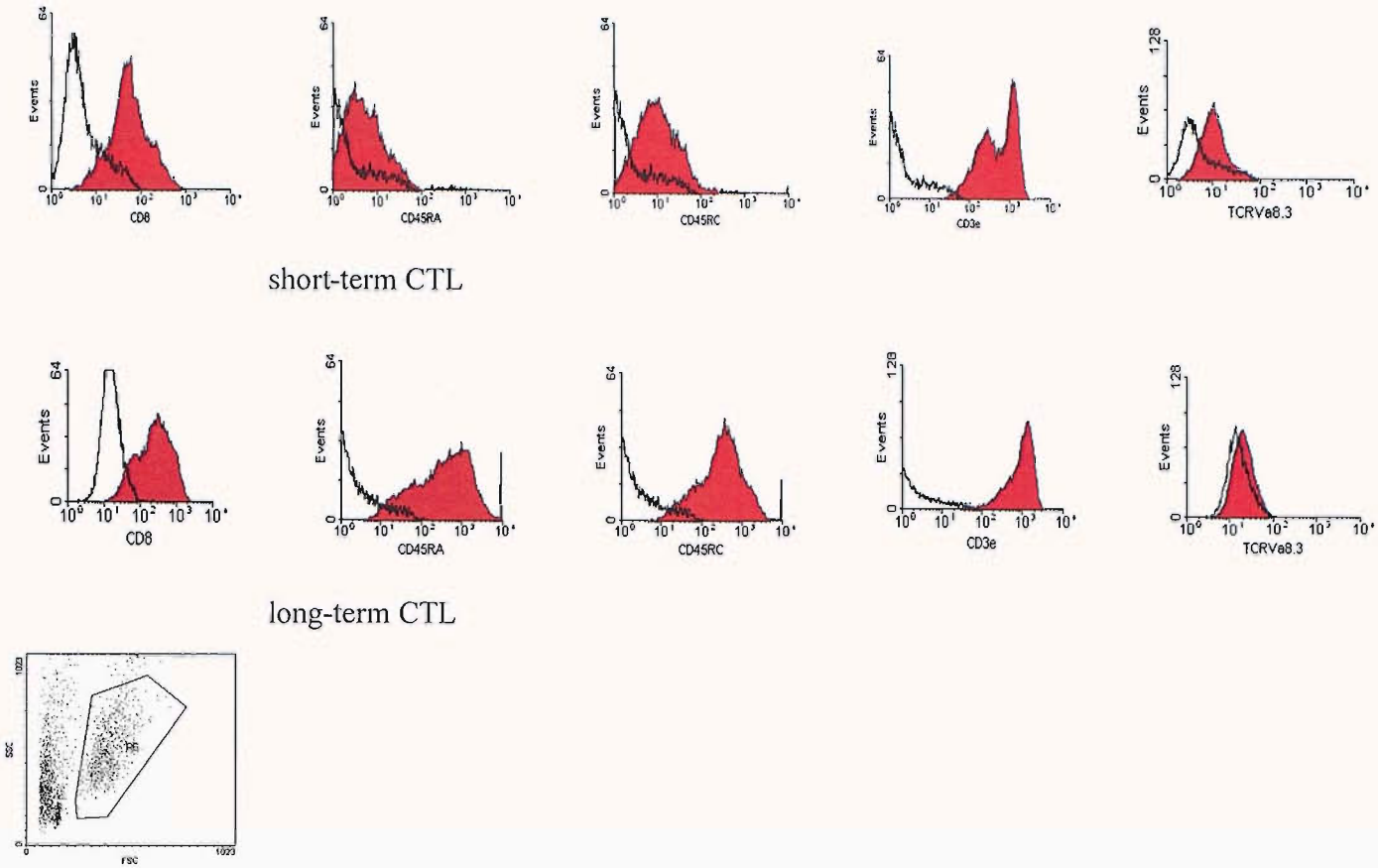
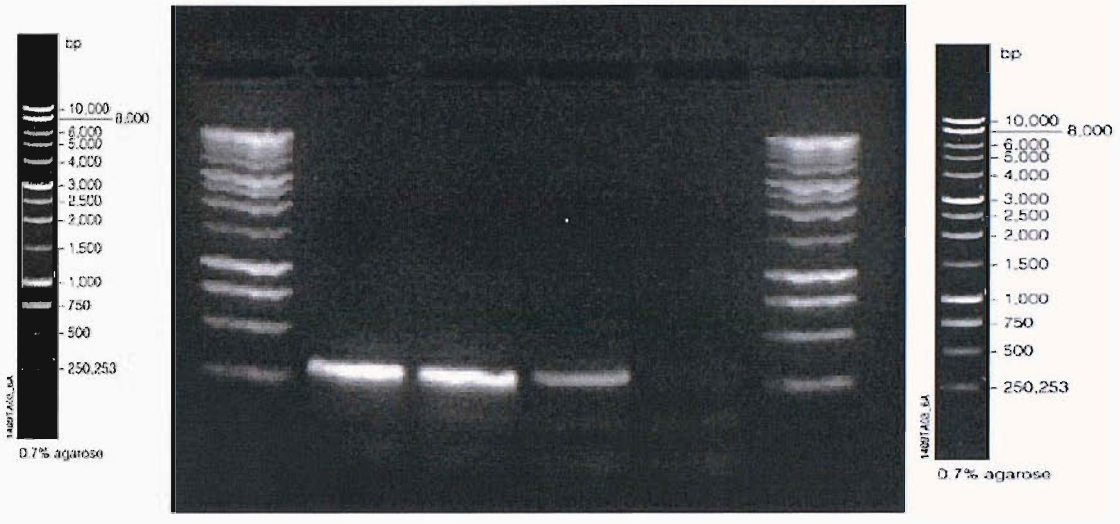


Figure 3.29. Phenotypic analysis of CTL clone 2 from both earlier culture and long-term culture

T cells from short-term culture (upper panel) and long-term culture (lower panel) of CTL clone 2 were stained by various mAb and were analysed with FACS.



1 2 3 4

Figure 3.30. Persistent expression of TCRVβ8.3 in T cells from long-term CTL clone 2 in RT-PCR. .

RT-PCR with primers of TCRVβ8.3 and CBR was carried out with mRNA extracted from short-term CTL clone 2 (1), long-term CTL clone 2 (2), and Balb/C spleen cells (3); CTL clone 9 (4) as a negative control.

3.2.7. Generation of an antigen-specific, LacZ-inducible T-T hybridoma cells

CTL has been used successfully to identify from cDNA libraries [2, 105, 172]. The way to success for this method is the availability of a large number of CTL with stable reactivity that is reliable over a period of *in vitro* maintenance. To this end, generation of immortalized hybridoma cells is preferential to making CTL clone.

In order to generate antigen-specific TCR-controlled LacZ-inducible T-T hybridoma cells, tumour specific CD8+ T cells from immunized mice in our animal model [16] were harvested and used to fuse with the fusion partner, BWZ.36/CD8α cells, by standard PEG protocols detailed as in *materials and methods*. We have tried using different panels of tumour-specific CD8+ T cells that were prepared with different methods. Table 4 summarizes the difference and results of our trials.

Table 4. Summary of attempts to make hybridoma cells from tumour-specific CD8+ T cells

	T cells Fused	Cell numbers		Number of wells		
		T cells	BWZ.36CD8α	Plated	Positive growth	Tumour-specific/ tested
1	Splenocytes [a]	1 X 10^7	1 X 10^7	384	78	0
2	Splenocytes [b]	1 X 10^7	1 X 10^7	192	51	4
3	CTL cell line 1[c]	5 X 10^6	5 X 10^6	96	0	0
4	CTL clone3[d]	5 X 10^6	5 X 10^6	96	12	5(1) [e]

a. Splenocytes without pre-activation.

b. Splenocytes with *in vitro* pre-activation by irradiated CT26 tumour cells in the presence of IL-2 for 5 days.

c. CD8+ T cells were harvested from CTL line *1* that was *in vitro* cultured for 3 months, and used on

the 5th day after re-stimulation.

d. CD8+ T cells were harvested from CTL clone *3* that was *in vitro* culture for 6 months, and used on the 5th day after re-stimulation.

e. 5 hybridoma cells were in the same clonality, overall designated as H-3-4-17

No hybridoma was selected in the first trial, in which CD8+ T cells were prepared from immunized mice and used directly in fusion without *in vitro* re-stimulation. In our second trial, the procedure was modified. Spleen cells from immunized mice were *in vitro* re-stimulated once with irradiated CT26 tumour cells in the presence of IL-2. On the 5th day after re-stimulation, the viable mononuclear cells were depleted of CD4+ T cells and the CD8+ enriched blast cells were used in fusion. Consequently, 51 growing hybridoma cells were obtained in the second fusion. Among these, 4 putative positive tumour-specific hybridoma cells, designated as C2, F3, G3, and G5, as shown by their specific reactions against CT26 and A20 tumour cells in CPRG assays (see figure 3.31).

However, the hybridoma cells were found to be very unstable in our culture even after subcloning by limiting dilution. Within one month of culture, all the putative positive hybridoma cells rapidly lost the specificity to tumours without evidence of infection by mycoplasma. Malarkannan and colleagues (2001) [200] have reported that their hybridoma cells were stable in culture for more than one month in their hands. It is possible that the genetic characteristics of hybridoma cells are varied from cell to cell. However, it is hard to determine the difference between their hybridoma and ours. .

In the third fusion, we tried to generate hybridoma cells using CTL blast cells derived

from long-term cultured T cell line. There were only a few reported cases of CD8+ hybridoma cells made by fusion of CTLs derived from long term T cell line or clone, perhaps because it is difficult to fuse such a pre-established T cell. Still, Shoichi.[262] have shown in their experiments that T-T hybridoma cells could be successfully made from cloned T cells that have been *in vitro* cultured with specific antigen and APCs in the presence of IL-2. The resulting hybridoma cells and matched cloned T cells differed subtly in their capacity to mediate killing function and cytokine production. In our experiment, however, we failed to make hybridoma cells from our T cell line that has been *in vitro* cultured and re-stimulated with irradiated CT26 tumour cells in the presence of IL-2 and IL-15 for more than 4 months.

Alternatively, the cloned T cells were considered to be better candidates for fusion than long-term cultured T cell line due to purification of cloned cells and increased population of reactive T cells. In our fourth trial, we prepared CD8+ blast cells from our CTL clone 3 and used to fuse with BWZ.36/CD8α cells on the 5th day after re-stimulation by irradiated CT26 tumour cells. Using CD8+ T cells from CTL clone 3, we have obtained a tumour-specific T-T hybridoma cells that are specific to both CT26 and A20 tumour cells, but not syngeneic MCFL or monkey kidney Cos-7 cells (see figure 3.32, hybridoma cells H-3-4-17).

As their parental CD8+ T cells, the phenotypic expressions of these hybridoma cells were shown to be CD3+CD8+ with TCRVβ8.3 (figure 3.33). The tumour specific TCR reactions induced the accumulation within hybridoma cells of intracellular β-galactosidase that can be measured by CPRG assay. In addition, the TCR-controlled LacZ activities were H-2-D^d restricted because they can only be blocked by an anti-H-2-D^d mAb, but not by anti- H-2-K^d mAb. Binding of CD8 molecules on hybridoma

cells by mAb against CD8α could partially block the stimulation of hybridoma cells by specific CT26 tumour cells. On the other hand, we found that our hybridoma cells neither have cytotoxic killing effect nor secrete TNF towards specific tumour cells.

As expected, these hybridoma cells were known to be very unstable in culture. Their tumour-specificity was lost in a certain percentage of cells in each generation and was disappeared eventually after about 4-6 weeks of culture. It was not a worthwhile strategy to rescue the lost specificity by limiting dilution. A reasonable approach is to expand a large pool of cells as early as they are generated after cell fusion, freeze down significant amount of cells at -80^{o}C, and draw on frozen stock regularly during subsequent experiments.

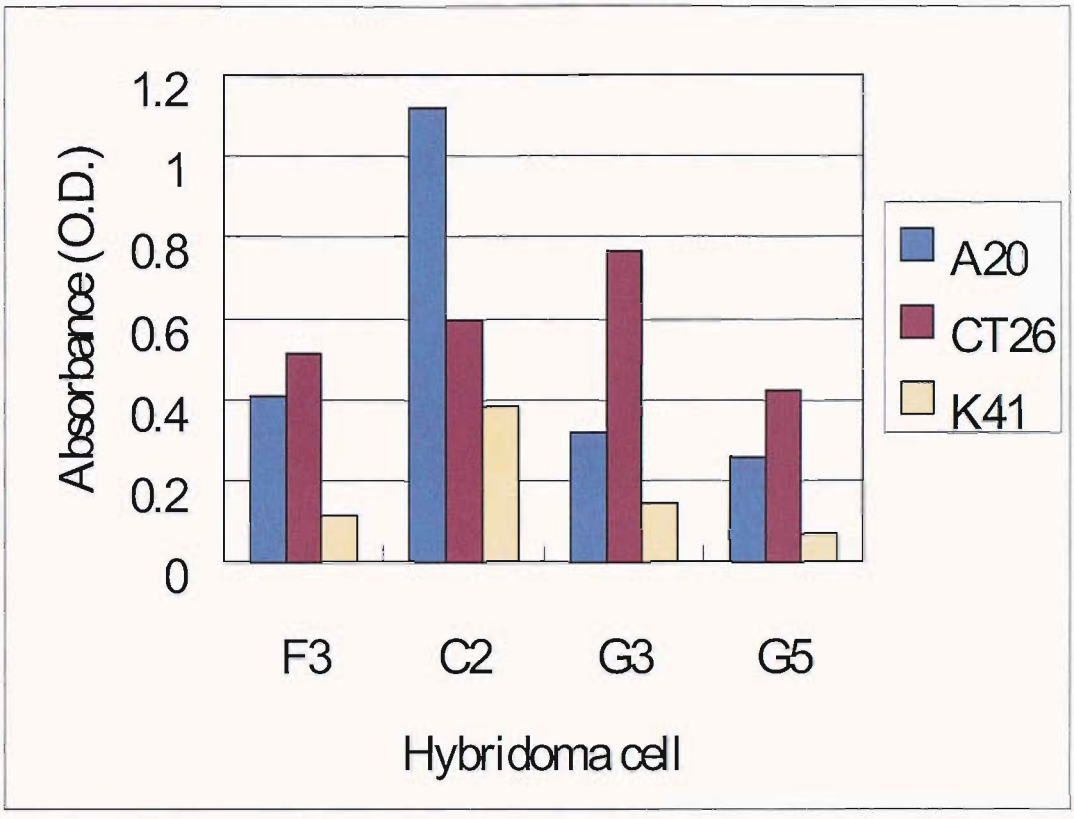


Figure 3.31. Antigen-induced LacZ activity against A20 and CT26 tumour cells of 4 positive hybridoma cells (F3, C3, G3 and G5) in the first fusion trial.

1 X 10^5 cells/well of hybridoma cells were cultured with 1 X 10^5 of target cells (A20 or CT26 tumour cells). After a 20-h incubation at 37°C, the TCR induced LacZ activity was measured by CPRG assay (see *Materials and Methods*). Murine embryonic fibroblast cells, K42, were used as a negative control. Each point represents an average of duplicates.

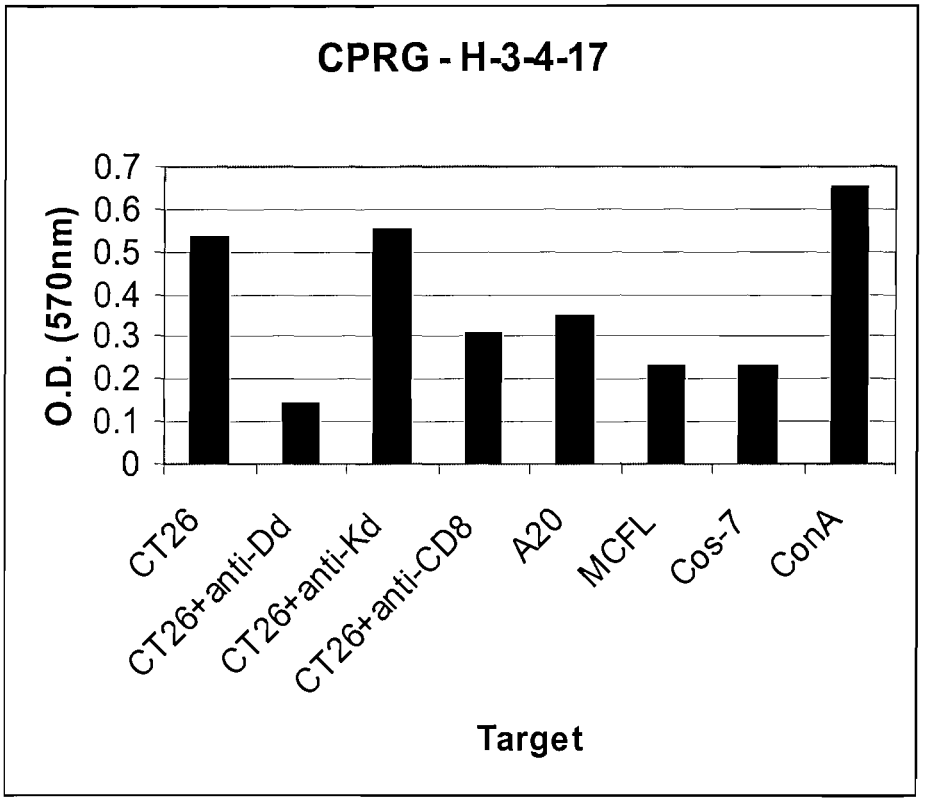


Figure 3.32. CPRG assay for the tumour-specific reactivity and H-2 restriction of hybridoma cells H-3-4-17.

Hybridoma cells (1 X 10^5 cells/well) were cultured with 1 X 10^5 of target cells (A20, CT26 tumour cells and MCFL cells, Cos-7 cells as negative controls) with or without the presence of monoclonal antibodies against MHC class I molecules H-2D^d and H-2K^d, and CD8α (in a final concentration of 10μg/ml). ConA was added in a concentration of 7.5ng/ml as a positive control. After 20 hours of incubation at 37^{O}C, the TCR-controlled LacZ activities within hybridoma cells were measured by a CPRG assay (see *Materials and Methods*). Each point represents an average of duplicates.

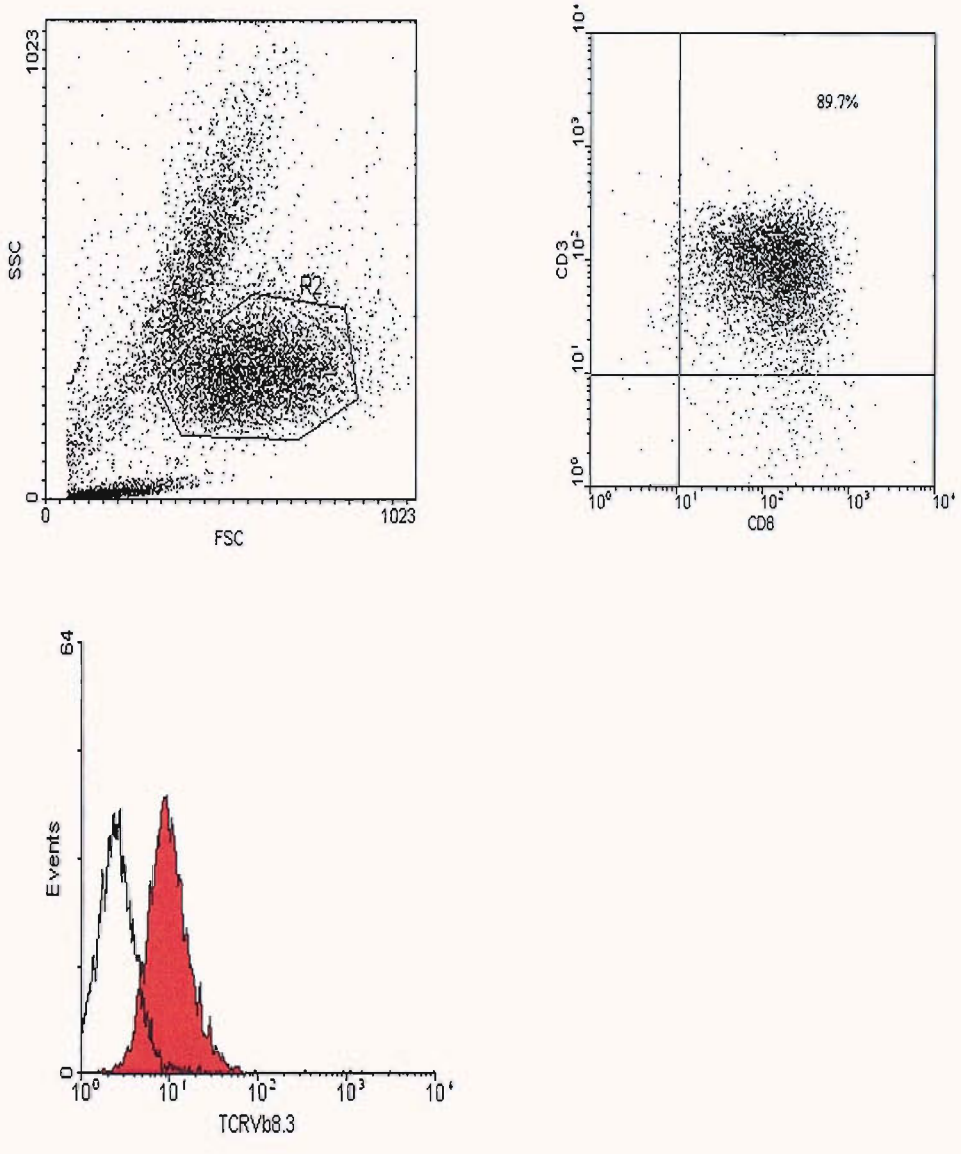


Figure 3. 33. Phenotypic expressions of hybridoma cells H-3-4-17.

Hybridoma cells, H-3-4-17, were stained with anti-CD3ε mAb-biotin and anti-CD8a mAb-FITC or anti-TCRVβ8.3-FITC followed by streptavidine-PE (indirect immunofluorescent staining for CD3ε). T Cells that were stained with an irrelevant IgG2 mAb-FITC plus streptavidine-PE were used as a negative control. FACS profile presents dots obtained by analysis of 10^4 events.

3.3. Discussion.

These data demonstrate that cross-reactive antigen-specific CD8+ T cell lines and CTL clones can be established *in vitro* from Treg depleted immunized mice (summary in table 5 below), suggesting a proportion of responding CD8+ T cells seen during immunization in the absence of CD25+ regulatory T cells develop into effector memory cells that may be *ex vivo* propagated and reactivated on exposure to tumour cells in the presence of IL-2 and IL-15.

Table 5. Summary of antibody blocking experiments of CTL against CT26 tumour cells

	HB-27(28-14-8S) anti-H-2L^d	HB-79 (34-1-2S) Anti-H-2K^d/D^d	HB-159(SF1-1.1) Anti-H-2K^d	HB-102(34-5-8S) Anti-H-2D^d
CTL line1	***Partial***	***Complete***		
Long-term CTL line 1	***Complete***	***No***		
CTL line 2	***No***	***Complete***		***Complete***
CTL clone 1	***Complete***	***No***		
CTL clone 2			***No***	***Complete***
CTL clone 3			***No***	***Complete***
CTL clone 6			***No***	***Complete***
CTL clone 9			***Complete***	***No***

Note: The availability of T cell lines/clones and blocking MHC class I monoclonal antibodies varied. Because of this, it was not possible to test all combinations of antibodies and T cell lines/clones

Two CD8+ T cell lines cytotoxic to both CT26 and A20 tumour cells were generated However, at least three CTL clones (CTL clone 1, 3 and 9) were isolated from T cell

line 1, whereas only one clone (CTL clone 2) was separated from T cell line 2 (see appendix 1). One factor might be important for this different outcome that CTL line 2 was depleted of CD4+ T cells in the beginning of cell culture, whereas CD4+ T cells from immunized mice were retained in culture of CTL cell line 1 for 2 months. As documented in many papers, CD4+ helper T cells are important for the development of antigen-specific CD8+ memory T cells [291, 292].

It seems that a mixture of multiple clones of CD8+ cytotoxic cells specific to different antigenic peptides with various H-2^d restriction were stimulated in our immunized mice in the absence of CD25+ regulatory T cell. At least 4 CTL clones were isolated from 2 CD8+ T cell lines, including H-2-L^d restricted AH1 specific CTL clone 1, H-2-D^d restricted CTL clone 2 and clone 3, and H-2-K^d restricted CTL clone 9.

Cross-reactivity of these clones was confined on a panel of tumour cell lines. CTL clone 2 was responsive to CT26, A20 and BCL1 tumour cells. CTL clone 3 was shown to produce TNF in response to CT26, A20, BCL1 and RENCA tumour cells. CTL clone 9 was reactive to CT26, A20 and BCL1 in a TNF assay. In contrast, the CTL clone 1 appeared to retain specificity to CT 26 via recognition of the H-2-L^d restricted gp70-derived epitope AH1 (not BCL1 without pulsing with AH1 peptide).

CD8+ T cells from immunized mice in our culture gave a higher response to CT26 than to A20 in Chromium-release assay, TNF/WEHI assay or ELISPOT. This was in line with the report that CD8+ T cells from immunized mice were able to reject the challenge of CT26 tumour cells, but partially protect from the challenge of A20 tumour cells. According to literature, effective T cell activation depends on a complex interplay between inherent TCR and ligand/MHC binding kinetics and the epitope

density on the APC [315, 316]. However, the reason underlying this fact needs to be further investigated.

These findings were in agreement with the previous report [16] that shared tumour-associated antigens among tumours from different histological sources were uncovered in mice vaccinated with CT26 tumour cell in the absence of CD25+ regulatory T cells. It is also in line with the comparison that mice immunized with CT26/GM-CSF could generate immunity against only AH1, whereas immunization in the absence of CD25+ regulatory T cells could prefer the generation of multiple clones of T cells with different specificity to shared tumour antigens..

In our T cell cultures, AH1-specific CTL were also isolated from long-term T cell line 1. The mechanism of replacement of predominant cellular population in our long-term culture T cells was unknown. Furthermore, our CTL clone 2 developed decreased reactivity against CT26 tumour cells in term of TNF production and cytotoxicity after *in vitro* culture for more than 18 months. Though its pattern of tumour target recognition remained stable, downregulation in TCRVα gene expression was noted along with changes in the expressions of CD45RA and CD45RC. Further investigation is needed to examine the influence of *ex vivo* conditions on T cell cultures.

In addition, class-I-restricted T-T hybridoma cells generated as described in chapter 3.2.6 have been shown to be relatively conveniently maintained by standard culture conditions. Large numbers of hybridoma cells can be easily obtained and readily used for antigen hunting using the CPRG assay. However, these cells were shown to be genetically unstable and growing of hybridoma cells for less than one month would be

essential to ensure their function.

These techniques have provided a basis for the *in vitro* culture of antigen-specific CD8+ T cells, and also for the screening of CT26 cDNA libraries in order to identify the shared tumour rejection antigens operating in our animal model.

4. Adoptive Transfer of CTL Clone 2

4.1. Introduction

In contrast to vaccine therapies, where reagents are usually more readily available, adoptive cellular therapy using antigen-specific T cells can be relatively labour-intensive, but provides more rigorous control over specificity, phenotype and magnitude of the intended immune response. The CD8+ T cells from CTL clone 2 were shown cytotoxic to CT26 tumour cells in an *in vitro* chromium-releasing cytotoxicity assay even after culture *ex vivo* for nearly one year. We next tried to test their in vivo cytotoxicity against the challenge of CT26 tumour cells.

As suggested by Croft and colleagues [46], CD8+ cytotoxic cells fall generally into two subpopulations based on the cytokine secretion. Type 1 CD8+ T cells (Tc1) secrete IFN-γ and TNF-α, whereas type 2 CD8+ T cells (Tc2) secrete IL-4, IL-5 and IL-10 [46, 47]. One study indicates that both types of CD8+ T cells could kill target cells *in vitro*, however, only tumour-specific Tc1 cells can deliver protective antitumour immunity *in vivo* [48]. This prompted us to investigate the cytokine profile and phenotypes of the *ex vivo* growing cloned CD8+ T cells.

In another study reported by Sallusto and colleagues (1999) [317], CD8+ T cells can be roughly divided into four sub-groups according to their phenotypic expressions of CCR7 and CD45RA. CD8+ T cells were identified as naïve (CD45RA+CCR7+),

central memory (CD45RA-CCR7+), effector memory (CD45RA-CCR7-), and effector (CD45RA+CCR7-) cells. These subpopulations of T cells have not been fully characterized. However, the differential expression of chemokine receptor (CCR7) and adhesion molecules (CD62L) on memory T cells could be linked to their trafficking and antitumour activity *in vivo*. Among these, naïve and central memory T cells are usually found in lymphoid organ, whereas effector memory T cells are found in peripheral non-lymphoid tissue and mucosa sites [317, 318].

In addition, although the appropriate protocol for in vitro activation of CD8+ T cells remains to be determined, it was suggested in another study by Kemp and colleagues (2005) [319] that Tc1 activated by anti-CD3 and anti-CD28 mAb had better *in vivo* anti-tumour activity than that activated by antigen/MHC. We would like to verify this factor in adoptive transfer of our cloned CTL.

4.2. Results

4.2.1. Phenotypic and functional characterizations of T cells from CTL clone 2

As shown in figure 4.1, FACS analysis of live cells revealed that T cells from CTL clone 2 were CD3+, CD8+, CD45RA ^{low}, CD45RC ^{low}, CD44 ^{mid} and CCR7-. These CTL expressed a classical effector memory cell type [317]. It has been suggested that CCR7- memory cells express receptors for migration to inflamed tissues and display immediate effector function. In contrast, CCR7+ memory cells express lymph-node homing receptors and lack immediate effector function, but are efficiently stimulated by dendritic cells and differentiate into CCR7- effector cells upon secondary stimulation.

It has been shown by ELISA assays that T cells from CTL clone 2 can produce high amount of IFN-γ but not IL-5 or IL-10 against CT26 tumour cells (figure 4.2). Thus, according to the cytokine profile, these CD8+ T cells could be classified into the subset of Tc1 [45, 46], which was generally documented with higher in vivo antitumour functions [47].

As showed in a panel of experiments, the phenotypic expressions (figure 4.1), cytokine profiles (figure 4.2) and proliferation rates (figure 4.3) were similar for T cells from CTL clone 2 that were activated by irradiated CT26 and spleen cells or by anti-CD3/anti-CD28, except that the latter expressed CD44 ^{high}, produced lower level of IFN-γ upon TCR engagement.

It was found that CD8+ T cells had higher percentage of cellular death after activation by bead-bound anti-CD3/anti-CD28 than after activation by CT26/spleen cells. The latter fact might be owing to over-stimulation.

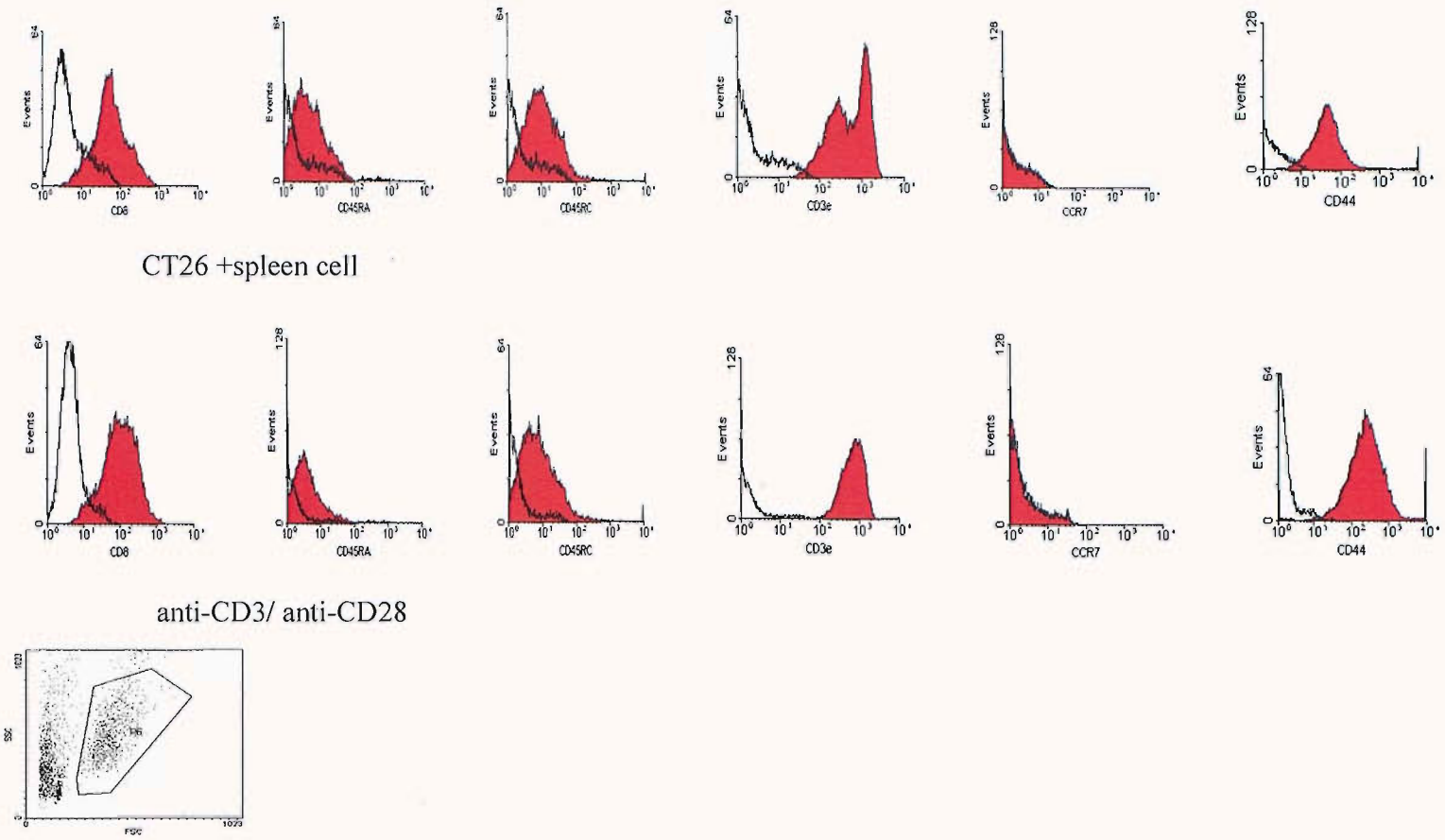


Figure 4.1 Phenotypic analysis of CTL clone 2 after in vitro stimulation by CT26 cells /spleen cells or anti-CD3/anti-CD28

T cells from CTL clone 2 were acivated by irradiated CT26 cells /spleen cells (upper panel) or anti-CD3/anti-CD28 (lower panel). 5 days later, T cells were harvested and stained by various mAb. Staining cells were analyzed with FACS.

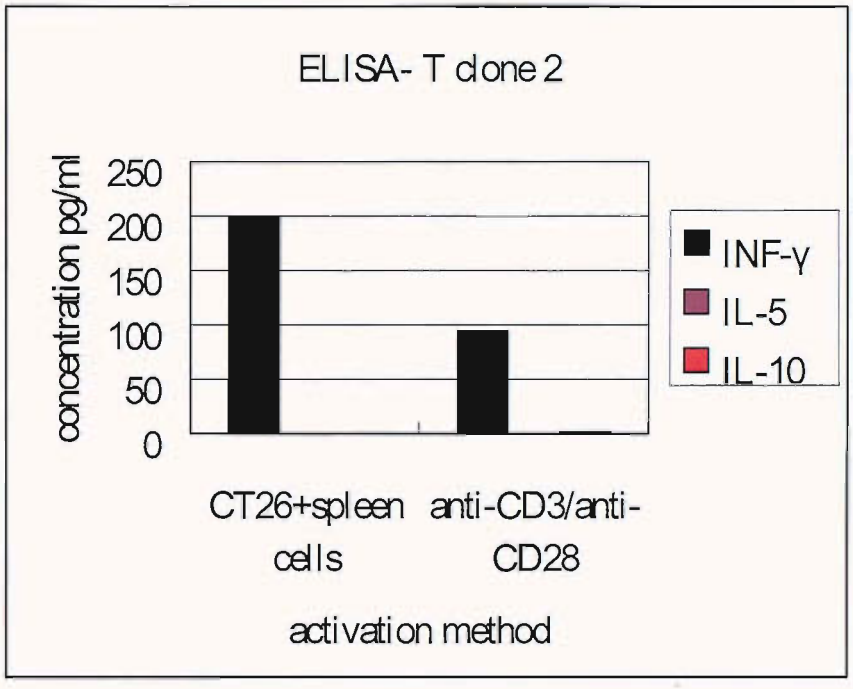


Figure 4.2. Cytokine profiles of T cells from CTL clone 2 after varied *in vitro* stimulation

1 X 10^6 of T cells from CTL clone 2 with 1 X 10^6 irradiated syngeneic spleen cells with 5 X 10^5 irradiated CT26 tumour cells/spleen cells (left) or Dynabead-coated anti-CD3e/anti-CD28 (right). 48-72 hours later, supernatant of each culture was harvested and analyzed by cytokine-specific ELISA assay using the appropriate antibody pairs. Standard curves were generated using recombinant cytokines.

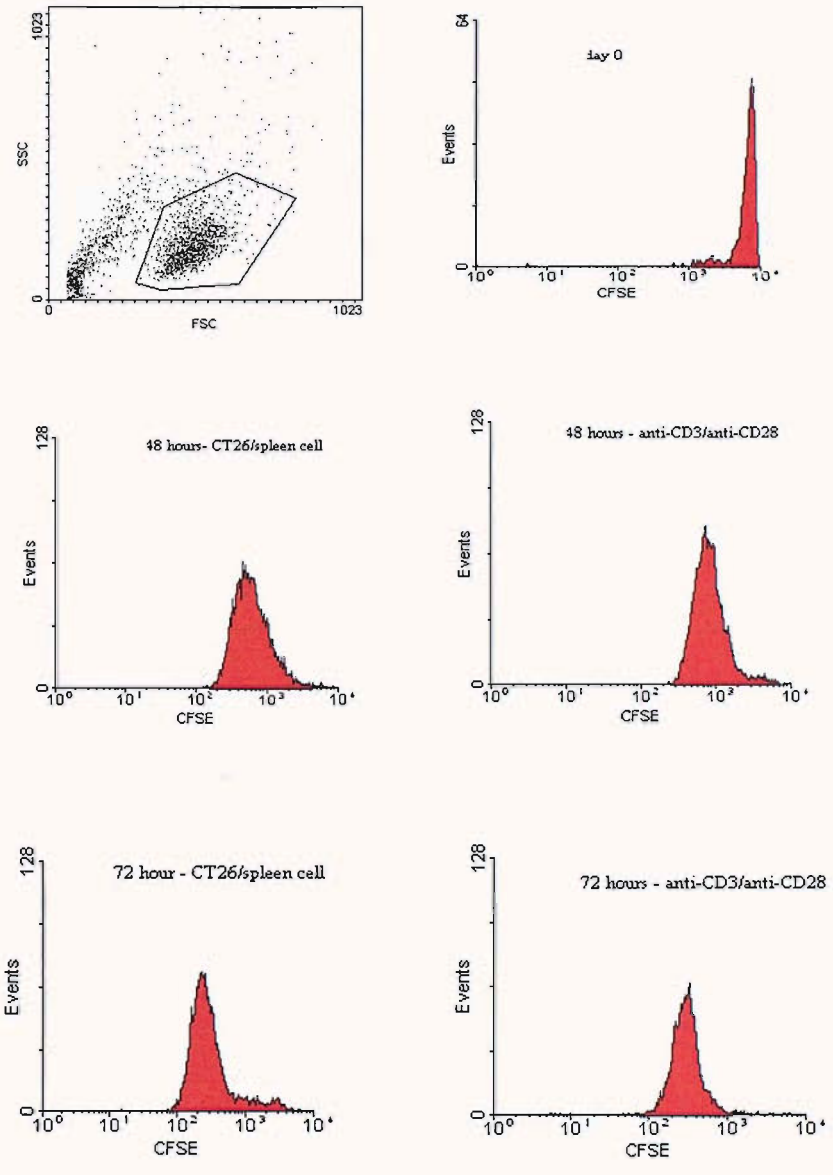


Figure 4.3. CTL clone 2 divide at similar rate after activation by irradiated CT26/spleen cell or anti-CD3/anti-CD28

CTL cells from CTL clone 2 were incubated with 5μM of Carboxy-fluorescence diacetate succinimidyl ester (CFSE) for 10 minutes at room temperature before culture with either irradiated CT26 tumour cells with spleen cells or Dynabead-coated ant-CD3/anti-CD28. Cells were harvest at different times (48 and 72 hour later) and CFSE fluorescence intensity measured by flow cytometry.

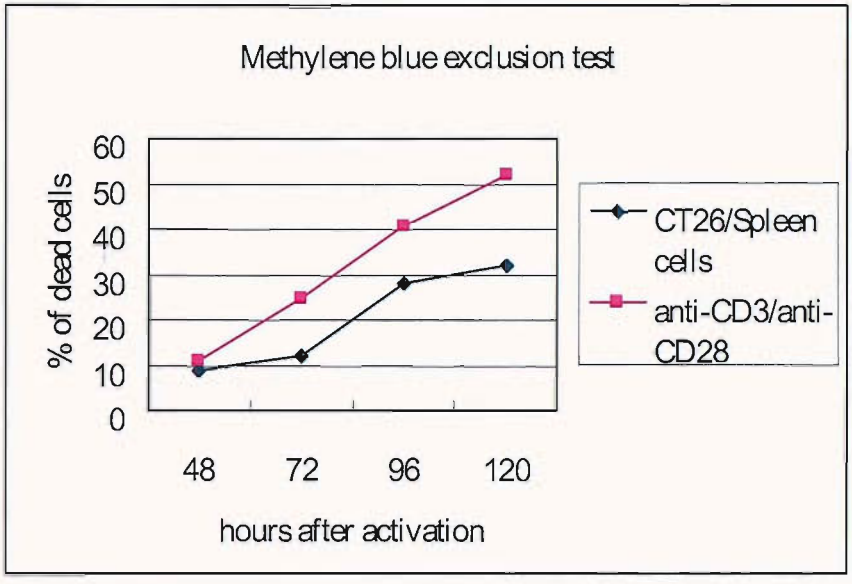


Figure 4.4 Percentage of cell death in T cells from CTL clone 2 after activation

T cells from CTL clone 2 were *in vitro* activated by irradiated CT26 and spleen cells (♦) or anti-CD3/anti-CD28 (■). At different time points (48hr, 72hr, 96hr and 120hr), cells were harvested and methylene blue exclusion was performed.

4.2.2. T cells from CTL clone 2 protected SCID mice poorly against CT26 after adoptive transfer.

CD8+ T cells from CTL clone 2 were activated by irradiated CT26 tumour cells/spleen cells or bead-bound anti-CD3/anti-CD28 in the presence of IL-2/IL-15 and were harvested on post-activation 5^{th} day for adoptive transfer. 5 X 10^6 cells of CTL in both groups were transferred into SCID mice via tail vein. On the next day, recipient SCID mice were challenged with 1 X 10^5 cells of CT26 tumour cell by subcutaneous injection.

In a parallel ^{51}Chromium-release assay (figure 4.5), it was shown that the in vitro cytotoxicity of CTL towards CT26 tumour cells was obvious in both activation groups. Whereas, T cells that were stimulated by CT26 tumour cells and spleen cells had slightly better killing effect against CT26 tumour cells in all tested E/T ratios. .

However, all the recipient SCID mice of both groups after adoptive transfer with activated CTL were shown unable to reject CT26 tumour cells in our experiment (figure 4.6). Nevertheless, although it was not significant in statistics, adoptive transfer with CTL clone 2 after activation with irradiated CT26 tumour cells and spleen cells can slightly delay the growth of the transplanted tumour cell and prolong the survival of recipient SCID mice.

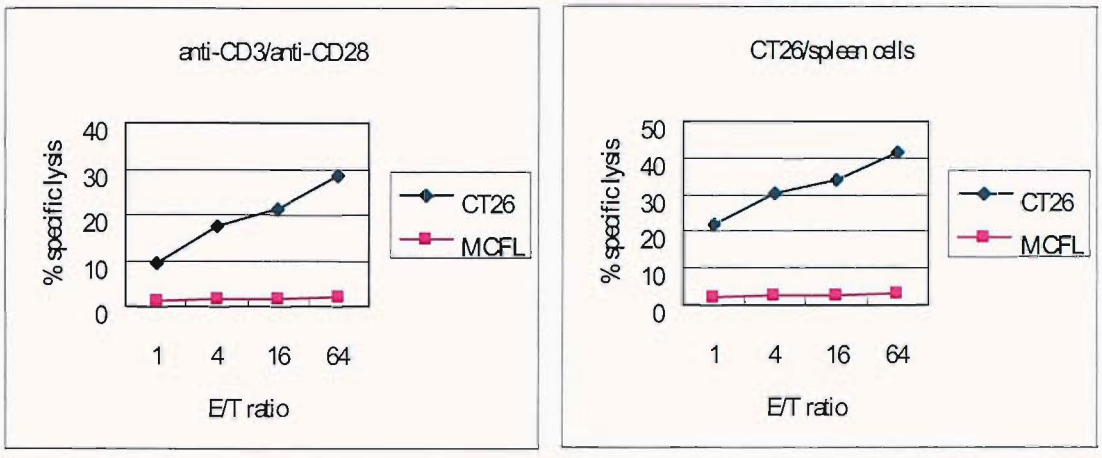


Figure 4.5. ***in vitro*** **cytotoxicity of T cells from CTL clone 2 after activation by irradiated CT26/spleen cell or anti-CD3/anti-CD28**

T cells from CTL clone 2 were activated by either irradiated CT26 tumour cells with syngeneic spleen cells (right panel) or anti-CD3/anti-CD28 (left panel). 5 days later, CTL cells were harvested and tested for cytotoxicity toward CT26 tumour cells (♦) by 4-hour ^{51}Cr-releasing assay. MFCL cells (■) were used as negative control.

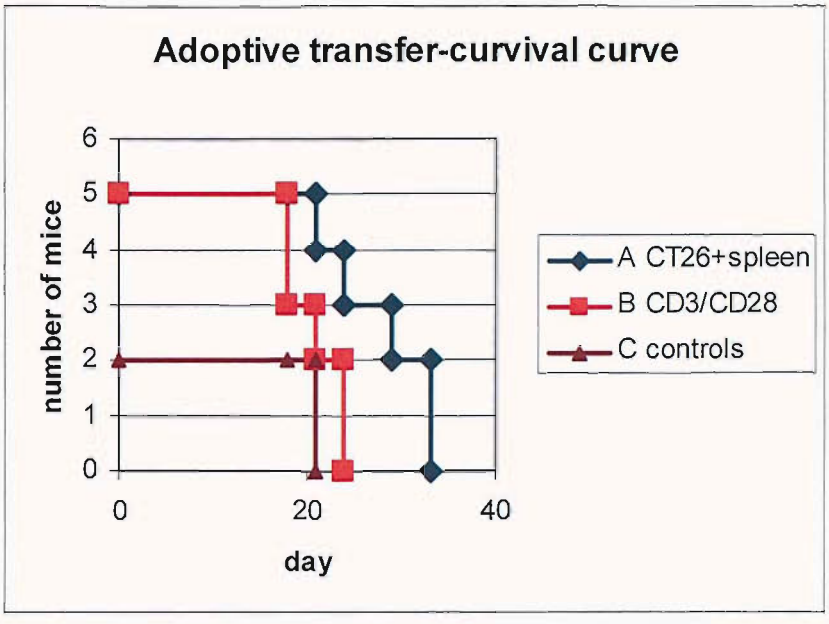


Figure 4.6. Adoptive transfer of CTL clone 2 in SCID mice and tumour challenge.

CD8+ T cells from CTL clone 2 were activated by either irradiated CT26 tumour cells with syngeneic spleen cells (n=5) (♦) or anti-CD3/anti-CD28 (n=5) (■) for 5 days. Then 5 X 10^6 cells per mouse of CTL cells were transferred in SCID mice via tail vein. On the second day, 1 x 10^5 cells per mouse of live CT26 tumour cells were inoculated in mice by subcutaneous injection. Mice without adoptive transfer of T cells were used as a control (n=2) (▲). The numbers of mice survived were measured in correlation with the numbers of day after tumour challenge.

4.3. Discussion

In our experiments, as few as 4 x 10^5 of purified CD8+ T cells harvested from immunized mice have been shown able to reject the challenge of CT26 tumour cells in recipient SCID mice (see chapter 3.2.1). However, our cloned CTL derived from the above tumour-reactive CD8+ T cells were unable to render recipient mice protection from CT26 tumour cells.

There are many potential reasons for the failure of adoptively transferred CTL to protect recipient SCID mice from tumour challenge. The survival and proliferation for such long-term cloned T cells could be very much dependent on the presence of some unknown *in vitro* factor, such as the exogenous IL-2 or IL-15 [320], as in some experiments of adoptive cellular transfer, IL-2 or IL-15 were used as an adjuvant to improve the *in vivo* function or survival of transferred CTL [79, 95, 320], .

CTL cells generated in our *ex vivo* culture system expressed phenotype of effector memory cells, in which CCR7- could possibly led to the accumulation of T cells in liver or lung instead of homing to tumour site where their effector functions could be realized [317, 318].

In addition, adoptive transfer with CTL of single clone in SCID mice would possibly led to the development of antigen-loss variant of tumours. As mentioned in many published papers [321, 322], the correlation between tumour regression responses and determinant spreading suggests that immune diversification should represent a key goal for therapeutic cancer vaccination strategies. A broadened antitumour T cells response might promote the elimination of tumour antigen-loss variant cells. Some

might argue that rapid time-course in therapy would not allow the genetic modification, suggesting this might not be the case.

Another possibility is that this CTL clone might have very low avidity. As seen in chromium-release assay, less than 50% specific lysis toward CT26 tumour cells was observed at E:T ratio of 50:1 rather than 1:1, which would be more usual for a antiviral CTL with high K_A.

However, all these need to be further investigated in the future. This might in part explain the discrepancy between our CTL clone 2 and the original purified CD8+ T cells from immunized mice.

5. Identification of a Novel Tumour Antigen Recognized by CD8+ T Cells from Treg-depleted Immunized Mice

5.1. Introduction.

The constructs of CT26 cDNA library contain mixture of DNA plasmids with various sizes of inserts of cDNA from tumour cells. These DNA plasmids are then transformed into competent E. coli bacteria for cloning and amplification. In general, a minimum of around one million colonies with average insertion sizes of 0.3 - 5.0 kb can largely represent the transcription of a cell line.

To facilitate antigen identification, the cDNA library is fractionated into smaller pools. This can be done conveniently by plating the library on a large LB agar supplemented with indicated selective antibiotic and by cutting the agar piece by piece in such a way as to gather an average of 100 colonies per piece. The clones of cDNA libraries in each fraction are then amplified in LB broth and plasmid extracted for transfection into appropriate recipient cells along with cDNA encoding indicated H-2 molecule (see figure 5.1) [323]. The expression cloning system was tested by CTL using TNF/WEHI assay or hybridoma cells using CPRG assay.

In any positive suspected, it is necessary to re-confirm the positive result by co-transfecting in a panel of recipient cells with irrelevant MHC class I gene. After that, the truly positive fraction of colonies of the cDNA libraries could be recovered from frozen tube and subsequently divided into smaller fraction. As a result, the same screening procedure is repeated until a single positive clone of cDNA is identified.

After a single clone is identified from cDNA libraries, the genes encoding the tumour antigens are then recovered from the plasmid by DNA sequencing. The complete genomic sequence of the identified cDNA can be surmised by comparing with the established database for genome DNA (GenBank) in computer programs (such as BLAST: http:// www.ncbi.nlm.nih.gov/BLAST).

The next step is to isolate the antigenic epitopes from the identified antigens that are specifically recognized by the CTL clone. One approach is based on the finding that the binding of antigenic peptides to the restricted MHC class I molecules relies on their interaction with certain anchor residues of 9-11 amino acids in pocket of the MHC groove [302, 303]. Peptides carrying these potential anchor residuals can be deduced from the nucleotide sequence of a given antigen by computer programs (such as http://www.imtech.res.in/cgibin/propred1, http://www.syfpeithi.de/, http://bimas.dcrt.nih.gov/molbio/hla_bind/). The predicted epitopes are thereafter synthesized and tested for their ability to sensitize to lysis by CTL target cells expressing the relevant MHC class I molecules.

Another approach to identify the region of the gene that codes for the antigenic peptide is to generate a panel of deleted clones by exonuclease digestion. The ability of these clones to produce the antigen of interest is then tested by transfection into recipient cells and recognition by CTL.

Alternatively, subgenic fragments of the identified nucleotide sequence can be tested to localize the region encoding the antigenic peptides. The sub-segments can be generated by PCR amplification that narrows down the sequence, or by endonuclease digestion within the sequence.

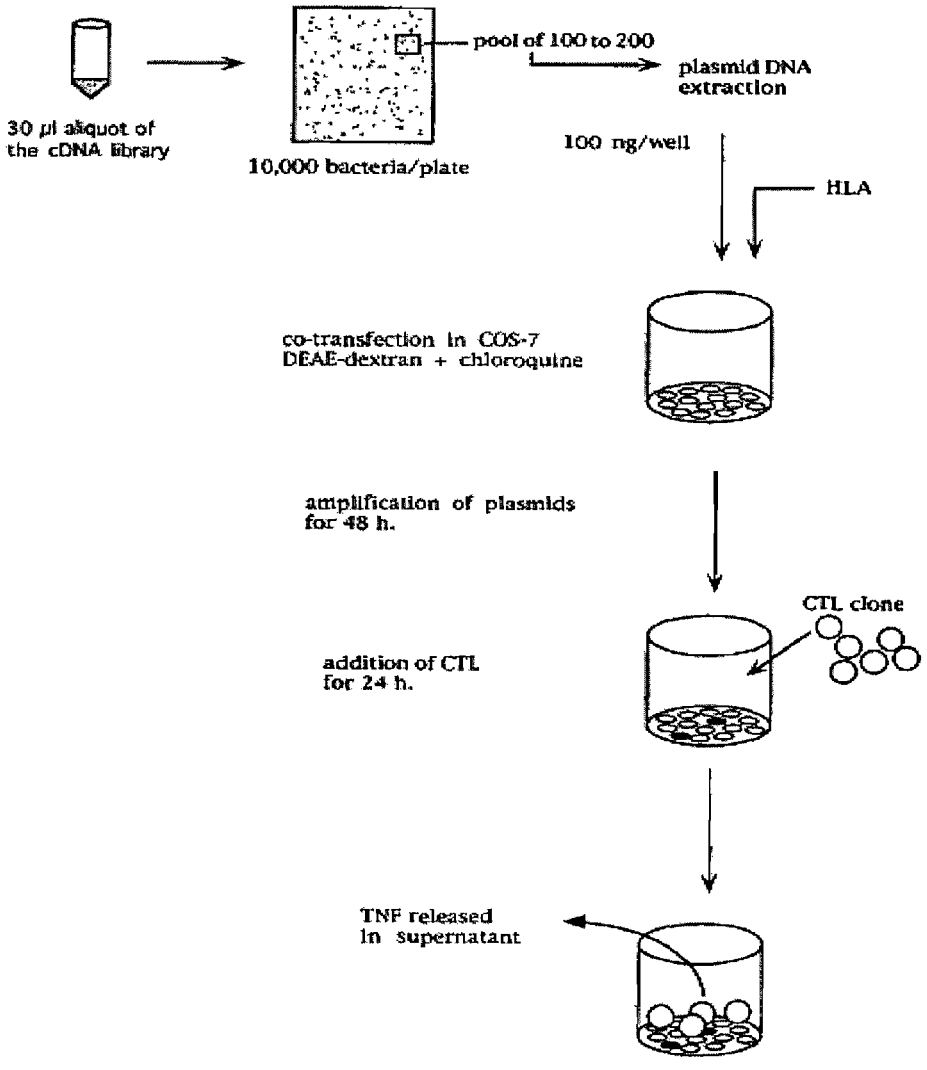


Figure 5.1. Strategy for screening of the tumour cDNA libraries.

COS cells seeded in 96 microwells are co-transfected with the relevant HLA and 100 ng of plasmid DNA extracted from a fraction of around 100 bacteria. After 2 days of plasmid replication, transfectants are incubated with the appropriate CTL and then tested for the expression of the antigen by measuring the amount of TNF released in the supernatant. (Copy from **Etienne De Plaen**, *et al.* Identification of gene coding for tumour antigens recognized by cytolytic T lymphocytes. ***Methods: A companion to methods in enzymology*** 1997; **12:** 125-142).

5.2. Results

5.2.1. Set up of transfection and expression cloning system for the screening of cDNA libraries.

Transient transfection of recipient cells with plasmid containing cDNA from tumour cDNA libraries has been shown to be a feasible strategy for the expression cloning of mammalian tumour genes [180, 205, 323]. Among a variety of transfection methods, DEAE-dextran and chloroquine coupled with a shock treatment by DMSO is a simple and cost-effective method that gives significant and consistent high transfection efficiency with low toxicity [212]. DEAE-dextran is widely used for gene transfer into mammalian cells [213, 214]. Transient transfection following DEAE-dextran protocol allows the recipient cells to express the introduced gene in 24-48 hours.

To set up the transfection-expression system, the first priority is to choose appropriate recipient cells. As mentioned, the criteria for recipient cells include; 1) whether the recipient APCs will stimulate T cells activity without transfection and result in an unacceptably high background reaction; 2) whether the antigen processing of the recipient cells is identical or similar to the antigen-bearing cells; 3) whether the recipient cells are readily transfectable and efficient in expressing the indicated protein.

We have tested the transfection efficiency in several cell lines using a plasmid DNA containing gene encoding for H-2-D^d molecules and another plasmid, pEGFP-N1, which was used as a substitute for cDNA libraries. Transfection with pEGFP-N1 allowed us to verify the transfection efficiency by flow cytometry analysis of cellular

expression of green fluorescent protein (GFP). As summarized in **table 6**, only Cos-7 cells and L cells could allow acceptable transfection efficiency. However, L cells transfected with H-2-D^d alone would stimulate T cells from our CTL clone 2 and lead to a high background (data not shown). As a result, Cos-7 cells were preferentially used as recipient cells for expression cloning system [302].

Table 6. Transfection efficiency in different cell line

Transfection(a)	Cos-7	WOP	MCFL	L cell	Ltk-/D^d(b)	Cos-7/Dd (c)
H-2-D^d	31%	<1%	<1%	27%	>90%	>90%
pEGFP-N1	51%	<1%	<1%	29%	2%	40%
H-2-D^d +GFP	31%	<1%	<1%	23%	<1%	32%

(a). Transfection was performed by DEAE-dextran and Chloroquine as in *Materials and Methods*.

(b). Ltk-/D^d cells are Ltk- cells with stable transfection of H-2-D^d.

(c). Cos-7/D^d cell are Cos-7 cells with stable transfection of H-2-D^d.

We next moved to find out appropriate conditions for the achievement of maximal transfection efficiency. In **table 7** and **table 8**, we summarized the results of transfection efficiencies of Cos-7 cells under varied concentrations of DEAE-dextran and Chloroquine, and varied duration of incubation in transfection medium. According to these experiments, we have set up an optimal protocol, in which 200μg/ml DEAE-dextran and 200μM Chloroquine was used in transfection medium with incubation of 90 minutes. In several independent experiments, we have performed transfection in Cos-7 cells using this protocol and varied expression rates

between 25% and 35% for both GFP and H-2-D^d were obtained.

Table 7. Transfection efficiency in varied concentration of DEAE-dextran and Chloroquine

Chloroquine / DEAE-dextran	400μM	300	200	100
400 μg/ml	GFP 39% H-2-D^d 9%	GFP 40% H-2-D^d 9%	GFP 39% H-2-D^d 15%	GFP 35% H-2-D^d 12%
300	H-2-D^d 12%	GFP 40% H-2-D^d 12%	GFP 40% H-2-D^d 17%	H-2-D^d 15%
200	GFP 43% H-2-D^d 17%	GFP 42% H-2-D^d 19%	**GFP 47%** **H-2-D^d 23%**	GFP 42% H-2-D^d 12%
100	H-2-D^d 15%	GFP 39% H-2-D^d 17%	GFP 39% H-2-D^d 17%	GFP 38% H-2-D^d 15%

(a). Cos-7 cells were cultured in transfection medium containing plasmid for 2 hours.

Table 8. Transfection efficiency with varied duration

Duration (a)	4 hours	3 hours	2 hours	1 hours
Efficiency	GFP 36.57%	GFP 41.24%	**GFP 50.5%**	GFP 48.29%

(a). Cos-7 Cells were cultured in transfection medium (DEAE-dextran 200μg/ml, Chloroquine 200μM) containing pEGFP-N1.

5.2.2. Sensitivity of TNF/WEHI assay and CPRG assay in antigen hunting

We next determined the sensitivity of CTL or T-T hybridoma cells for detection of antigen-presenting cells. To mimic the cellular mixture in pool of transfectants, we have designed an experiment, in which increasing numbers of CT26 target cells were mixed with 20000 of non-target Cos-7 cells in a TNF/WEHI assay or CPRG assay.

As in figure 5.2, TNF/WEHI assay using T cells from CTL clone 2 was shown to be highly sensitive in that as few as 20-40 CT26 tumour cells in a pool of 20000 Cos-7 cells could be detected by 5000 CTL cells, suggesting 0.1 to 0.2 % of the transfectants expressing antigen of interest could be detectable. To estimate this numerically, if a 30% transfection efficiency of both H-2-D^d and cDNA libraries could be achieved in Cos-7 cells; the efficiency was equally divided by 100 because 100 clones of cDNA libraries were fractionated as a pool in primary screening. That is, any positive antigen could be detectable if expressed in about 0.3% or more of transfected Cos-7 cells. Therefore, the wanted antigen could be theoretically identified by our T cells in this system.

Similarly for our hybridoma cells, as shown in figure 5.3, at least 312 antigen-presenting cells expressing the antigen of interest were required to be differentially detected by our hybridoma cells (H-3-4-17) over a pool of Cos-7 cells, suggesting that our hybridoma cells could detect a positive colony only if there are more than 1.5% of the transfected cells expressing antigen of interest.

The sensitivity of hybridoma cells very much depends on the affinity of TCR on hybridoma cells to antigen of interest. In comparison with another commercialized

hybridoma cells, B3Z cells that are specific ovalbumin (OVA), our hybridoma cells are far less sensitive.

In practice, we have utilized T cells from CTL clone 2 and hybridoma cells, H-3-4-17, derived from CTL clone 3 as probes in parallel to screen through our prepared CT26 cDNA libraries for identification of the CTL-reactive tumour antigens. We decided that a screen of 2 X 10^5 cDNA clones would be the "endpoint" for a primary screen. We were unable to isolate any one positive fraction in the primary screen using hybridoma cells, and all subsequent screening was carried out with T cell clones.

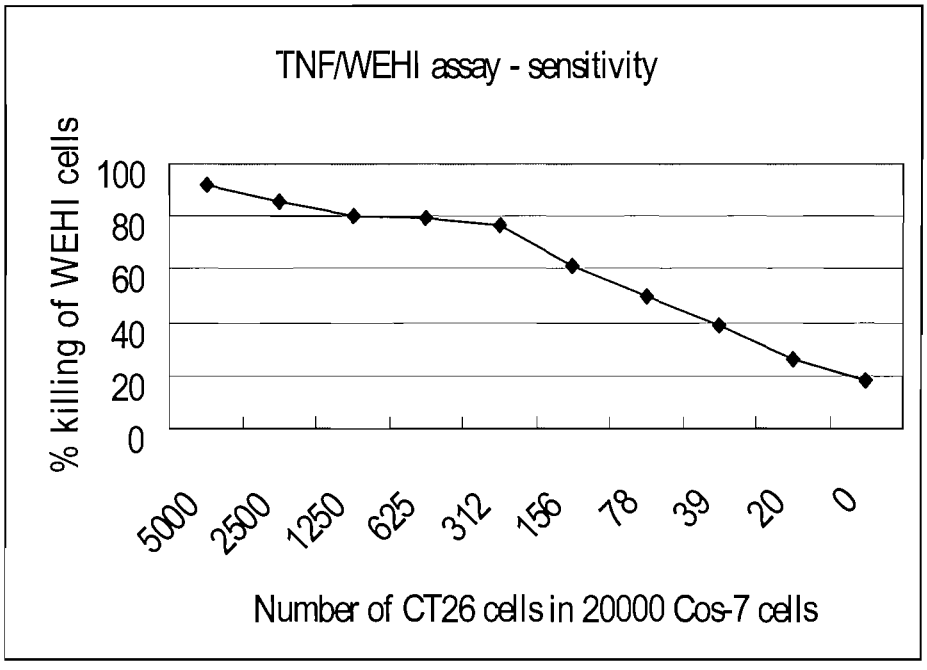


Figure 5.2. Sensitivity of T cells from CTL clone 2 in a TNF/WEHI assay

Different numbers of T cell-reactive CT26 tumour cells were added in duplicates of 96-well plate containing 20000 of Cos-7 cells in each well. T cells from CTL clone 2 were added in a concentration of 5000 cells/well. The antigen-specific TNF production of T cells was examined by TNF/WEHI assay.

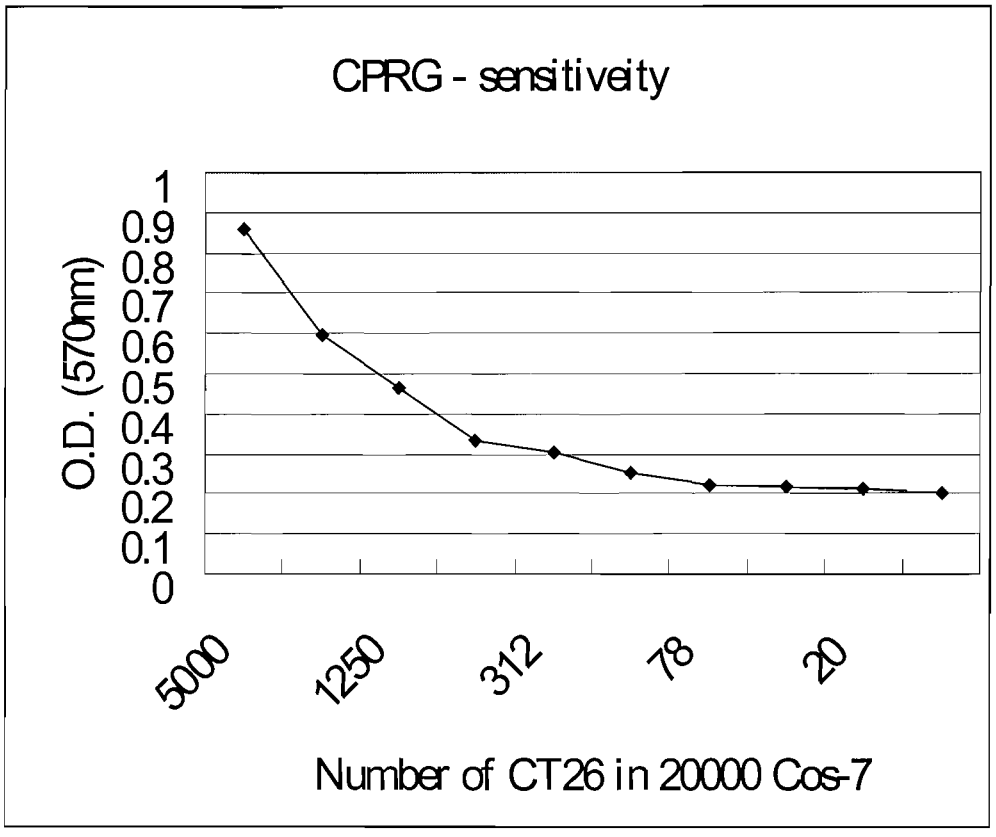


Figure 5.3. Sensitivity of hybridoma cells H-3-4-17 in a CPRG assay

Different numbers of CT26 tumour cells were added in duplicates of 96-well flat bottom plate containing 20000 of Cos-7 cells in each well. Hybridoma cells, H-3-4-17, were added in a concentration of 1 X 10^5 cells/well. The TCR induced intracellular LacZ activities were examined by CPRG assay.

5.2.3. A murine leukemia virus antigen was identified as a novel tumour antigen recognized by cross-reactive CTL clone 2.

The CT26 cDNA library was divided into pools of about 100 bacterial colonies, 100ng of DNA from each pool was transfected by DEAE-dextran/Chloroquine protocol together with 100ng of plasmid containing H-2-D^d gene into 20000 Cos-7 cells. 48 hour later, the transfectants were screened by 5000 T cells from CTL clone 2 using TNF/WEHI assay. Any positive cDNA pool was repeatedly divided into smaller pools until a single clone was identified. A summary of the screen is presented in Appendix 2.

A single positive cDNA clone, P20E8A1, was isolated after screening about 2 X 10^5 cDNA colonies. To confirm that P20E8A1 encodes the CTL-defined antigen, it was transfected along with H-2-D^d or H-2-K^d cDNA into Cos-7 cells and the transfectants were tested with or without the presence of mAb against H-2-D^d. The P20E8A1 transfected Cos-7 cells were shown to be repeatedly reactive to CD8+ T cells from CTL clone 2. Recognition was H-2-D^d restricted because the TNF production of CTL cells occurred only if P20E8A1 was co-transfected with H-2-D^d. In addition, the CTL reaction can be blocked by anti- H-2-D^d mAb (figure 5.4).

Clone P20E8A1 was known to contain a cDNA insert of about 2000-2500 bp. However, for some unknown reason, we were unable to sequence the insert using the indicated pair of primers (T7 promoter and SP6 promoter) for the suggested vector pcDNA3 (appendix 3). We have confirmed that the insert cDNA of interest was cloned in site of EcoR1 and the neighbouring restriction sites of HindIII and XhoII that flank the cloning position were intact. To enable sequencing, we re-cloned the

insert into a known vector, pcDNA3.1/V5-His (appendix 4). Therefore, the insert could be sequenced using the suggested primers, T7 promoter (5') and BGH reverse (3') (see appendix 5).

A search of the National Center for Biotechnology Information and GenBank data bases (BLAST: http:// www.ncbi.nlm.nih.gov/BLAST) has allowed the identification of one protein that contains a sequence with over 97% identity to the insert cDNA. The readable sequence of the identified cDNA clone, P20E8A1 (67-1037) was identical to a sequence of an endogenous ectotropic Murine Leukemia Virus (MuLV) *pol/env* gene (5515-6086), which includes overlap of both the reverse transcriptase gene (*pol*) encoding the sequence of amino acids (797-1037) and envelope glycoprotein gene (*env*) with an encoding sequence of amino acids (1-120) (figure 5.5).

To search for CD8+ T cell-reactive epitopes within the MuLV *pol/env* antigen operating in our system, computer software (http://www.imtech.res.in/cgibin/propred1) was used for the prediction from the identified cDNA sequence. Consequently, nearly a hundred of epitope candidates were deduced. As shown in **table 9**, twelve candidate nonamers were selected according to the scoring results that estimate half time of disassociation of a molecule containing this predicted sequence.

Table 9. H-2-D^d peptide motif search result

Rank	Gene	Subsequence Residue Listing	Score (estimate of half time of disassociation of a molecule containing this subsequence)	designated

1	*env*	**WHPLIVLLI**	200	WI
2	*pol*	**NGPAFTSQV**	120	NV
3	*pol*	**KGPYTVLLT**	48	KT
4	*pol*	**HGLTPYEIL**	24	HL
5	*env*	**WWPDLTPDL**	24	WL
6	*env*	**NSPHQVFNL**	24	NL-1
7	*pol*	**NSPSLQAHL**	20	NL-2
8	*pol*	**AGTRDWVLL**	20	AL
9	*env*	**LGGVNPVTL**	20	LL
10	*env*	**SKPEFNQVN**	12	SN
11	*pol*	**QNPLKIRLT**	10	QT
12	*env*	**LGNSPHQVF**	6	LF

Our previous data would indicate that around 50% of these peptides will bind to class I molecules with high K_A, but the algorithm is unable to predict which one will be. Peptides were synthesized and used in CTL sensitization test to determine the CTL-reactive epitope within MuLV *pol/env* antigen in our vaccination model.

However, in the primary screening by using MCFL cells as target cells for pulsing with those synthetic peptides and testing their ability to sensitize CTL clone 2 in both TNF/WEHI assay and ^{51}Chromium- release assay, we were unable to identified a positive antigenic peptide from these deduced synthetic peptides (figure 5.6 and figure 5.7)

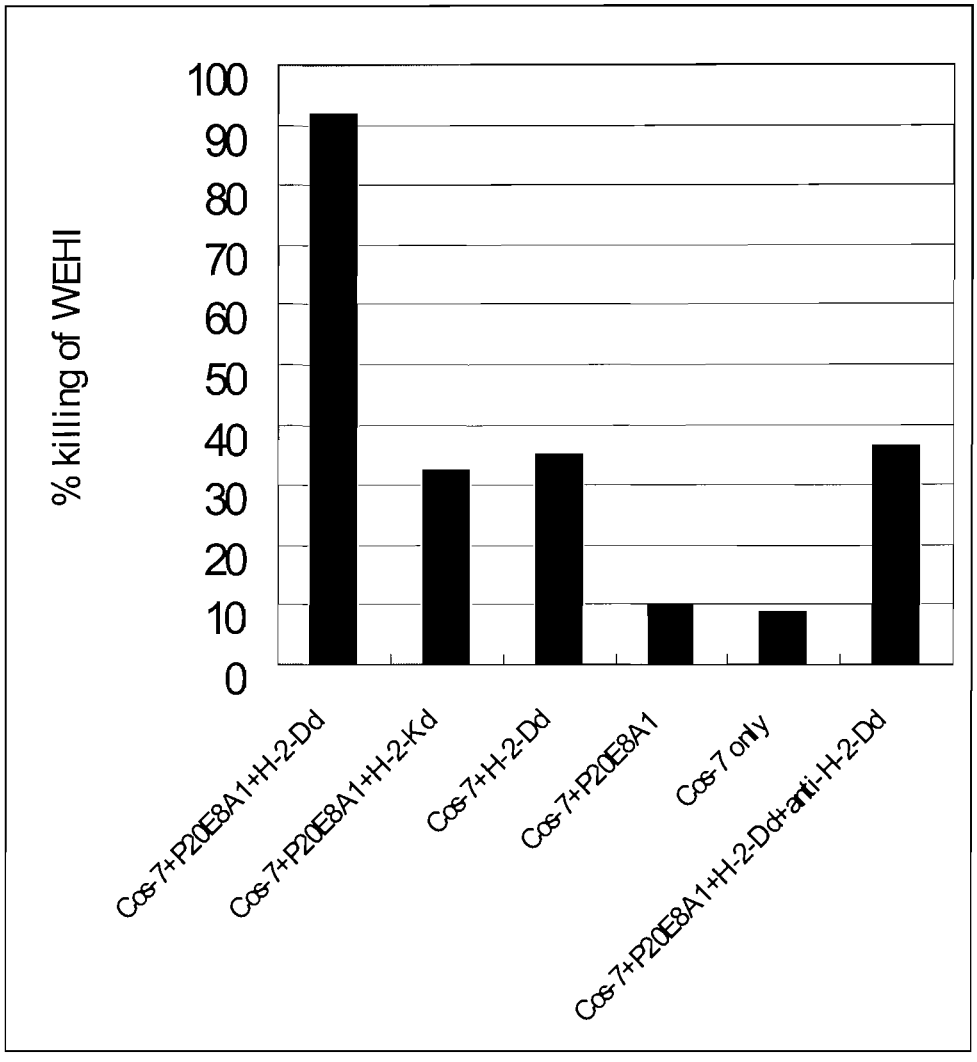


Figure 5.4. Clone P20E8A1 encodes the CT26 tumour antigen recognized by CTL clone 2 in H-2-D^d restriction.

P20E8A1 was transfected along with H-2-D^d or H-2-K^d DNA into Cos-7 cells and the transfectants were tested with or without the presence of mAb against H-2-D^d by T cells from CTL clone 2 using TNF/WEHI assay.

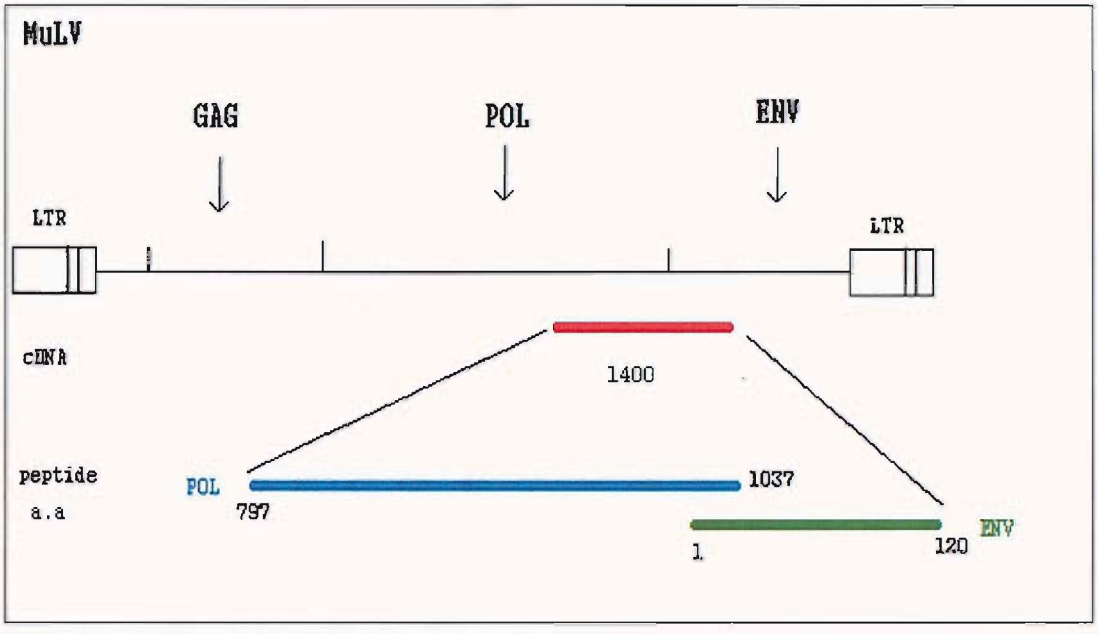


Figure 5.5. Schematic illustration of the region of MuLV gene that was in homology with the identified clone of tumour cDNA (BLAST) and its protein translation.

Full-length MuLV is represented by LTR-gag-pol-LTR (LTR: proviral long terminal repeats that are derived from sequences at the ends of viral RNA).

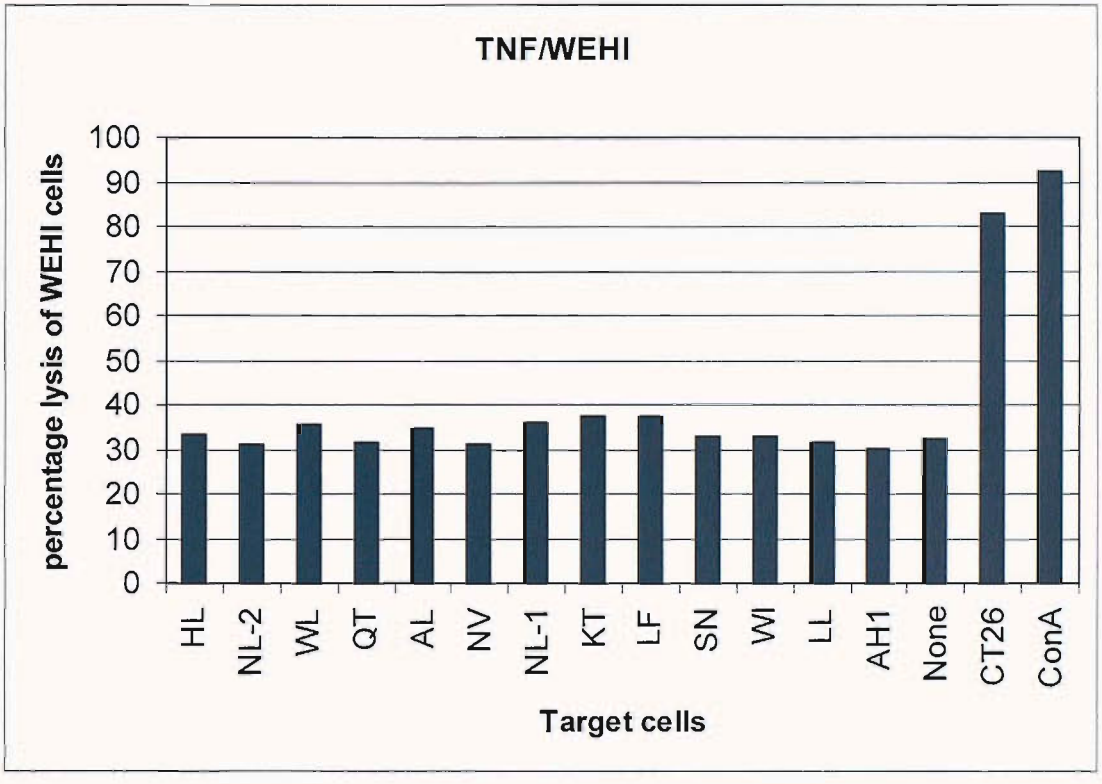


Figure 5.6. Peptide sensitization test by CTL clone 2 in a TNF/WEHI assay

10000 MCFL cells were incubated with 10-20 μM of synthetic nonamers (as detailed in table 9) for 1 hour in serum-free medium. Then 5000 of CTL were added for a TNF/WEHI assay). MCFL cells pulsed with AHI or with no peptide were used as negative controls. CT26 tumour cells and 7.5 ng/ml of ConA in CTL culture were used as positive controls.

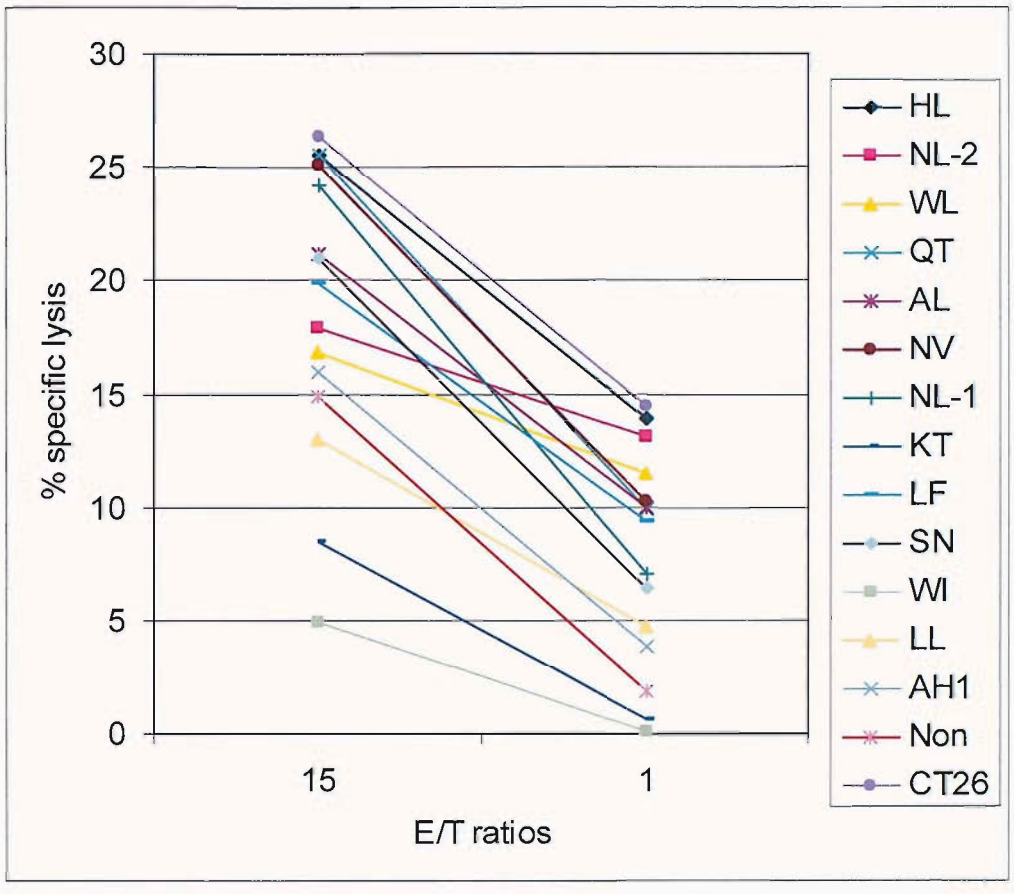


Figure 5.7. Peptide sensitization test by CTL clone 2 in a ^{51}Chromium-release assay

10000 MCFL cells were incubated with 15μM of nonamers (as detailed in table 8) for 1 hour in serum-free medium, and then were labelled with ^{51}chromium as standard procedure. CTL were added in indicated ratios for a 4-hour ^{51}Chromium-release assay. MCFL cells pulsed with AHI or with no peptide were used as negative controls. CT26 tumour cells were used as a positive control.

5.3. Discussion

It was hard to compare the sensitivity of CTL clone 2 and hybridoma cells, H-3-4-17, in antigen hunting since they recognized different antigens. In our experiment, we were unable to identify a reactive antigen using our hybridoma cells (see full dataset in appendix 2). Except for the known relatively high level of antigen-presenting cells required for the recognition of H-3-4-17 hybridoma cells as described in chapter 5.2.2, a relatively high background observed in our Cos-7 expression cloning system might be another obstacle contributing to the difficulty using hybridoma cells in antigen hunting.

On the contrary, tumour-reactive CTL clone was sensitive for identification of tumour-associated antigens in our hand. These *in vitro* CTL divided rapidly after re-stimulation, thus sufficient yield could be achieved for use at 5000-10000 cells per well in a 96-well format. Antigen-specific TNF production from CTL was merely dependent on interaction via antigen/MHC complex on transfected Cos-7 cells without the presence of other costimulatory molecules, such as B-7 and LFA-1, and was readily measured using TNF/WEHI assay.

However, one of the drawbacks in using CTL as probes in antigen hunting is that they need to be re-stimulated regularly every 10-14 days. Besides, there is cyclic fluctuation in the function of CTL. Thus, in our experiment, CTL was preferentially used for screen of the expression cloning cDNA in TNF/WEHI assay between 4-7 days after re-stimulation.

The successful rate in antigen identification varied. First of all, it is very much

dependent on the affinity of TCR of selected CTL to the antigen of interest. Secondly, the quality of cDNA library generated would influence the faithfulness of the proteins for tumour cells of interest. Thirdly, the transfection and expression efficiency of cDNA libraries in recipient cells are essential for the expression cloning system. Furthermore, there are many biological mechanisms that would change the final properties of proteins expressed, such as posttranslational protein splicing or chemical modification [324]. Therefore, the expressed antigen might be different from libraries made from cDNA.

cDNA Library are constructed from mRNA that is extracted from cells of interest, followed by several steps, including first-strand cDNA synthesis by reverse transcriptase, second-strand cDNA production, restriction by endonuclease enzymes, ligation by adaptor, and finally clone into an appropriate vector (see Appendix 6). Each step of construction is crucial for the quality of cDNA library.

It has been suggested that the prepared recombinant cDNA library can be fractionated according to size before cloning into vectors and transformation into competent bacteria to avoid the growth competition between bacterial colonies with different sizes of cDNA [320]. As bacteria with short plasmid could have growth superiority.

It was also crucial in the choice of recipient cells for expression cloning system. In our screening, we used Cos-7 cells due to their good transfection efficiency and low background for our CTL. The Cos-7 cells are relatively easy to maintain in culture and to transfect; therefore this Cos-7 cell expression system has been extensively developed by Seed and colleagues for the rapid cloning of DNA encoding cell surface molecules recognized by monoclonal antibodies [179]. Furthermore, it has been

proposed for cloning of cDNA libraries in the identification of CTL-defined antigens [180].

In our expression cloning system, Cos-7 cells were transiently co-transfected with both H-2-D^d and cDNA libraries. Using GFP instead of cDNA libraries, we have achieved 25-35% of transfection efficiencies for both GFP and H-2-D^d in several independent transfections. There was no obvious benefit using Cos-7 with stable transfection of H-2-D^d compared with co-transfection system, since stable transfectants expressed a lower amount of antigens (see table 6).

Using both set of CTL clone 2 with TNF/WEHI assay, and hybridoma cells, H-3-4-17, derived from CTL clone 3, with CPRG, we have screened 2 X 10^5 fractionated colonies of CT26 tumour cDNA library (see appendix 2). A gene encoding for murine leukemia virus antigen (*pol/env*) has been identified as CTL clone 2-reasctive antigen operating in our animal model of immunization.

The technique of identification of tumour-associated antigens through screening of CT26 cDNA libraries by CTL is new to our laboratory. This provides a basis for further application of genetic approach of antigen hunting in the future.

In the primary sensitization test by CTL clone 2, we failed to identify the antigenic epitope from the deduced synthetic nonamers in TNF/WEHI assay and ^{51}Chromium-release assay, in which MCFL cells were pulsed with those synthetic nonamers and used as target cells. However, although the quality of assay was relatively poor; the result in chromium-release assay has indeed shown that several peptides might be the antigenic epitope (see figure 5.7 above).

There are several possibilities that influence the successful rate in identified such an antigenic epitope from the identified MuLV antigen. First of all, we may have incomplete coverage of the transfected target gene with synthetic peptide due to the factor that around 700bp of insert appeared to be lost upon recloning, perhaps because of a possible endonuclease cleavage internal to the original insert cDNA. Second, the pulsed synthetic peptide, if it bound to H-2 molecules with very low affinity, might be removed in the washing steps or in the step of incubation with CTL. In the latter concern, chromium-release assay could be better than TNF/WEHI assay because only 4-hour incubation is needed. Third, MCFL cells might not be an appropriate target cells for sensitization test because of too low level of MHC class I expression. The density of MHC class I expressed on target cells is crucial for peptide sensitization test. In addition, there are some mechanisms, such as post-translational protein splicing and chemical modification that would confound simple epitope prediction algorithm.

For repeating the peptide sensitization test, it is necessary to assure that all length of functional insert cDNA has been re-cloned into new vector before sequencing. This can be simply done by transfection of Cos-7 cells with the re-cloned plasmid and testing its ability to activate the TNF production by CTL clone 2. H-2^d cells other than MCFL cells could be used as target cells in chromium-release assay, such as BCL1 or H-2-D^d transfected Cos-7 cells. However, it is important to verify the expression of H-$2D^d$ molecules over those selective targets cells before test.

Other alternative methods, such as transfection of panel of deleted clones or subgenic segments generated by endonuclease cleavage or PCR amplification can used in case of failure in the deduction method.

6. General Discussion and Future Direction

We showed here that multiple clones of CTL that are specific to CT26 and are cross-reactive to several other tumours were stimulated in BALB/c mice vaccinated with CT26 tumour cells in the absence of CD25+ regulatory T cells. At least 4 distinct CTL clones with different antigen-specificity were isolated from immunized BALB/c mice, AH1-specific CTL clone 1, H-2-D^d restricted CTL clone 2 and CTL clone 3, and H-2-K^d restricted CTL clone 9. Whereas only one clone of CTL that is specific to AH1 in H-2-L^d restriction was known in mice vaccinated with CT26/GM-CSF [286, 287].

The activation of multiple CTL especially found in the absence of CD25+ T regulatory cells could be a particular scenario in our animal model, in which suppression from regulatory T cells was eliminated before vaccination. The roles of CD4+CD25+ regulatory T cells in the maintenance of self tolerance have been highlighted in many studies (see chapter 1.4.1). Presumably, all antigens on CT26 tumour cells, self or tumour-specific, could be recognized by host CD8+ T cells. However, there was no evidence of autoimmune disease found in our immunized mice.

Another possible explanation might be owing to the effect of determinant spreading that developed in a high frequency of tumour regression, in which antigenic determinants distinct from an inducing determinant become additional targets of an ongoing immune response [325, 326].

Among these CTL clones, CTL clone 2 was shown cross-reactive against CT26, A20

and BCL1 tumour cells in H-2-D^d restriction. In a genetic approach using CTL clone 2 and screening through around 2 X 10^5 clones of CT26 tumour cDNA library, we have successfully identified a gene encoding murine leukemia virus *pol/env* antigen operating in our mouse immunization model.

Murine leukemia virus (MuLV) belongs to a group of retroviruses that replicate via reverse transcription and infect a wide range of vertebrate species. During the evolution of vertebrate, some of the genetic structural features of these retroviruses have been stably integrated into DNA of the host's germline and, thereby, were transmitted to the next generation in a Mandelian fashion [327]. These genomes are known as endogenous retroviruses (ERV). The integrated DNA copy of this genome, or provirus, consists of the sequence U3-R-U5, referred to as the long terminal repeat (LTR), followed by the structural genes gag-pol-env and flanked at the 3' terminus by an LTR (see figure 5.5). The LTR contains sequences that serve to initiate, terminate, and perhaps modulate transcription of viral RNA [328].

Endogenous ecotropic murine leukemia virus-related sequences are widely dispersed in many inbred strains of mice. As implied by the name, the earliest isolates of these agents possessed leukemia-inducing activity [329], although now the term MuLV is frequently applied to all replication competent and replication-defective viruses that show some sequence homology to the original strains. The early isolates differ from recent fresh isolates from inbred mice in their increased leukemogenic potential. Three of these early lymphomagenic viruses, including Moloney (M-MuLV), Rauscher (R-MuLV) and Friend (F-MuLV), are serologically distinguishable from MuLV recovered from inbred mice [330].

The elegant genetic experiments of Rowe and colleagues (1972) first localized sequences encoding ecotropic MuLV to specific chromosomes and demonstrated their endogenous nature by genetic crosses of inbred mice [331]. BALB/c mice, which have been classified as a strain of low-leukemic mice, contain a single, genome-length, endogenous ecotropic provirus [332, 333]. The expression has been mapped to a segment of chromosome 5, designated *Cv-1* [334, 335]. It is now clear that endogenous proviruses exist in nonexpressed and expressed states. In vivo, few BALB/c mice express ecotropic virus before 6 month of age; approximately 50% of animals express moderate titres of virus by 12 months of age [336, 337].

Two general models have been proposed to account for the probabilities of expression of ERV. One asserts that surrounding cellular DNA sequences play a crucial role in expression. The other model asserts that signals governing virus expression are intrinsic to the viral genome. However, no definitive evidence could be obtained for either model [330].

As a result, while the genome of many ERVs is interrupted by termination codons, deletions or frame shift mutations that render ERVs defective in the capability to generate complete infectious virions, some ERVs are transcriptionally active and recent studies reveal protein expression or particle formation by ERVs [338, 339]. In addition, although infectious endogenous retroviruses were never seen in human beings, they were widely found in vertebrate and non-human primates.

Murine leukemia viruses (MuLV) were known to induce a variety of haematological tumours in different strains of mice. A variety of tumour cell lines transformed by MuLV gene products include the Friend MuLV-induced erythroleukemia FBL-3, the

Moloney MuLV-induced T cell lymphoma MBL-2, and the Rauscher MuLV-induced T cell lymphoma RBL-5 and its derivatives RMA and RMA-S.. Among these tumours, two CTL epitopes and one CD4+ helper cell epitope were molecularly identified with viral origin [340, 341, 342].

Members of murine ERVs are preferentially expressed in fresh tumour or cell lines rather than in normal cells. Recently, the murine colorectal carcinoma CT26 derived from BALB/c mice and melanoma B16 derived from C57BL/6 mice have found to express the endogenous retroviral gene products *gp*70 [287] and P15E [343]. Among these, AH1, an H-2-L^d restricted peptide derived from gp70 (423-431), is known to be the reactive CTL epitopes in BALB/c mice immunized with CT26/GM-CSF [286, 287].

These ERV sequences are estimated to comprise up to 1% of human genome DNA and derived from ancestral encounters with retrovirus [344, 345, 346]. The relationship between ERV and the development of human tumours or autoimmune disease has been extensively investigated, but the field remains rather controversial [347]. The expression of human endogenous retroviral gene products in some cancer types has been reported, including HERV *gag* gene product in the sera of patients with human seminoma [348] and renal cell carcinoma [349], and HERV-derived *pol* gene product in human breast cancer [350] and colorectal carcinoma [351]. In addition, expression levels of the retroviral gene products can be correlated with diseases in some human malignancies [352].

The underlying mechanism leading to cell transformation upon integration or infection of retrovirus early in cellular evolution is thought to be the activation of proto-oncogenes or inactivation of tumour suppressor genes as a consequence of

either transcriptional activation or retrotransposition of proviral DNA with subsequent insertion into host genome. It has been suggested that cells carrying proviral insertions that confer some selective advantages will preferentially grow out and contribute to tumourigenesis [353]. However, despite these observations, the real significance of ERV expression in tumour cells is far from clear.

ERV genome is transmitted as part of the host's genome, thus it is expected that the host is tolerant to ERV antigens as self. In exogenous infection, functional impairment of CD8+ T cells by regulatory T cells during persistent retroviral infection was found [354]. In addition, timed ablation of CD4+ regulatory T cells has been suggested as a new therapy for murine retroviral infection [355]. These facts have implied that some elements of retrovirus could induce immune tolerance to retrovirus.

Mangeney and colleagues (1998) demonstrated that introduction of an *env* gene derived from an infectious murine retrovirus can induce tumour escape from immunosurveillance [356]. Recently, the author further reported that the endogenous retrovirus is essential for a regulatory T cell-mediated suppression of tumour immunity against murine melanoma *in vivo* [357]. Similar effects were observed with full-length envelope gene of a human endogenous retrovirus to promote tumour escape [358].

.

The exact mechanisms of murine leukemia virus-induced tumour cells to escape from host immune detection have yet to be elucidated. ERV have evolved ways to avoid immune detection either by altering their own genome or the host environment [359]. In addition, the intrathymic replication of MuLV during thymocyte maturation and immune selection plays an important role in T cell repertoire development and

immune inhibition in peripheral tolerance [359, 360].

Other findings dispute this simplified view. Cellular and humoral immune responses to human ERV (HERV) proteins have been reported in patients with certain tumours [361, 352]. Similarly, an effective immune response has been described in mice with tumours that express murine ERV (MERV) proteins [287, 288, 362, 363]. In addition, the crucial role of CTL as well as Th cells in the immune rejection of MuLV-induced tumours have been established [359, 364].

We demonstrated in our experiment that a MuLV *pol/env* antigen that are shared in several tumour types was uncovered in CT26-vaccinated and Treg-depleted BALB/c mice. However, it is not clear yet whether and how CD4+CD25+ regulatory T cells would involved in the maintenance of tolerance to MuLV-expressed tumour cells.

6.1. Future works

6.1.1. Identification of epitope

Since no antigenic epitope was identified in primary screen, we would like to first verify the coverage of target gene in recloned plasmid DNA before repeating the peptide sensitization test in a ^{51}chromium-release assay. The recloned P20E8A1 can be transfected into Cos-7 cells and tested the ability to activate CTL clone 2 in a TNF/WEHI assay.

Alternative method involving the generation of truncated gene segments by PCR amplification can be used to identify the antigenic epitope. Other mechanisms including proteasome splicing and chemical modification should be considered for

defining the epitope in case of failure in above methods.

6.1.2. Measurement of *pol/env* gene expression

The expression of gene producing MuLV *pol/env* antigenic peptide in normal cells, tumour cells and thymus endothelium could be evaluated by RT-PCR. This would help to verify the expression of this "shared" tumour-associated antigen among tumour cells of different histological origins. The effective length of MuLV (*pol/env*) antigen could be determined by narrowing down the full-length of identified sequence in a CTL sensitization assay [365].

Furthermore, it seems that the CD25+ regulatory T cells can prevent MuLV *pol/env* antigen from recognition by CD8+ T cells; there raises a possibility that MuLV *pol/env* antigen is indeed a "self" antigen. The quantitative measurement of antigen expression in normal murine cells and thymus could explain in part the role of CD25+ regulatory T cell operating in this system.

In our mouse model, manipulation of mice in a reciprocal protocol in which mice were vaccinated with A20 tumour cells in the absence of CD25+ regulatory T cells failed to establish cross-protection from CT26 tumour. Since the activation of TCR depends on the density of antigen expressed on APCs [316], the level of expression of MuLV *pol/env* antigen on CT26 and A20 tumour cells might in part explain this discrepancy.

To further dissect the difference of immune mechanism between our animal model and CT26/GM-CSF model. It is also worthwhile to known the density of expression of both gp70 and MuLV *pol/env* antigen on CT26 tumour cells.

In addition, Beatty and colleagues (2000) [366] demonstrated that IFN-γ secreted by tumour-infiltrated T cells promotes tumour (CT26) escape through the down-regulation of an immunodominant endogenous tumour antigen gp70. We would like to verify this fact and determine whether *pol* expression is similarly regulated.

6.1.3. Confirmation of crypticity

In our result, polyclonal CTL were activated in the absence of CD25+ regulatory T cells. We would like to known if the hidden effect of regulatory T cells is through the induction of functional unresponsiveness or the blocking of activation.

The antigenic peptide from MuLV antigen could be tested in a ELISPOT assay its ability to activate bulk CD8+ T cells from CT26 tumour cell-vaccinated mice with or without *in vivo* depletion of CD25+ regulatory T cells to confirm its crypticity *in vivo*. Alternatively, if an epitope/H-2-D^d tetramer is available, the bulk CD8+ T cells can be also analyzed by FACS for their TCR specificity.

6.1.4. Relationship between regulatory T cells and costimulation in T cell activation

It has been suggested, T cell activation is a threshold phenomenon and the threshold is tuned by the stimulatory experience of the cell [367]. T cell activation threshold (TAT) was defined as minimum TCR signal required to elicit the response [316]. Activation threshold tuning allows T cells to respond to foreign epitopes, while retaining tolerance to self-epitopes. Various mechanisms were suggested involved in T cell

activation threshold tuning, including both activated and inhibitory costimulatory receptors on T cells and intracellular signals [316].

Tolerance to autoantigens is maintained by regulatory T cells, which block T cell differentiation into effector cells, and by CTLA-4, which increases the activation threshold of T cells. Whereas, both signals of TCR and activated costimulatory receptor (such as CD28) are required for T cells activation. Thus, T cell activation could be activated in the presence of regulatory T cells through costimulation and deletion of regulation.

As aforementioned, T cell activation threshold depends on the binding dynamics of TCR. Thus, T cells with different affinity of TCR would be activated different mechanism. As AH-1-specific CTL were activated through a potent costimulation signals (GM-CSF), whereas they were not a predominant population in the polyclonal CTL that were stimulated in our immunization system that regulatory T cells were depleted before vaccination. It would be interesting to know whether high K_A CTL, such as anti-AH1 clone, are tolerized in the presence of excess costimulation/reduced suppression condition, in which low K_A CTL, such as anti-*pol* clone, preferentially expand.

6.1.5. Regulation of immunodominance by regulatory T cells

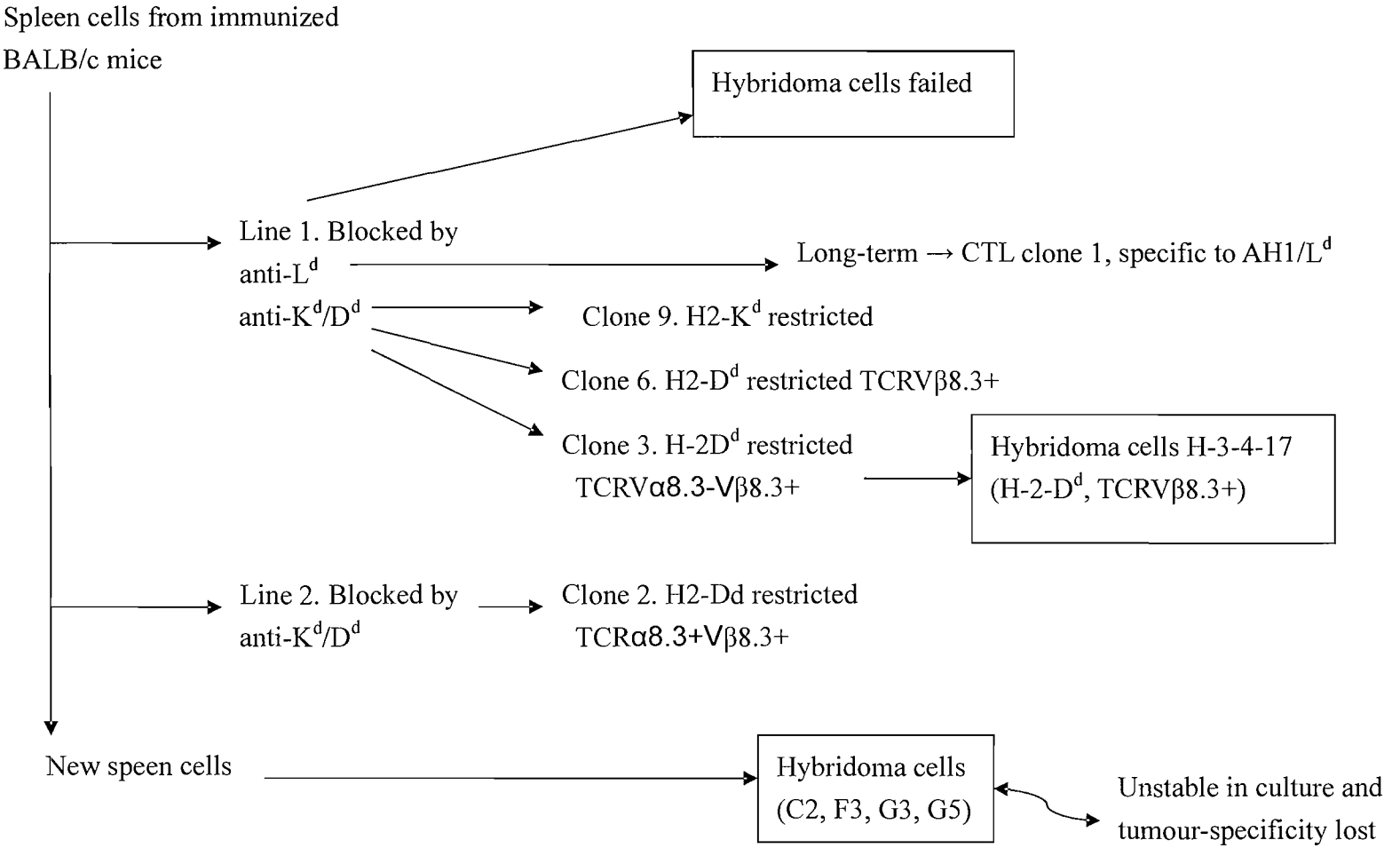
In addition, we have demonstrated in our animal model that immunodominance was regulated by regulatory T cells. We would like to compare the avidity of AH1-specific CTL from CT26/GM-CSF model and our 4 CTL in their *in vitro* responses to CT26 tumour cells with or without the presence of CD4+CD25+ regulatory T cells. This

could be further verified *in vivo* by vaccinated BALB/c mice with gp70 and MuLV *pol/env* antigen with or without depletion of CD25+ regulatory T cells.

6.1.6. The roles of CD4+ effector cells operating in Treg-depleted immunized BALB/c mice

In our animal vaccination model, CD4+ T cells and polyclonal CD8+ T cells were activated against CT26 tumour cells and other histologically different tumours. Since it was suggested that CD4+ T cell-derived IFN-γ is a dominant cytokine that is associated with CTL activity [290]. It is worthwhile to know if CD4+ helper and effector cells in vaccinated mice can recognize MuLV *pol/env* antigen.

Appendix 1. Lineage of CTL from Treg-depleted CT26-immunized mice



Appendix 2. Full dataset of the results in antigen screening

Plate (a)	CPRG assay - H-3-4-17 (**CTL clone 3**)		TNF/WEHI assay- **CTL clone 2**	
	Suspected positive	Re-confirm	Suspected positive	Re-confirm
1	Nil		Nil	
2	Nil		Nil	
3	Nil		Nil	
4	Nil		H5, G4	Nil
5	Nil		Nil	
6	Nil		H1	Nil
7	Nil		A5, A7,F12,G7,H7	Nil
8	Nil		D7	Nil
9	Nil		H1	Nil
10	H8, H9	Nil	B2,C2,D2,E2	Nil
11	C5,C7,D5,D8,F3,F7 (b)	D8 (c)	F8,F11	Nil
12	Nil		A4,A9,A11,A12,H11,H12	Nil
13	Nil		H3,F12	Nil
14	B3,C7	Nil	A3,H2,H3	Nil
15	Nil		B8,E9,F5,G6,G8,G11	Nil
16	Nil		Nil	
17	Nil		B3	Nil
18	A11,A12,E8	Nil	A7,B8,D9,E2,E8,E9	Nil
19	Nil		A12,C12,D11,E12,G8,H3,H5, H8,H10,H11	Nil
20	Nil		C4,E8 (d)	E8 (e)

(a): Each plate contains cDNA colonies in a 96-well format with around 100 colonies in each well, that is there are around 10000 colonies in each plate

(b): Plate 11 screened by H-3-4-17 in a CPRG assay, represented by O.D (average of duplicate).

	!	2	3	4	5	6	7	8	9	10	11	12
A	0.261	0.23	0.233	0.225	0.253	0.233	0.296	0.284	0.259	0.264	0.243	0.224
B	0.299	0.251	0.288	0.289	0.296	0.248	0.289	0.296	0.277	0.27	0.283	0.248
C	0.26	0.268	0.278	0.304	**0.342**	0.258	**0.357**	0.298	0.286	0.282	0.28	0.248
D	0.276	0.292	0.296	0.299	**0.32**	0.26	0.297	**0.365**	0.294	0.274	0.288	0.242
E	0.289	0.242	0.295	0.288	0.291	0.237	0.285	0.267	0.274	0.269	0.271	0.23
F	0.286	0.249	**0.319**	0.267	0.288	0.242	**0.331**	0.297	0.285	0.252	0.282	0.249
G	0.26	0.278	0.255	0.247	0.28	0.227	0.289	0.281	0.257	0.245	0.284	0.236
H	0.273	0.234	0.244	0.226	0.227	0.234	0.286	0.273	0.256	0.265	0.238	0.279

(c): from plate 11, a reactive clone, P11D8G8, was isolated. However, the sequence of this cDNA was known to be unreadable or non-sense.

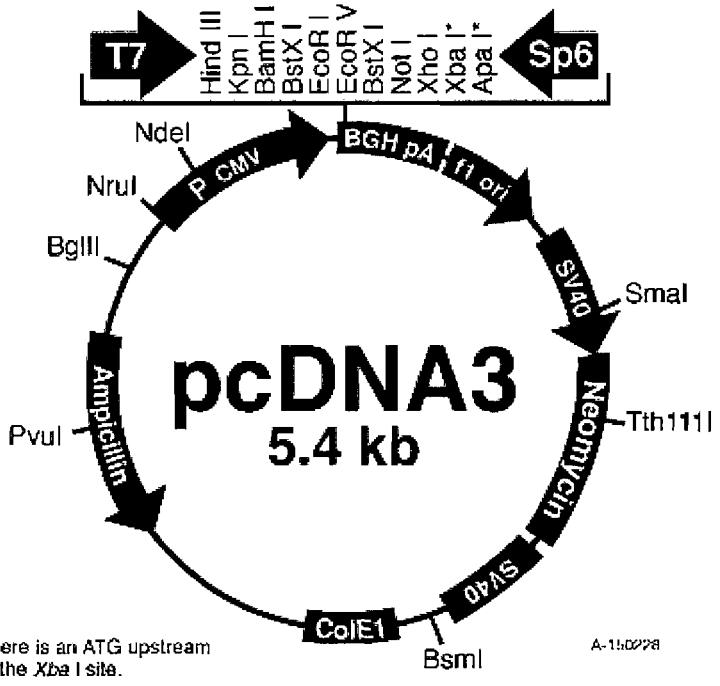
(d): Plate 20 screened by CTL clone 2 in TNF/WEHI assay, represented by percent killing of WEHI cells

	1	2	3	4	5	6	7	8	9	10	11	12
A	21	17	22	18	12	18	15	5	5	5	9	11
B	15	15	14	15	15	12	15	6	7	12	10	8
C	19	18	22	**32**	22	13	15	6	3	10.	11	6
D	18	20	21	20	15	13	3	6	2	8	12	12
E	21	22	16	16	11	9	15	**36**	6	0	7	11
F	16	13	24	18	17	9	17	10	6	2	9	11
G	13	19	14	16	18	13	2	9	16	12	5	12
H	12.	10	20	17	23	16	15	8	20	10	16	16

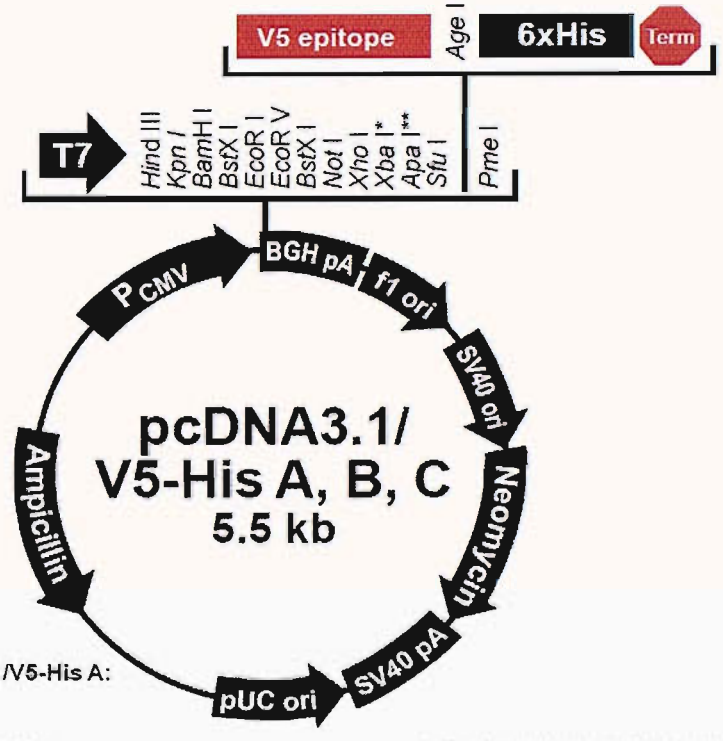
(e): From plate 20, a CTL-reactive clone, P20E8A1, was isolated.

Appendix 3. Map of plasmid vector pcDNA3

CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker bases 889-994
Sp6 promoter bases 999-1016
BGH poly A bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin ORF: bases 2151-2945
SV40 poly A bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin ORF bases 4450-5310



Appendix 4. Map of plasmid vector pcDNA3.1/V5-His



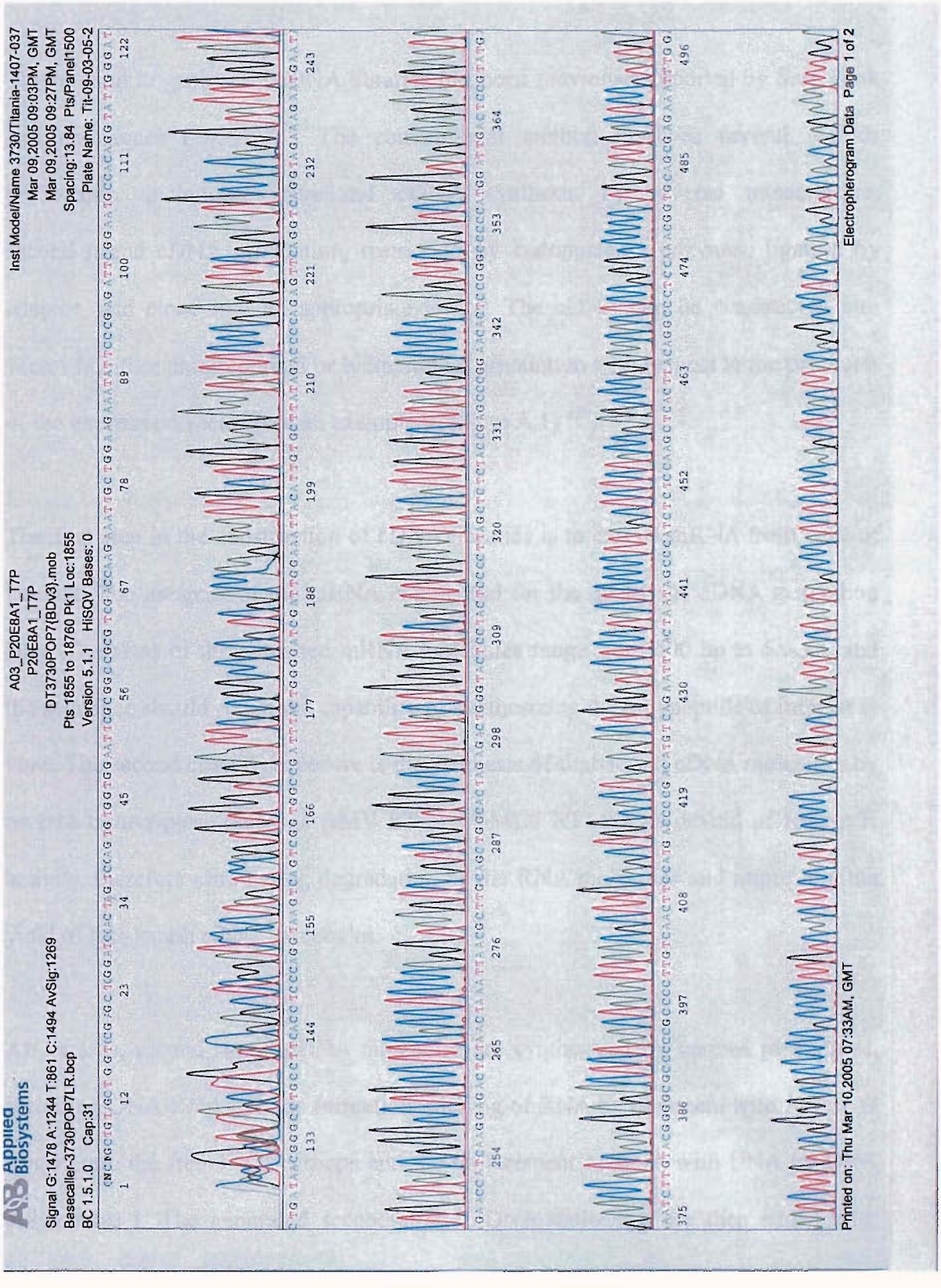
Comments for pcDNA3.1/V5-His A:
5502 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 902-999
V5 epitope: bases 1000-1041
Polyhistidine (6xHis) tag: bases 1051-1068
BGH reverse priming site: bases 1091-1108
BGH polyadenylation signal: bases 1090-1304
f1 origin of replication: bases 1357-1780
SV40 promoter and origin: bases 1845-2169
Neomycin resistance gene: bases 2205-2999
SV40 polyadenylation signal: bases 3018-3256
pUC origin: bases 3688-4361 (C)
Ampicillin resistance gene: bases 4506-5366 (C)
(C) = complementary strand

* After the *Xho* I site, there is a unique *BstE* II site, but no *Xba* I or *Apa* I sites in version C.

** There is a unique *Sac* II site between the *Apa* I site and the *Sfu* I site in version B only.

Appendix 5. DNA sequencing of clone P20E8A1



Appendix 6. Construction of a cDNA library.

The method to generate a cDNA libraries has been previously reported by Sambrook and colleagues (1989) [368]. The conventional method involves several tedious procedures, including first-strand cDNA synthesis by reverse transcriptase, second-strand cDNA production, restriction by endonuclease enzymes, ligation by adaptor, and clone into an appropriate vector. The cDNA can be constructed into vector in either unidirectional or bidirectional orientation with respect to the promoter of the expression vector (see an example in figure A.1) [320].

The first step in the construction of cDNA libraries is to extract mRNA from cells of interest. The integrity of the mRNA is essential for the quality of cDNA generation later. The sizes of the extracted mRNA molecules range from 400 bp to 5.0 kb, and the sequence should retain the capability of synthesizing the polypeptide of interest *in vitro*. The second crucial procedure is the synthesis of first-strand cDNA molecules by reverse transcriptase (such as AMV RT or M-MLV RT) that is devoid of RNase H activity, therefore eliminating degradation of the RNA molecules and improving the yield of full-length cDNA molecules.

Afterwards, second-strand cDNA molecules are synthesized by various procedures, including DNA-RNA hybrids formation, nicking of RNA by treatment with RNase H to generate the free 3'-OH groups and the replacement of RNA with DNA by DNA polymerase I. The generated second-strand cDNA molecules are then added with adaptors (such as BstXl adaptor), cleaved the ends by indicated restriction enzymes

and ligated into the cloning site of appropriate replication-competent expression vectors (such as plasmid, cosmid, phage or virus vector) site in a non-directional, bi-directional or uni-directional orientation.

The constructs of the plasmid DNA, containing various sizes of inserts of cDNA from tumour cells, are then transformed into competent E. coli bacteria for cloning and amplification. In general, a panel of nearly a million of colonies can represent almost the whole tumour cDNA library. Therefore, the pool of bacteria is plated on a large LB agar supplemented with indicated selective antibiotic, and is divided into smaller fractions by cutting the agar piece by piece. The clones of cDNA libraries in each fraction are then amplified in LB broth and plasmid extracted for the next stage of transfection into recipient cells and screening by CTL or hybridoma cells (see Figure 10)[318].

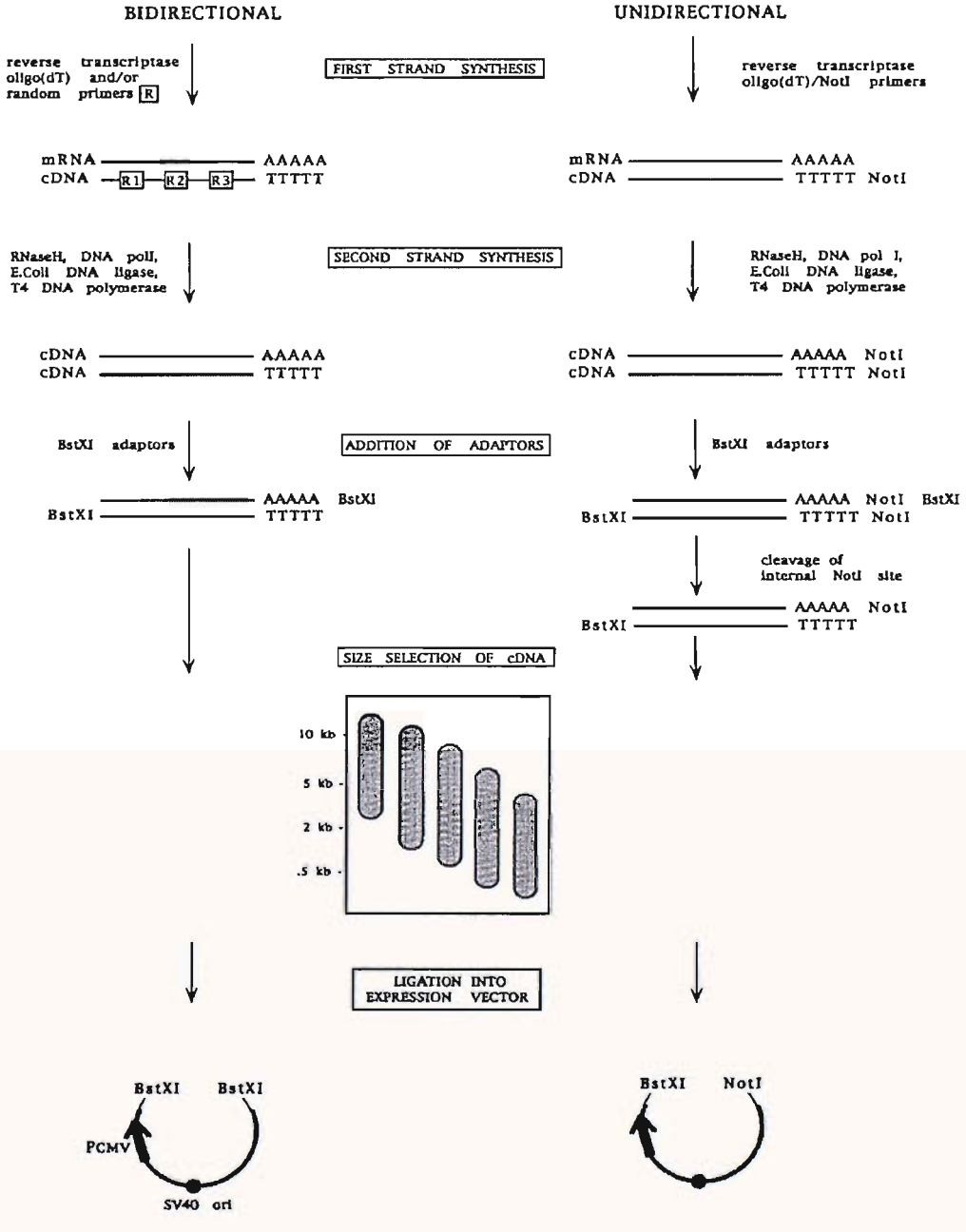


Figure A.1. Schematic diagram of the procedure used to construct bidirectional and unidirectional cDNA libraries.

Starting from mRNA extracted from cells of interest, first-strand cDNA molecules are synthesis by reverse transcriptase, second-strand cDNA molecules were produced by PCR, then after adaptors ligation, the cDNA inserts are cloned into an appropriate vector. (Copy from **Etienne De Plaen,** *et al.*

Identification of gene coding for tumour antigens recognized by cytolytic T lymphocytes. ***Methods: A companion to methods in enzymology*** 1997; **12:** 125-142).

Reference

1. **Ehrlich P.,** (The state of the art of cancer research). Ned. Tijdschr Geneeskd 1909; 5: 273.
2. **Van der Bruggen P,** *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. ***Science*** 1991; **254:** 1634-1637.
3. **Jager D.,** *et al.* Immune responses to tumour antigens: implications for antigen-specific immunotherapy of cancer. ***J. Clin. Pathol.*** 2001; **54:** 669-674.
4. **Mandelboim O.,** *et al.* CTL induction by a tumour-associated antigen octapeptide derived from a murine lung carcinoma. ***Nature*** 1994; **369:** 67-71.
5. **Rosenberg S. A.,** *et al.* Treatment of patients with metastatic melanoma with autologous tumour-infiltrating lymphocytes and interleukin-2. ***Cell Biochem. Suppl.*** 1994; 86:
6. **Nestle F. O.,** *et al.* Vaccination of melanoma patients with peptide- or tumour lysate-pulsed dendritic cells. ***Nat. Med.*** 1998; **4:** 328-332.
7. **Rosenberg R. A.,** *et al.* Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. ***Nat. Med.*** 1998; **4:** 321-327.
8. **Jager E.,** *et al.* Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. ***Proc. Natl. Acad. Sci. USA*** 2000; **97:** 12198-12203.
9. **H. W. H. G. Kessels,** *et al.* Circumventing T-cell tolerance to tumour antigens. ***Biologicals*** 2001; **29:** 277-283.
10. **Sakaguchi S.,** *et al.* Immunological self tolerance maintained by activated T cells expressed IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune disease. ***J. Immunol.*** 1995; **155:** 1151-1164.
11. **Takahashi, T.,** *et al.* Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergy / suppressive state. ***Int Immunol.*** 1998; **10**: 1969-1980.
12. **Thornton, A. E.,** and **Shevach, E. M.** CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin-2 production. ***J. Exp. Med.*** 1998; **188**: 287-296.
13. **Shimizu, J.,** *et al.* Induction of tumour immunity by removing CD25+CD4+ T

cells: a common basis between tumour immunity and autoimmunity. ***J.Immunol.*** 1999; **163**: 5211-5218.

14. **Onizuka, S.,** *et al.* Tumour rejection by *in vivo* administration of anti-CD25 interleukin-2 receptor alpha) monoclonal antibody. ***Cancer Res.*** 1999; **59**: 3128-3133.
15. **Sutmuller, R. P.,** *et al.* Synergism of cytotoxic T lymphocyte-associated antigen-4 blockade and deletion of CD25+ regulatory T cells in anti-tumour therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. ***J. Exp. Med.*** 2001; **194**: 823-832.
16. **Golgher, D.,** *et al.* Deletion of CD25+ regulatory cells uncovers immune responses to shared murine tumour rejection antigens. ***Eur. J. Immunol.*** 2002; **32**: 3267-3275.
17. **Coley WB**. The treatment of malignant tumours by repeated inoculation of erysipelas. With a report of ten original cases. ***Am J Med Sci*** 1893; **105**: 487-551.
18. **Thomas L.** Discussion of cellular and humoral aspects of the hypersensitivity states. Cellular and humoral aspects of the hypersensitivity states: a symposium held at New York Academy of Medicine 1959.
19. **Burnet F. M.** The concept of immunological surveillance. ***Progress in Experimental Tumour Research*** 1970; **13:** 1.
20. **Stutman O.** Tumour development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. ***Science*** 1974; **183:** 534-536.
21. **Foley E. J.** Antigenic properties of methylcholanthrene-induced tumours in mice of the strain of origin. ***Cancer Res.*** 1953; **13:** 835-837.
22. **Prehn R. T.,** *et al.* Immunity to methylcholanthrene-induced sarcoma ***J. Natl. Cancer Inst.*** 1957; **18:** 769-778.
23. **Kobayashi H.,** *et al.* Inhibition of metastasis in rats immunized with xenogenized autologous tumour cells after excision of the primary tumour. ***J. Natl. Cancer Inst.*** 1975; **54:** 997-999.
24. **Van Pel A. and Boon T.** Protection against nonimmunogenic mouse leukemia by an immunogenic variant obtained by mutagenesis. ***Proc. Natl. Acad. Sci. USA*** 1982; 79: 4718-4722.
25. **Dighe A. S**. *et al.* Enhanced in vivo growth resistance to rejection of tumours expressing dominant negative IFN-γ receptors. ***Immunity***1994; **1:** 447-456.
26. **van den Broek M. F.,** *et al.* Decreased tumour surveillance in perforin-deficient mice. ***J. Exp. Med.*** 1996: **184:** 1781-1790.
27. **Peterson R.** An emerging cancer risk: organ transplantation. ***Cancer Nurs***. 1993; **16:** 468-472. .

28. **Svennevig J. L.,** *et al.* Lymphoid infiltration and prognosis in colorectal carcinoma. ***British J. Cancer*** 1984; **49:** 375-377.
29. **Whiteside T. L.,** Tumour infiltrating lymphocytes in human malignancies. ***Austin, Tex: RG Landes Co***; 1993.
30. **Smyth M. J.,** *et al.* A fresh look at tumour immunosurveillance and immunotherapy. ***Nat. Immunol.*** 2001; **2:** 293-299.
31. **Lanier LL.** Renaissance for the tumour immunosurveillance hypothesis. ***Nat. Med.*** 2001; **7:** 1178-1180.
32. **Smyth M. J.,** *et al.* Differential tumour surveillance by natural killer (NK) and NKT cells. ***J. Exp. Med.*** 2000; 191: 661-668.
33. **Hobohm U.** Fever and cancer in perspective. ***Cancer Immunol. Immunother.*** 2001; **50:** 391-396.
34. **Medzhitov R. and Janeway C. J.** The toll receptor family and microbial recognition. ***Trends Microbiol.*** 2000; **8:** 452-456.
35. **Akira S.,** *et al.* Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2001; 2: 675-680.
36. **Girardi M.,** *et al.* Regulation of cutaneous malignancy by γδ T cells. **Science** 2001; **294:** 606-609.
37. **Shankaran V.,** *et al.* IFN-γ lymphocytes prevent primary tumour development and shape tumour immunogenecity. ***Nature*** 2001; **410:** 1107-1111.
38. **Mihm M.,** *et al.* Tumour infiltrating lymphocytes in lymph node melanoma metastases – a histopathologic prognosis indicator and an expression of local immune responses. ***Lab Invest.*** 1996; **74:** 43-47.
39. **Lee P. P.,** *et al.* Characterization of circulating T cells specific for tumour-associated antigens in melanoma patients. ***Nat. Med.*** 1999; **5:** 677-685.
40. **Pittet M. J.,** *et al.* Expansion and functional maturation of human tumour antigen-specific CD8+ T cells after vaccination with antigenic peptide. ***Clin. Cancer Res.*** 2001; 7: 796s-803s.
41. **Svane I. M.,** *et al.* The role of cytotoxic T-lymphocyte in the prevention and immune surveillance of tumours: lessons from normal and immunodeficient mice. ***Med. Oncol.*** 1999; **16:** 223-238.
42. **Barth R.,** *et al.* Interferon γ and tumour necrosis factor have a role in tumour regression mediated by murine CD8+ tumour-infiltrating lymphocytes. ***J. Exp. Med.*** 1991; **173:** 647-658.
43. **Rosenberg S.,** *et al.* A new approach to the adoptive immunotherapy of cancer with tumour-infiltrating lymphocytes. ***Science*** 1986; **233:** 1318-1321.
44. **Lynch D. and R. Miller** Interleukin-7 promotes long-term in vitro growth of antitumour cytotoxic T lymphocytes with immunotherapeutic efficacy in vivo. ***J.***

Exp. Med. 1994; **179:** 31-42.

45. **Seliger B.**, *et al.* HLA class I antigen abnormalities and immune escape by malignant cells. ***Semin. Cancer Biol.*** 2002; **12:** 3-13.
46. **Croft M.,** *et al.* Generation of polarized antigen specific CD8 effector populations: reciprocal actions of IL-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. ***J. Exp. Med.*** 1994; **180:** 1715-1728.
47. **Sad S.,** *et al.* Cytokine induced differentiation of precursor mouse CD8 T cell into cytotoxic CD8 T cells secreting th1 or th2 cytokines. ***Immunity*** 1995; **2:** 271-279.
48. **Kemp R. A. and Ronchese F.** Tumour-specific Tc1, but not Tc2, cells deliver protective antitumour immunity. ***J. Immunol.*** 2001; **167:** 6497-6502.
49. **Dobrzanski, M, J.,** *et al.* Tc1 and Tc2 effector cell therapy elicit long-term tumour immunity by contrasting mechanisms that result in complementary endogenous type I antitumour responses. ***J. Immunol.*** 2004; **172:** 1380-1390.
50. **Zitvogel L.,** *et al.* Therapy of murine tumours with tumour-peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. ***J Exp. Med.*** 1996; **183:** 87-97.
51. **Gong J.,** *et al.* Induction of antitumour activity by immunization with fusions of dendritic and carcinoma cells. ***Nat. Med.*** 1997; **3:** 558-561.
52. **Goedegebuure P. and T. Eberlein** The role of CD4+ tumour-infiltrating lymphocytes in human solid tumours. ***Immunol. Res.*** 1995; **14:** 119-131.
53. **Toes, R. E.,** *et al.* CD4+ T cells and their role in antitumour immune responses. ***J. Exp. Med.*** 1999; **189:** 753-756.
54. **Von Mensdorff-Pouilly S.** *et al.* Survival in early breast cancer patients is favourably influenced by a natural humoral response to polymorphic epithelial mucin. ***J. Clin. Oncol.*** 2000; **18:** 574-583.
55. **Livingston P. O.,** et al. Improved survival in stage III melanoma patients with GM2 antibodies: a randomized trial of adjuvant vaccination with GM2 ganglioside. ***J. Clin. Oncol.*** 1994; **12:** 1036-1044.
56. **Cesano A., Gayko U.** CD20 as a target of passive immunotherapy. ***Semin. Oncol.*** 2003; **30:** 253-257.
57. **Riethmuller G.,** *et al.* Monoclonal antibody therapy for resected Duke's colorectal cancer: seven-year outcome of a multicenter randomized trial. ***J. Clin. Oncol.*** 1998; **16:** 1788-1794.
58. **Vogel C. L.,** *et al.* Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER-2-overexpressing metastatic breast cancer. ***J. Clin. Oncol.*** 2002; **20:** 719-726.
59. **Jeffrey S.,** *et al.* Targeted therapies for cancer 2004. ***Am. J. Clin. Pathol.*** 2004;

122: 598-609.

60. **Cheng W. F.,** *et al.* CD8+ T cells, NK cells and IFN-gamma are important for control of tumour with downregulated MHC class I expression by DNA vaccination. ***Gene Ther.*** 2003; **10:** 1311-1320.
61. **Ma H. L.** *et al.* IL-21 activates both innate and adaptive immunity to generate potent antitumour responses that require perforin but are independent of IFN-gamma. ***J. Immunol.***2003; **171:** 608-615.
62. **Fernandez N. C.,** *et al.* Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumour immune responses *in vivo*. ***Nat. Med.*** 1999; **5:** 405-411.
63. **Whitside T. L.** Apoptosis of immune cells in the tumour microenvironment and peripheral circulation of patients with cancer: implications for immunotherapy. **Vaccine** 2002**; 20 suppl 4:** A46-51**.**
64. **Cerwenka A.,** *et al.* Ectopic expression of expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumour *in vivo*. ***Natl. Acad. Sci. U.S.A.*** 2001; **98:** 11521-11526.
65. **Vujanovic N. L.,** *et al.* Non-secretory apoptotic killing by human natural killer cells. ***J. Immunol.*** 1996; **15:** 1117-1126.
66. **Kelly J. M.** *et al.* Induction of tumour-specific T cell memory by NK cell-mediated tumour rejection. ***Nat. Immunol.*** 2002; **3:** 83-90.
67. **Smyth M. F. and Gogfrey D. I.** NKT cells and tumour immunity: a double-edged sword. ***Nat. Immunol.*** 2000; **1:** 459-460.
68. **Atkins M. B.,** *et al.* High-dose recombinant interleukin-2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. ***J. Clin. Oncol.*** 1999: **17**: 2105-2116.
69. **Lotem M.,** *et al.* Interleukin-2 improves tumour response to DNP-modified autologous vaccine for the treatment of metastatic malignant melanoma. ***Br. J. Cancer*** 2004; **90:** 773-780.
70. **Nanni P.,** *et al.* Combined allogeneic tumour cell vaccination and systemic interleukin-12 prevents mammary carcinogenesis in Her-2/neu transgenic mice. ***J. Exp. Med.*** 2001; **194:** 1195-1205.
71. **Meazza R.,** *et al.* Gene transfer of a secretable form of IL-15 in murine adenocarcinoma cells: effect on tumourigenicity, metastatic potential and immune response. ***Int. J. Cancer*** 2000; **87:** 574-581.
72. **Tatsumi T.,** *et al.* Interleukin-18 gene transfer expands the repertoire of antitumour Th1-type immunity elicited by dendritic cell-based vaccines in association with enhanced therapeutic efficacy. ***Cancer Res.*** 2002: **62:**

5853-5858.

73. **Soiffer R.,** *et al.* vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumour immunity in patients with metastatic melanoma. ***Proc. Natl. Acad. Sci. USA*** 1998; **95:** 13141-13146.
74. **Jaffee E. M.,** *et al.* A phase I clinical trial of lethally irradiated allogeneic pancreatic tumour cells transfected with the GM-CSF gene for the treatment of pancreatic adenocarcinoma. ***Hum. Gene Ther.*** 1998; **9:** 1951-1971.
75. **Gowans J.L., Uhr J. W.** The carriage of immunological memory by small lymphocytes in the rat. ***J Exp Med.*** 1966; **124(5)**:1017-1030.
76. **Childs R.,** *et al.* Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. ***N. Engl. J. Med.*** 2000; **343:** 750-756.
77. **Goedegebuure P. S.,** *et al.* Adoptive immunotherapy with tumour-infiltrating lymphocytes and interleukin-2 in patients with metastatic melanoma and renal cell carcinoma: a pilot study. ***J. Clin. Oncol.*** 1995; 13;: 1939-1949.
78. **Yannelli J. R.,** *et al.* Growth of tumour-infiltrating lymphocytes from human solid cancer: summary of a 5-year experience. ***Int. J. Cancer*** 1996; **53:** 413-421.
79. **Khanna R.,** *et al.* Activation and adoptive transfer of Epstein-Barr virus-specific Cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. ***Proc. Natl. Acad. USA.*** 1999; **96:** 10391-10396.
80. **Burns D. M. and Crawford D. H.** Epstein-Barr virus-specific cytotoxic T-lymphocytes for adoptive immunotherapy of post-transplant lymphoproliferative disease. ***Blood Rev.*** 2004; **18(3)**: 193-209.
81. **Moser B. and Loetscher P.** Lymphocyte traffic control by chemokines. ***Nat. Immunol.*** 2001; **2:** 123-128.
82. **Cawthon A. G. and Alexadnder-Miller M. A.** Optimal co-localization of TCR and CD8 as a novel mechanism for the control of functional avidity. ***J. Immunol.*** 2002; **169:** 3492-3498.
83. **Slifka M. K. and Whitton J. L.** Functional avidity maturation of CD8 (+) T cells without selection of higher affinity TCR. ***Nat. Immunol.*** 2001; **2:** 711-717.
84. **Margulies D. H.** TCR avidity: it's not how strong you make it, its how you make it strong. ***Nat. Immunol.*** 2001; **2:** 669-670.
85. **Dudley M. E. and Rosenberg S. A.** adoptive-cell-transfer therapy for the treatment of patients with cancer. ***Nat. Rev. Cancer*** 2003; **3:** 666-675.
86. **F De Paoloa,** *et al.* Restored T cell activation mechanism in human

tumour-infiltrating lymphocytes from melanoma and colorectal carcinoma after exposure to interleukin-2. ***Brit. J. Cancer*** 2003; **88:** 320-326.

87. **Nagagomi H.,** *et al.* Decreased expression of the signaling-transducing ζ chains in tumour-infiltrating T cells and NKT cells of patients with colorectal carcinoma. ***Cancer Res.*** 1993; **53:** 5610-5612.
88. **Torsten E.** *et al.* Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumour-infiltrating lymphocytes in patients with oral carcinoma. ***Clin. Cancer Res.*** 2002; **8:** 3137-3145.
89. **Ling W.,** *et al.* Impaired activation of NFκB in T cells from a subset of renal cell carcinoma patients is mediated by inhibition of phosphorylation and degradation of the inhibitor, IκBα. ***Blood*** 1998; **92:** 1334-1341.
90. **Uzzo R. G.,** *et al.* Alteration in NFκB activation in T lymphocytes of patients with renal cell carcinoma. ***J. Natl. Cancer Inst.*** 1999; **91:** 718-721.
91. **Finke J. H., et al.** Loss of T-cell receptor ζ chain and $p56^{lck}$ in T-cells infiltrating human renal cell carcinoma. ***Cancer Res.*** 1993: **53:** 5613-5616.
92. **Finke J. H.,** *et al.* Tumour-induced sensitivity to apoptosis in T cells from patients with renal cell carcinoma: role of nuclear factor kappa B suppression. ***Clin .Cancer Res.*** 2001; **12:** 43-50.
93. **Dworacki G.,** *et al.* Decreased ζ chain expression and apoptosis in CD3+ peripheral blood T lymphocytes of patients with melanoma. ***Clin. Cancer Res.*** 2001; **7:** 947s-957s.
94. **Dudley M. E.,** *et al.* Cancer regression and autoimmunity in patients after clonal repopulation with antitumour lymphocytes. ***Science*** 2002; **298:** 850-854.
95. **Dudley M. E.,** *et al.* A phase I study of nonmyeloablative chemotherapy and adoptive transfer of autologous tumour antigen-specific T lymphocytes in patients with metastatic melanoma. ***J. immunother.*** 2002; **25:** 243-251.
96. **Gromet MA.,** *et al.* The regressing thin malignant melanoma: a distinctive lesion with metastatic potential. ***Cancer*** 1978; **42:** 2282-2292.
97. **Knuth A.,** *et al.* T cell responses to human malignant tumours. ***Cancer Surv.*** 1992; **13:**39-52.
98. **Jager D.,** *et al.* Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer. ***J. Clin. Pathol.*** 2001; **54:** 669-674.
99. **Knut A.,** *et al.* Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection. ***Proc. Natl. Acad. Sci. USA*** 1989; **86:** 2804-2808.
100. **Wolfel T.,** *et al.* Lysis of human melanoma cells by autologous cytolytic T cell clones. Identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. ***J. Exp. Med.*** 1989; **170:**

797-810.

101. **Knuth A.,** *et al.* T-cell-mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin-2-dependent T-cell culture. ***Proc. Natl. Acad. Sci. USA*** 1984; **81:** 3511-3515.
102. **Boon T.,** *et al.* Tumour antigens recognized by T lymphocytes. ***Annu. Rev. Immunol.*** 1994; **12**: 337-365.
103. **Van den Eynde, B.,** *et al.* A new family of genes coding for an antigen Recognized by autologous cytolytic T lymphocytes on a human melanoma. ***J. Exp. Med.*** 1995; **182**: 689-698.
104. **Sahin U.** *et al.* Human neoplasms elicit multiple specific immune responses in the autologous host. ***Proc. Natl. Acad. Sci. USA*** 1995: **92:** 11810-11813.
105. **Van den Eynde, B.,** *et al.* The gene coding for a major tumour rejection antigen of tumour P815 is identical to the normal gene of syngeneic DBA/2 mice. ***J. Exp. Med.*** 1991; **173**: 1373-1384.
106. **Akiko Uenaka and Eiichi Nakayama** Murine leukaemia RL♂ and sarcoma Meth A antigens recognized by cytotoxic lymphocyte. ***Cancer Sci.*** 2003; **94 (11):** 931-936.
107. **Gaugler B.,** *et al.* Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocyte. ***J. Exp. Med.*** 1994; **179**: 921-930.
108. **Brichard, V.,** *et al.* The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanoma. ***J. Exp. Med.*** 1993; **178**: 489-495.
109. **Coulie, P.,** *et al.* A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanoma. ***J. Exp. Med.*** 1994; **180:** 35-42.
110. **Bakker, A.,** *et al.* Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumour-infiltrating lymphocytes. ***J Exp. Med.*** 1994; **179**: 1005-1009.
111. **Coulie, P.,** *et al.* A mutated intron sequence codes for an antigenic epitope recognized by cytolytic T lymphocytes on a human melanoma. ***Proc. Natl. Acad. Sci. USA*** 1995; **92:** 7976-7980.
112. **Monach, P.,** *et al.* A unique tumour antigen produced by a single amino acid substitution. ***Immunity*** 1995; **2:** 45-59.
113. **Wolfel, T.,** *et al.* A $p16^{INK4a}$-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. ***Science*** 1995; **269:** 1281-1284.
114. **Robins, P.,** *et al.* A mutated β-catenin gene encodes a melanoma-specific antigen recognized by tumour infiltrating lymphocytes ***J. Exp. Med.*** 1996; **183**:

1185-1192.

115. **Wagner W. M.,** *et al.* The abl/bcr gene product as a novel leukemia-specific antigen with peptides spanning the fusion region of abl/bcr can be recognized by both CD4+ and CD8+ T lymphocytes. ***Cancer Immunol. Immunother.*** 2003; **52:** 89-96.
116. **Thompson M. P. and Kurzrock R.** Epstein-Barr virus and cancer. ***Clin. Cancer Res.*** 2004; **10:** 803-821.
117. **Gilbo, E.** The makings of a tumour rejection antigen. ***Immunity*** 1999; **11**: 263-270
118. **Longo D. L.,** *et al.* Isolation of HTLV-transformed B-lymphocyte clone from patient with HTLV-associated adult T-cell leukemia. ***Nature*** 1984; **310**: 505-506.
119. **Disis M.,** *et al.* Oncogenic proteins as tumour antigens. ***Current Opin. Immunol.*** 1996; **8:** 637-642.
120. **Michael A.,** *et al.* Mucins in cancer: protection and control of the cell surface. ***Nature Reviews Cancer*** 2004; 4: 45-60.
121. **Rammensee H. C.,** *et al.* MHC molecules as peptide receptors. ***Curr. Opin. Immunol.*** 1993; **5:** 35-44.
122. **Marchand M.,** *et al.* Tumour regression s observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. ***Int. J. Cancer*** 1999; **80:** 219-230.
123. **Jager E., Jager D., Knuth A.** Clinical cancer vaccine trials. ***Curr. Opin. Immunol.*** 2002; **14:** 178-182.
124. **Thurner B.,** *et al.* Vaccination with MAGE-3A1 peptide-pulse mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. ***J. Exp. Med.*** 1999; **190:** 1669-1678.
125. **Panelli M.C.,** *et al.* Phase I study in patients with metastatic melanoma of immunization with dendritic cells presenting epitopes derived from melanoma-associated antigens MART-1 and gp100. ***J. immunother.*** 2000; **23:** 487-498.
126. **Slingluff CL Jr.,** *et al.* Immunologic and clinical outcomes of vaccination with a multiepitope melanoma peptide vaccine plus low-dose interleukin-2 administered either concurrently or on a delay schedule. ***J. Clin. Oncol.*** 2004; **22:** 4474-4485.
127. **Reddy S. L.** A phase I trial of a PSA peptide vaccine for patients with prostate cancer. ***Proc. Am. Soc. Clin. Oncol.*** 2003; **22:** 757.
128. **Dillman R. O.** Monoclonal antibody therapy for lymphoma: an update. ***Cancer***

Pract. 2001; **9**: 71-80.

129. **Grillo-Lopez A. J.** Anti-CD20 mAb: modifying therapeutic strategies and outcomes in the treatment of lymphoma patients. ***Expert. Rev. Anticancer Ther.*** 2002; **2:** 323-329.
130. **Slamon D. J.,** *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. ***N. Engl. J. Med.*** 2001; **344:** 783-792.
131. **Ligibel J. A. and Winer E. P.** Trastuzumab/chemotherapy combinations in metastatic breast cancer. ***Semin. Oncol.*** 2002; **29:** 38-43.
132. **Richard B. S. Roden,** *et al.* Vaccination to prevent and treat cervical cancer. ***Human Pathology*** 2004; **35:** 971-982.
133. **Mukherji, B.,** *et al.* Induction of antigen-specific T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. ***Proc. Natl. Acad. Sci. USA*** 1995; **92:** 8078-8082.
134. **Kripke M. L.** Antigenicity of murine skin tumours induced by ultraviolet light. ***J. Natl. Cancer Inst.*** 1974; **53:** 1333-1336.
135. **Uenaka A. and Nakayama E.** Murine leukaemia RL♂ and sarcoma Meth A antigens recognized by cytotoxic lymphocyte. ***Cancer Sci.*** 2003; **94 (11):** 931-936.
136. **Wortzel R. D.** et al. Multiplicity of unique tumour-specific antigens expressed on a single malignant cell. ***Nature*** 1983; **304:** 165-167.
137. **Ward P. L.,** *et al.* Tumour antigens defined by cloned immunological probes are highly polymorphic and are not detected on autologous normal cells. ***J. Exp. Med.*** 1989; **170:** 217-232.
138. **Van den Eynde B.**, et al. Presence on a human melanoma of multiple antigens recognized by autologous CTL. ***Int. J. Cancer*** 1989; **44:** 634-640.
139. **Pieper R.** *et al.* Biochemical identification of a mutated humane melanoma antigen recognized by CD4 (+) T cells. ***J. Exp. Med.*** 1999; **189:** 756-766.
140. **Pierre van der Bruggen,** *et al.* Tumour-specific shared antigenic peptides recognized by human T cells. ***Immunological Review*** 2002; **188:** 51-64.
141. **Wang RF.** Tumour antigens discovery: perspectives for cancer therapy. ***Mol. Med.*** 1997; **3 (11):** 716-731.
142. **Greiner J.,** *et al.* Simultaneous expression of different immunogenic antigens in acute myeloid leukemia. ***Exp. Hematol.*** 2000; **28(12):**1413-1422.
143. **Chene P.** Targeting p53 in cancer. ***Curr. Med. Chem. Anti-Canc. Agents***. 2001; **1:** 151-161.
144. **Andersen M. H. and thor H. P.** Survivin--a universal tumour antigen. ***Histol. Histopathol.*** 2002; **17(2):** 669-675

145. **Fong L. and Small E. J.** Immunotherapy for prostate cancer. ***Semin. Oncol.*** 2003; **30:** 649-658.
146. **Brandle, D.,** *et al.* The shared tumour-specific antigen encoded by mouse gene P1A is a target not only for cytolytic T lymphocytes but also for tumour rejection. ***Eur. J. Immunol.*** 1998; **28:** 4010-4019.
147. **Wolfers, J.,** *et al.* Tumour-derived exosomes are a source of shared tumour rejection antigens for CTL cross-priming. ***Nature medicine*** 2001; **7(3)**: 297-303.
148. **Dranoff G.** Coordinated tumour immunity. ***J Clin Invest.*** 2003; **111:** 1116-1118.
149. **Curcio C.** *et al.* Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas. ***J Clin Invest.*** 2003; **111:** 1161-1170.
150. **Zeng G.,** *et al.* Generation of NY-ESO-1-specific CD4+ and CD8+ T cells by a single peptide with dual MHC class I and II specificities: a new strategy for vaccine design. ***Cancer Res.*** 2002; **62:** 3630-3635.
151. **Nishikawa H.,** *et al.* Role of SEREX-defined immunogenic wild-type cellular molecules in the development of tumour-specific immunity. ***Proc. Natl. Acad. Sci. USA*** 2001; **98:** 14571-14576.
152. **Old L. J., Chen Y. T.** New paths in human cancer serology. ***J. Exp. Med.*** 1998; **187:** 1163-1167.
153. **Jager D.** *et al.* Identification of tumour antigens as potential target antigens for immunotherapy by serological expression cloning. ***Cancer Immunol. Immunother.*** 2004; **53(3):** 144-147.
154. **Li G.** *et al.* Identification of tumour antigens by serological analysis of cDNA expression cloning. ***Cancer Immunol. Immunother.*** 2004; **53(3):**139-143.
155. **Jager E.**, *et al.* Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. ***J. Exp. Med.*** 1998; **161:** 265-270.
156. **Cox A.L.,** *et al.* Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. ***Science*** 1994; **264:** 716-719.
157. ***Uenaka A.***, *et al.* Induction of a unique antigen peptide pRL1 on BALB/c RL♂ leukemia recognized by cytotoxic T lymphocytes and its relation to the *Akt* oncogene. ***J. Exp. Med.*** 1994; **180:** 1599-1607.
158. **Naour F. L.,** *et al.* Identification of tumour-associated antigens using proteomics. ***Technol. Cancer Res. Treat.*** 2002; **1:** 257-262.
159. **Radvanyi L.** Discovery and immunologic validation of new antigens for therapeutic cancer vaccines. ***Int. Arch Allergy Immunol.*** 2004; **133:** 179-197.
160. **Pascolo S.** *et al.* A MAGE-A1 HLA-A A*0201 epitope identified by mass

spectrometry. ***Cancer Research*** 2001; **61:** 4072-

161. **Zhang L.**, *et al.* Gene expression profiles in normal and cancer cells. ***Science*** 1997; **276:** 1268-1272.
162. **Velculescu V. E.,** *et al.* Serial analysis of gene expression. ***Science*** 1995; **270:** 484-487.
163. **Lockhart D. J.,** *et al.* Genomics, gene expression and DNA array. ***Nature*** 2000; **405:** 827-836.
164. **Yutaka Kawakami,** *et al.* Identification of human tumour antigens and its implications for diagnosis and treatment of cancer. ***Cancer Sci.*** 2004; **95:** 784-791.
165. **Liang P.,** *et al.* Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. ***Science*** 1992; **257:** 967-971.
166. **Lee S. W.,** *et al.* Positive selection of candidate tumour-suppressor genes by subtraction hybridization. ***Proc. Natl. Acad. Sci. USA.*** 1991; **88:** 2825-2829.
167. **Scott D. M.,** *et al.* Identification of a mouse male-specific transplantation antigen, H-Y. ***Nature*** 1996; **376**: 695-698.
168. **Zuberi A.,** *et al.* Positional cloning and molecular characterization of an immunodominant cytotoxic determinant of the mouse H3 minor histocompatibility complex. ***Immunity*** 1998; **9:** 687-698.
169. **Boon T. and van der Bruggen P.** Human tumour antigens recognized by T lymphocyte. ***J. Exp. Med.*** 1996; **183:** 725-729.
170. **Ito K.,** *et al.* Identification of bladder cancer antigens recognized by IgG antibodies of a patient with metastatic bladder cancer. ***Int. J. Cancer*** 2004; **108:** 712-724.
171. **Vonderheide R. H.,** *et al.* The telomerase catalytic subunit is a widely expressed tumour-associated antigens recognized by cytotoxic T lymphocytes. ***Immunity*** 1999; **10:** 673-679.
172. **Uyttenhove C.,** *et al.* Escape of mouse mastocytoma P815 after nearly complete rejection is due to antigen-loss variants rather than immunosuppression. ***J. Exp. Med.*** 1983; **157:** 1040-1052.
173. **Brunner K. T.,** *et al.* Antigen-specificity of the cytolytic T lymphocyte (CTL) response to murine sarcoma virus-induced tumours. II. Analysis of the clonal progeny of CTL precursors stimulated in vitro with syngeneic tumour cells. ***J. Immunol.*** 1980; **124:** 1627-1634.
174. **Maryanski J. L.,** *et al.* Immunogenic variants obtained by mutagenesis of mouse mastocytoma P815. III. Clonal analysis of the syngeneic cytolytic T lymphocyte response. ***Eur. J. Immunol.*** 1982; **12:** 401-406.
175. **Mukherji B. and MacAlister T. J.** Clonal analysis of cytotoxic T cells response

against human melanoma. ***J. Exp. Med.*** 1983; **158:** 240-245.

176. **Knuth A.,** *et al.* T-cell mediated cytotoxicity against autologous malignant melanoma: Analysis with interleukin-2-dependent T-cell culture. ***Proc. Natl. Acad. U.S.A.*** 1984; **81:** 3511-3515.
177. **Herin M.,** *et al.* Production of stable cytolytic T-cell clones directed against autologous human melanoma. ***Int. J. Cancer*** 1987; **30:** 390-396.
178. **Traversari C.,** *et al.* Transfection and expression of a gene encoding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes. ***Immunogenetics*** 1992; **35**: 145-152.
179. **Seed B. and Aruffo A.** Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. ***Proc. Natl. Acad. Sci. USA.*** 1987; **84:** 3365-3369.
180. **Karttunen J.,** *et al.* Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. ***Proc. Natl. Acad. Sci. USA.*** 1992; **89:** 6020-6024.
181. **Brichard V.,** *et al.* A tyrosinase nonapeptide presented by HLA-B44 is recognized on a human melanoma by autologous cytolytic T lymphocytes. ***Eur. J. Immunol.*** 1996; **26:** 224-230.
182. **Kawakami Y.,** *et al.* Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumour. ***Proc. Natl. Acad. Sci. USA.*** 1994; **91:** 3515-3519.
183. **Boel P.,** *et al.* BAGE, a new gene encoding an antigen recognized on human melanoma by cytolytic T lymphocytes. ***Immunity*** 1995; **2:** 167-175.
184. **Brunner K. T.,** *et al.* Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vivo; inhibition by isoantibody and by drugs. ***Immunology*** 1968; **14,** 181-196.
185. **Sheehy M. E.,** *et al.* A novel technique for the fluorometric assessment of T lymphocyte antigen-specific lysis. ***J. Immunol. Methods*** 2001; **249:** 99-110
186. **Lui L.,** *et al.* Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. ***Nat. Med.*** 2002; **8:** 185-189.
187. **Uenaka A.,** *et al.* ELISPOT cloning of tumour antigens recognized by cytotoxic T-lymphocytes from a cDNA expression library. ***Cancer Immun.*** 2001; **1:** 8.
188. **Scheibenbogen C.,** *et al.* Identification of known and novel immunogenic T-cell epitopes from tumour antigens recognized by peripheral blood T cells from patients responding to IL-2-based treatment. **Int. J. Cancer** 2002; **98:** 409-412.
189. **Nissen-Meyer J.,** *et al.* Comparison of recombinant tumour necrosis factor and the monocyte-derived cytotoxic factor involved in monocyte-mediated

cytotoxicity.
Cancer Res. 1987; **47(9):** 2251-2258.

190. **Espevik T. and Nissen-Meyer J.** A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumour necrosis factor from human monocytes. ***J. Immunol. Methods*** 1986; 95: 99-105.
191. **Mosmann T.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. ***J. Immunol. Methods*** 1983; **65:** 55-63.
192. **Green L. M.,** *et al.* Rapid colorimetric assay for cell viability: Application to the quantification of cytotoxic and growth inhibitory lymphokines. ***J. immunol. Methods*** 1984; **70:** 257.
193. **Danizot F. and Lang R.** Rapid colorimetric assay for cell growth and survival. ***J. Immunol. Methods*** 1986; **89:** 271.
194. **Nabholz, M.,** *et al.* Cytolytically active murine T-cell hybrids. ***Nature*** 1980; **287**: 437-440.
195. **Ozaki S.,** *et al.* Production of T-T hybrids from T cell clones: direct comparison between cloned T cells and T hybridoma cells derived from them. ***J. Immunol.*** 1988; **141:** 71-78.
196. **Karttunen J. and Shastri N.** Measurement of ligand-induced activation in single viable T cells using the LacZ reporter gene. ***Proc. Natl. Acad. Sci. USA.*** 1991; **88:** 3972-3976.
197. **Crabtree G.** Contingent genetic regulatory events in T lymphocyte activation. ***Science*** 1989; **243:** 355.
198. **Ullman K.,** *et al.* Transmission of signals from T lymphocyte antigen receptor to the gene responsible for cell proliferation and immune function. ***Annu. Rev. Immunol.*** 1990; **8:** 421.
199. **Sanderson, S. and Shastri, N.** LacZ inducible, antigen/MHC-specific T cell hybrids. ***Int. Immunol.*** 1994; 6: 369-376.
200. **Malarkannan, S.,** *et al.* Generation and analysis of LacZ inducible T cell hybrids. ***Methods Mol. Biol.*** 2001; 156: 265-272.
201. **Kenneth L.,** *et al.* Generation of class I MHC-restricted T-T hybridomas. ***J. Immunol.*** 1990; **145:** 804-811.
202. **Wong F. S.,** *et al.* Identification of an MHC class I-restricted autoantigen in type I diabetes by screening an organ-specific cDNA library. ***Nature Medicine*** 1999; **5:** 1026-1031.
203. **Malarkannan S.** *et al.* Alloreactive CD8+ T cells can recognize unusual, rare, and unique processed peptide/MHC complexes. ***J. Immunol.*** 1996; **157:** 4464-4467.

204. **Fotedar A.,** *et al.* Fine specificity of antigen recognition by T cell hybridoma clones specific for poly-18: a synthetic polypeptide antigen of defined sequence and conformation. ***J. Immunol.*** 1985; **135:** 3028-3033.
205. **Mendoza L. M.,** *et al.* Identification of CD8+ T-cell-stimulating antigen gene in cDNA libraries. ***Methods Mol. Biol.*** 2001; **156:** 255-263.
206. **Lohr M.,** *et al.* Microencapsulated, CYP2B1-transfected cells activating ifosfamide at the site of the tumour: the magic bullets of the 21st century. ***Cancer Chemother Pharmacol.*** 2002; **49:** S21-24.
207. **Metzelaar M. J.,** *et al.* CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. ***J. Biol. Chem.*** 1991; **66(5):** 3239-3245.
208. **Robeva A. S.,** *et al.* Rapid expression cloning of receptors using epitope-tagged ligands and high-speed cell sorting. ***Cytometry A.*** 2003; **51(2):** 59-67.
209. **Darrow T. L.,** *et al.* The role of HLA class I antigens in recognition of melanoma cells by tumour-specific cytotoxic T lymphocytes. Evidence for shared tumour antigen. ***J. immunol.*** 1989; **142:** 3329-3335.
210. **Wölfel T.,** *et al.* Lysis of human melanoma cells by autologous cytolytic T cell clones. Identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. ***J. Exp. Med.*** 1989; **170:** 797-810.
211. **Wölfel T.,** *et al.* Analysis of antigens recognized on human melanoma cells by A2-restricted cytolytic T lymphocytes (CTL). ***Int. J. Cancer*** 1993; **55:** 237-244.
212. **Lopata M. A.,** *et al.* High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. ***Nucleic Acids Res.*** 1984; 12: 5707-5017.
213. **Schenborn E.T. and Goiffon V.** DEAE-dextran transfection of mammalian cultured cells. ***Methods Mol Biol.*** 2000; 130: 147-153.
214. **Pari G. S. and Keown W. A.** Experimental strategies in efficient transfection of mammalian cells. Calcium phosphate and DEAE-dextran. ***Methods Mol Biol.*** 1997; **62:** 301-306.
215. **Jager E.,** *et al.* Immunoselection in vivo: independent loss of MHC class I and melanoma differentiation antigen expression in metastatic melanoma mediated by antigen-specific CTL. ***Int. J. Cancer*** 1997; **71:** 142-147.
216. **Kaklamanis, L.,** *et al.* Loss of major histocompatibility complex-encoded transporter associated with antigen presentation (TAP) in colorectal cancer. ***Am. J. Pathol.,*** 1994; **145**: 505-509.
217. **Alain P. Vicari,** *et al.* Tumour escape from immune surveillance through

dendritic cell inactivation. ***Cancer Biology*** 2002; **12:** 33-42.

218. **Carreno B. M. and Collin M.** The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. ***Ann. Rev. Immunol.*** 2002; **20:** 29-53.
219. **Coyle A. J. and Gutierrez-Ramos J. C.** The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. ***Nat. Immunol.*** 2001; **2:** 203-209.
220. **Zang X.,** *et al.* B7x: a widely expressed B7 family member that inhibits T cell activation. ***Proc. Natl. Acad. Sci. U S A.*** 2003; **100 (18):** 10388-10392.
221. **Iwai Y.** *et al.* Involvement of PD-L1 on tumour cells in the escape from host immune system and tumour immunotherapy by PD-L1 blockade. ***Proc. Natl. Acad. Sci. USA*** 2002; **99:** 12293-12297.
222. **Dong H.**, *et al.* Tumour-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. ***Nat. Med.*** 2002; **8:** 793-800.
223. **Mellor A. L. and Munn D. H.** Tryptophan catabolism and T-cell tolerance: mmunosuppression by starvation? ***Immunol. Today*** 1999; **20:** 469-473.
224. **Uyttenhove C.,** *et al.* Evidence for a tumoural immune resistance mechanism based on cryptophan degradation by indoleamine 2, 3-dioxygenase. ***Nat Med.*** 2003; **9:** 1269-1274.
225. **Munn D. H. and Mellor A. L.** IDO and tolerance to tumours. ***Trends in molecular medicine*** 2004; **10:** 15-18.
226. **Voorzanger N.,** *et al.* Interleukin (IL)-10 and IL-6 are produced in vivo by non-Hodgkin's lymphoma cells and act as cooperative growth factors. ***Cancer Res.*** 1996; **56:** 5499-5505.
227. **Elgert, K. D.,** *et al.* Tumour-induced immune dysfunction: the macrophage connection. ***J. Leukoc. Biol.,*** 1998; **64**: 275-290.
228. **Staveley-O'Carroll, K.,** *et al.* Induction of antigen-specific T cell anergy: An early event in the course of tumour progression. ***Proc. Natl. Acad. Sci. USA.*** 1998; 95: 1178-1183.
229. **Fowell, D.,** *et al.* Subsets of CD4+ T cells and their roles in the induction and prevention of autoimmunity. ***Immunol. Rev.*** 1991; **123:** 37-64.
230. **Sakaguchi, S.,** and **N. Sakaguchi.** Thymus, T cells and autoimmunity: various causes but a common mechanism of autoimmune disease. In Autoimmunity: Physiology and Disease. **A. Coutinho** and **M.Kazatchkine**, eds.Wiley-Liss, 1994, New York, p. 203.
231. **Groux H.,** *et al.* A CD4 (+) T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. ***Nature*** 1997; **389:** 737-742.
232. **Zhang X.,** *et al.* Activation of CD25+CD4+ regulatory T cells by oral antigen

administration. ***J. Immunol.*** 2001; **167:** 4245-4253.

233. **Nishizuka Y. and Sakakura T.** Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. ***Science*** 1969; **166:** 753-755.
234. **Dieckmann, D.,** *et al.* Ex vivo isolation and characterization of CD4+CD25+ T cells with regulatory properties from human blood. ***J. Exp. Med.*** 2001; **193**: 1303-1310.
235. **Jonuleit, H.,** *et al.* Identification and functional characterization of human CD4+CD25+ T cells with regulatory properties isolated from peripheral blood. ***J. Exp. Med.*** 2001; **193:** 1285-1294.
236. **Stephens, L. A.,** *et al.* Human CD4+CD25+ thymocyte and peripheral T cells have immune suppressive activity in vitro. ***Eur. J. Immunol.*** 2001; **31**: 1247-1254.
237. **Sakaguchi N.** Naturally arising Foxp3-expressing CD4+CD25+ regulatory T cells in immunologic tolerance to self and non-self. ***Nat. Immunol.*** 2005. **6:** 345-352.
238. **Frontenot J. D. and Rudensky A.** A well adapted regulatory contrivance: regulatory T cell development and Forkhead family transcription factor Foxp3. ***Nat. Immunol.*** 2005; **6:** 331-337.
239. **Itoh M.,** *et al.* Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. ***J. Immunol.*** 1999; **162:** 5317-5326.
240. **Salomon, B.,** *et al.* B7/CD28 costimulation is essential for the homeostasis of The CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. ***Immunity*** 2000; **12**: 431-440.
241. **Read, S.,** *et al.* Cytotoxic T lymphocytes-associated antigen 4 plays an essential role in the function of CD25+CD4+ regulatory cells that control intestinal inflammation. ***J. Exp. Med.*** 2000; **192:** 295-302.
242. **Lepault, F.,** *et al.* Characterization of peripheral regulatory CD4+ T cells that prevent diabetes onset in non-obese diabetic mice. ***J. Immunol.*** 2000; **164**: 240-247.
243. **Suri-Payer E,** *et al.* CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. ***J. Immunol.*** 1998; **160:** 1212-1218.
244. **Viglietta V.,** *et al.* Loss of functional suppression by D4+CD25+ regulatory T cells in patients multiple sclerosis. ***J. Exp. Med.*** 2004; **199:** 971-979.
245. **Kriegel M. A.,** *et al.* Defective suppressor function of human CD4+CD25+ regulatory T cells in autoimmune polyglandular syndrome type II. **J. Exp. Med.**

2004; **199:** 1285-1291.

246. **Coutinho A.,** *et al.* Regulatory T cells: the physiology of autoreactivity in dominant tolerance and "quality control" of immune responses. ***Immunol. Rev.*** 2001; **182:** 89-98.
247. **Curotto M. A.,** *et al.* CD4+ regulatory T cells in autoimmunity and allergy. ***Curr. Opin. Immunol.*** 2002; **14:** 771-778.
248. **Gavin M., Rudensky A.** Control of immune homeostasis by naturally arising regulatory CD4+ T cells. ***Curr. Opin. Immunol.*** 2003; **15:** 690-696.
249. **Takahashi, T.,** *et al.* Immunologic self-tolerance maintained by CD4+CD25+ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. ***J. Exp. Med.*** 2000; **192**: 302-310.
250. **Fontenot JD.,** *et al.* Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. ***Nat. Immunol.*** 2003; **4:** 330-336.
251. **Khattri R.,** *et al.* An essential role for Scurfin in CD4+CD25+ T regulatory cells. ***Nat. Immunol.*** 2003; **4:** 337-342.
252. **Ramsdell F. and Ziegler S. F.** Transcription factors in autoimmunity. ***Curr. Opin. Immunol.*** 2003; **15:** 718-724.
253. **Cambineri E.,** *et al.* Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutation of FOXP3, a critical regulator of T-cell homeostasis. ***Curr. Opin. Rheumatol.*** 2003; **15:** 430-435.
254. **Jordan M. S.** *et al.* Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self peptide. ***Nat. Immunol.*** 2001; 2: 301-306.
255. **Shevach E. M.** CD4+CD25+ suppressor T cells: more questions than answers. ***Nat. Rev. Immunol.*** 2002; **2**: 389-400.
256. **Tang Q.,** *et al.* CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. **J. Immunol.** 2003; **171:** 3348-3352.
257. **Schimpl A.,** *et al.* IL-2 and autoimmune disease. ***Cytokine Growth Factor Rev.*** 2002; **13:** 369-378.
258. **Malek T. R.,** *et al.* CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rβ-deficient mice. Implications for the non-redundant function of IL-2. ***Immunity*** 2002; **17:** 167-178.
259. **Fisson S.,** *et al.* Continuous activation of autoreactive CD4+CD25+ regulatory T cells in the steady state. ***J. Exp. Med.*** 2003; **198:** 737-746.
260. **Walker LSK.,** *et al.* Antigen-dependent proliferation of CD4+CD25+ regulatory T cells in vivo. ***J. Exp. Med.*** 2003; **198:** 249-258.
261. **Yamazaki S.,** *et al.* Direct expansion of functional CD25+CD4+ regulatory T cells by antigen-processing dendritic cells. ***J. Exp. Med.*** **198:** 235-247.

262. **Joffre O.,** *et al.* Induction of antigen-specific tolerance to bone marrow allograft with CD4+CD25+ T lymphocytes. ***Blood*** 2004; **103:** 4216-4220.
263. **Piccirillo C., Shevach E.** Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. ***J. Immunol.*** 2001; **167:** 1137-1140.
264. **Asseman C.,** *et al.* An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. ***J. Exp. Med.*** 1999; **190:** 995-103.
265. **Powrie F.,** *et al.* A critical role for transforming growth factor-beta and interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB (low) CD4 (+) T cells. ***J. Exp. Med.*** 1996; **183:** 2669-2674.
266. **Seddon B. and Mason D.** Regulatory T cells in the control of autoimmunity: the essential role of transforming growth factor-beta and interleukin 4 in the prevention of autoimmune thyroiditis in rats by peripheral CD4 (+) CD45RC (-) cells and CD4 (+) CD8 (-) thymocytes. ***J. Exp. Med.*** 1999; **189:** 279-288.
267. **Piccirillo C. A. and Thornton A. M.** Cornerstone of peripheral tolerance: naturally occurring CD4+CD25+ regulatory T cells. ***Trends Immunol.*** 2004; 25: 374-380.
268. **Takeshi A.,** *et al.* Humane CD4+CD25+ regulatory T cells suppress NKT cell function. ***Cancer Research*** 2003; **63**: 4516-4520.
269. **Cederbom L.,** *et al.* CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. ***Eur. J. Immunol.*** 2000; **30:** 1538-1543.
270. **Thornton A. M. and Shevach E. M.** Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen-nonspecific. ***Immunol.*** 2000; **164:** 1137-1140.
271. **Houghton, A.** Cancer antigens: immune recognition of self and altered self. ***J. Exp. Med.*** 1994; **180**: 1-4.
272. **Yee C.,** *et al.* Melanocyte destruction after antigen-specific immunotherapy of melanoma: direct evidence of T cell-mediated vitiligo. ***J. Exp. Med.*** 2000; **192:** 1637-1643.
273. **Geissler M.,** *et al.* Immunotherapy directed against alpha-fetoprotein results in autoimmune liver disease during liver regeneration in mice. ***Gatrsoenterology*** 2001; **121:** 931-939.
274. **Ludewig B.,** *et al.* Immunotherapy with dendritic cells directed against tumour antigens shared with normal host cells results in severe autoimmune disease. ***J. Exp. Med.*** 2000; **191:** 795-803.
275. **Shimizu, J.,** *et al.* Induction of tumour immunity by removing CD25+CD4+ T cells: a common basis between tumour immunity and autoimmunity. **J.**

Immunol. 1999; **163:** 5211-5218.

276. **Woo, E. Y.,** *et al.* Regulatory CD4+CD25+ T cells in tumours from patients with early stage non-small cell lung cancer and late-stage ovarian cancer. ***Cancer Res.*** 2001; **61:** 4766-4772.
277. **Fumiko Ichihara,** *et al.* Increased populations of regulatory T cells in peripheral blood and tumour-infiltrating lymphocytes in patients with gastric and oesophageal cancers. ***Clinical Cancer Research*** 2003; **9:** 4404-4408.
278. **Kawaida H.,** *et al.* Distribution of CD4+CD25high regulatory T-cells in tumour-draining lymph nodes in patients with gastric cancer. ***J. Surg. Res.*** 2005; **124:** 151-157.
279. **Wolf A. M.,** *et al.* Increase of regulatory T cells in the peripheral blood of cancer patients. ***Clinical Cancer Research*** 2003; **9:** 606-612.
280. **Curiel T. J,** *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. ***Nat. Med.*** 2004; **10:** 942-949.
281. **Hiroyoshi Nishikawa,** et al. CD4+CD25+ T cells responding to serologically defined autoantigens suppress antitumour immune responses. ***Proc. Natl. Acad. Sci. USA*** 2003; 100: 10902-10906.
282. **Rakhmilevich, A. L.,** *et al.* Presence of CD4+ T suppressor cells in mice rendered unresponsive to tumour antigens by intravenous injection of irradiated tumour cells. ***Int. J. Cancer*** 1993: **55**: 338-343.
283. **Drebin, J. A.,** *et al.* Regulation of the immune response to antigens on the malignant cell surface. ***Springer Semin. Immunopathol.*** 1982; **5**: 175-192.
284. **Simon J. Prasad,** *et al.* Dendritic cells loaded with stressed tumour cells elicit long-lasting protective tumour immunity in mice depleted of CD4+CD25+ regulatory T cells. ***J. Immunol.*** 2005; **174:** 90-98.
285. **Hiroshi Nagai,** *et al. In vivo* elimination of CD25+ regulatory T cells leads to tumour rejection of B16F10 melanoma, when combined with interleukin-12 gene transfer. ***Experimental Dermatology*** 2004; **13:** 613-620.
286. **Dranoff G.,** *et al.* Vaccination with irradiated tumour cells engineered to secret murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific and long-lasting anti-tumour immunity. ***Proc. Natl. Acad. Sci. USA*** 1993; **90**: 3539-3543.
287. **Huang A. Y.,** *et al.* The immunodominant major histocompatibility complex class-I restricted antigen of a murine colon tumour derived from an endogenous retroviral gene product. ***Proc. Natl. Acad. Sci. USA*** 1996; **93**: 9730-9735.
288. **Yang J. C. and Perry-Lalley D.** The envelope protein of an endogenous murine retrovirus is a tumour-associated T-cell antigen for multiple murine tumours. ***J.***

Immunother. 2000; **23:** 177-183.

289. **Wang R.,** *et al.* Development of a retrovirus-based complementary DNA expression system for the cloning of tumour antigens. ***Cancer Res.*** 1998; **58:** 3519-3525.
290. **Noelia Casares,** *et al.* CD4+/CD25+ regulatory cells inhibit activation of tumour-primed CD4+ T cells with IFN-γ-dependent antiangiogenic activity, as well as long-lasting tumour immunity elicited by peptide vaccination. ***J. immunol.*** 2003; **171:** 5931-5939.
291. **Noelia Casares,** *et al.* Immunization with a tumour-associated CTL epitope plus a tumour-related or unrelated Th1 helper peptide elicits protective CTL immunity. ***Eur. J. Immunol.*** 2001; **31:** 1780-1789.
292. **Hiroyoshi Nishikawa,** *et al.* Role of SEREX-defined immunogenic wild-type cellular molecules in the development of tumour-specific immunity. ***Proc. Natl. Acad. Sci. U S A.*** 2001; **98**: 14571-14576.
293. **Griswold D. P.,** *et al.* A colon tumour model for anticancer agent evaluation. C***ancer*** 1975; **36:** 2441-2444.
294. **Corbett T. H.,** *et al.* Tumour induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. ***Cancer Res.*** 1975; **35:** 2434-2439.
295. **Thorbecke G. J.,** *et al.* Role of B cells in the stimulation of syngeneic mixed lymphocyte responses. ***Behring Inst Mitt.*** 1983; **(72):** 107-116.
296. **Illidge T.,** *et al.* A new in vivo and in vitro B cell lymphoma model, pi-BCL1. ***Cancer Biother. Radiopharm.*** 2000; **15:** 571-580.
297. **Murphy, G. P. and Hrushesky, W. J.** A murine renal cell carcinoma. ***J. Natl. Cancer Inst.*** 1973. **50:** 1013–1025.
298. **Nakamura, K.,** *et al.* Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. ***J. Cell Biol.*** 2000; **150:** 731–740.
299. **Ziegler-Heitbrock H. W. and Riethmuller G.** A rapid assay for cytotoxicity of unstimulated human monocytes. ***J. Natl Cancer Inst.*** 1984; **72(1):** 23-29.
300. **McMurray R. W.,** *et al.* T cell receptor V beta usage in murine experimental autoimmune thyroiditis. ***Cell Immunol.*** 1996; **172:** 1-9.
301. **Lyons A. B.** Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester. ***Immunol. Cell. Biol.*** 1999; **77:** 509-515.
302. **Gluzman Y.** SV40-transformed simian cells support the replication of early SV40 mutants. ***Cell*** 1981; **23:** 175-182.
303. **Falk K.,** *et al.* Allel-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. ***Nature*** 1991; **351:** 290-296.

304. **Madden D. G.,** *et al.* The structure of HLA-B27 reveals nonamer self peptides bound in an extended conformation. ***Nature*** 1991; **353:** 321-325.
305. **Bamford R. N.,** *et al.* The interleukin (IL)-2 receptor β-chain is shared by IL-2 and a cytokine, provisionally designated IL-T, that stimulates T-cell proliferation and induction of lymphokine-activated killer cells. ***Proc. Natl. Acad. Sci. USA*** 1994; **91:** 4940-4944.
306. **Grabstein K. H.,** *et al.* Cloning of a T cell growth factor that interacts with the β-chain of the interleukin-2 receptor. ***Science*** 1994; **264:** 965-968.
307. **Giri J. G.,** *et al.* Identification and cloning of a novel IL-15 binding protein that is structurally related to the α chain of the IL-2 receptor. ***EMBO J.*** 1995; **14:** 3654-3663.
308. **Donald M. Eicher** IL-2 and IL-15 manifest opposing effects on activation of nuclear factor of activated T cells. ***Cellular Immunology*** 2003; **223:** 133-142.
309. **Alves N. L.,** *et al.* IL-15 induces antigen-independent expansion and differentiation of human naïve CD8+ T cell in vitro. ***Blood*** 2003; **102 (7):** 2541-2546.
310. **Berard M.,** *et al.* IL-15 promotes the survival of naïve and memory phenotype CD8+ T cells. ***J. Immunol.*** 2003; **170:** 5018-5026.
311. **Chen Q.,** *et al.* Production of IL-10 by melanoma cells: examination of its role in immunosuppression mediated by melanoma. ***Int. J. Cancer*** 1994; **56:** 755-760.
312. **Oft M.,** *et al.* TGF-beta signalling is necessary for carcinoma cell invasion and metastasis. ***Curr. Biol.*** 1998; **8:** 1243-1252.
313. **Jeffrey J.,** *et al.* Prolonged culture of vaccine-primed lymphocytes results in decreased antitumour killing and change in cytokine secretion. ***Cancer Research*** 2004; **64:** 9124-9130.
314. **Kwong Y.,** *et al.* Phenotypic stability of a cytotoxic T-cell line directed against an immunodominant epitope of human carcinoembryonic antigen. ***Clin. Cancer Research*** 1997; **3:** 2439-2449.
315. **Gonzalez P. A.,** *et al.* T cell receptor binding kinetics required for T cell activation depend on the density of cognate ligand on the antigen-presenting cells. ***Proc. Natl. Acad. Sci. USA.*** 2005; **102:** 4824-4829.
316. **Hugo A. van den Berg and David A. Rand** Dynamics of T cell activation threshold tuning. ***Journal of Theoretical Biology*** 2004; **228:** 397-416.
317. **Sallusto F.,** *et al.* Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. ***Nature*** 1999; **401:** 708-712.
318. **Masopust D.** *et al.* Preferential localization of effector memory cells in nonlymphoid tissue. ***Science*** 2001; **291:** 2413-2417.

319. **Kemp R. A.,** *et al.* The phenotype of type 1 and type2 CD8+ T cells activated in vitro is affected by culture conditions and correlates with effector activity. 2005 in press.
320. **Roychowdhury S.,** *et al.* Failed adoptive immunotherapy with tumour-specific T cells: reversal with low-dose interleukin 15 but not low-dose interleukin 2. ***Cancer Research*** 2004; **64:** 8062-8067.
321. **Ribas A.,** *et al.* Determinant spreading and tumour responses after peptide –based cancer immunotherapy. ***Trends Immunol.*** 2003; **24 (2):** 58-61.
322. **Butterfield L. H.,** *et al.* Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. ***Clin. Cancer Res.*** 2003; **9(3):** 998-1008.
323. **Plaen E. D.,** *et al.* Identification of gene coding for tumour antigens recognized by cytolytic T lymphocytes. ***Methods: A companion to methods in enzymology*** 1997; **12:** 125-142.
324. **Ken-Ichi Hanada,** *et al.* Immune recognition of a human renal cancer antigen through post-translational protein splicing. ***Nature*** 2004; **427:** 252-256.
325. **Sercarz E. E**. Driver clones and determinant spreading. ***J. Autoimmun.*** 2000; **14:** 275-277.
326. **Vanderlugt C. L. and Miller S. D.** Epitope spreading in immune-mediated disease: implication for immunotherapy. ***Nat. Rev. Immunol.*** 2002; **2:** 85-95.
327. **Lower R.,** *et al.* The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequence. ***Proc. Natl. Acad. Sci. USA*** 1996; **93:** 5177.
328. **Shinnick T. M.,** *et al.* Nucleotide sequence of Moloney murine leukemia virus. ***Nature*** 1981; **293:** 543-548.
329. **Gross L.** Spontaneous leukemia developing in C3H mice following inoculation, in infancy, with Ak-leukemic extracts, or Ak-embryos. ***Proc. Soc. Exp. Biol. Med.*** 1951; **76:** 27-32.
330. **Risser R. and Horowitz J. M.** Endogenous mouse leukemia viruses. ***Ann. Rev. Genet.*** 1983; **17:** 85-121.
331. **Rowe W. P.,** *et al.* Genetic mapping of a murine leukemia virus-inducing locus of AKR mice. ***Science*** 1972; **178:** 860-862.
332. **Horowitz J. M. and Risser R.** A locus that enhances the induction of endogenous ecotropic murine leukemia viruses is distinct from genome-length ecotropic proviruses. ***J. Virol.*** 1982; **44:** 950-957.
333. **Jenkins N. A.,** *et al.* Organization, distribution, and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. ***J. Virol.*** 1982; **43:** 26-36.

334. **Nakagawa K. and Harrison L. C.** The potential rolo of endogenous retroviruses in autoimmunity. ***Immunol. Rev.*** 1996; **152:** 193-236.
335. **Taruscio D. and Mantovani A.** Factors regulating endogenous retroviral sequences in human and mouse. ***Cytogenet. Genome Res.*** 2004; **105:** 351-362.
336. **Peters R. L.,** *et al.* Prevalence of the group-specific (gs) antigen and infectious virus expressions of the murine C-type RNA viruses during the life span of BALB/cCr mice. ***Int. J. Cancer*** 1972; **10:** 283-289.
337. **McCubrey J. and Risser R.** Genetic interactions in the spontaneous production of endogenous murine leukemia virus in low leukemic mouse strains. ***J. Exp. Med.*** 1982; **156:** 337-349.
338. **Ihle J. N.,** *et al.* Genetic linkage of C3H/HeJ and BALB/c endogenous ecotropic C-type viruses of phosphoglucomutase-1 on chromosome 5. ***Science*** 1979: **204:** 71-73.
339. **Kozak C. A. and Rowe W. P.** Genetic mapping of a murine leukemia virus-inducing locus of BALB/c mouse to chromosome 5. ***Science*** 1979; **204:** 69-71.
340. **Sijts A. J.,** *et al.* Identification of an H-2K^{b} –presented Moloney murine leukemia virus cytotoxic T lymphocyte epitope that displays enhanced recognition in H-2D^{b} mutant bm 13 mice. ***J. Virol.*** 1994; **68:** 6038-6046.
341. **Chen W.,** *et al.* Identification of a gag-encoded cytotoxic T-lymphocyte epitope from FBL-3 leukemia shared by Friend, Moloney and Rauscher murine virus-induced tumours. ***J. Virol.*** 1996; **70:** 7773-7782.
342. **Iwashiro M.,** *et al.* Multiplicity of virus-encoded helper T-cell epitopes expressed on FBL-3 tumour cells. ***J. Virol.*** 1993; **67:** 4533-4542.
343. **White H. D.,** *et al.* An immunodominant Kb-restricted peptide from the P15E transmembrane protein of endogenous ecotropic murine leukemia virus (MuLV) AKR623 that restores susceptibility of a tumour line to anti-AKR/Gross MuLV cytotoxic T lymphocytes. ***J. Virol.*** 1994; **68:** 897-904.
344. **Ono M.,** *et al.* Nucleotide sequence of human endogenous retrovirus genome related to the mouse mammary tumour virus genome. ***J. Virol.*** 1986; **60:** 589-598.
345. **Ono M.** Molecular cloning and long terminal repeat sequences of human endogenous retrovirus genes related to types A and B retrovirus genes. ***J. Virol.*** 1986; **58:** 937-944.
346. **Medstrand P. and Blomberg J.** Characterization of novel reverse transcriptase encoding human endogenous retroviral sequences similar to type A and type B retroviruses: differential transcription in normal human tissues. ***J. Virol.*** 1993; **67:** 6778-6787.

347. **Lower R.** The pathogenic potential of endogenous retroviruses: facts and fantasies. ***Trends Microbiol.*** 1999; 7: 350.
348. **Sauter M.**, *et al.* Human endogenous retrovirus K10: expression of Gag protein and detection of antibodies in patients with seminoma. *J. Virol.* 1995; **69:** 414-421.
349. **Wahlstrom T.,** *et al.* Renal cell adenocarcinoma and retrovirus p30-related antigen excreted to urine. ***Lab. Investig.*** 1985; **53:** 464-469.
350. **Moyret C.** F., *et al.* Detection of reverse transcriptase activity in human breast tumours. ***Anticancer Res.*** 1988; **8:** 1279-1983.
351. **Moshier J. A.,** *et al.* mRNA from human colon tumour and mucosa related to the pol gene of an endogenous A-type retrovirus. ***Biochem. Biophys. Res. Commun***. 1986; **139:** 1071-1077.
352. **Boller K.**, *et al.* Characterization of the antibody response specific for the human endogenous retrovirus HTDV/HERV-K. ***J. Virol.*** 1997; **71:** 4581-4588.
353. **Mikkers H. and Berns A.** Retroviral insertional mutagenesis: tagging cancer pathways. ***Adv. Cancer Res.*** 2003; **88:** 53-99.
354. **Dittmer U.**, *et al.* Functional impairment of CD8 (+) T cells by regulatory T cells during persistent retroviral infection. ***Immunity*** 2004; **20:** 293-303.
355. **Beilharz M. W.,** *et al.* Timed ablation of regulatory CD4+ T cells can prevent murine AIDS progression. ***J. Immunol.*** 2004; **172:** 4917-4925.
356. **Mangeney M. and Heidmann T.** Tumour cells expressing a retroviral envelope escape immune rejection *in vivo*. ***Proc. Natl. Acad. Sci. USA*** 1998; **95:** 14920-14925.
357. **Mangeney M.**, *et al.* Endogenous retrovirus expression is required for murine melanoma tumour growth in vivo. ***Cancer Res.*** 2005; **65:** 2588-2591.
358. **Mangeney M.,** *et al.* The full-length envelope of an HERV-H human endogenous retrovirus has immunosuppressive properties. ***J. Gen. Virol.*** 2001; **82:** 2515-2518.
359. **Marshall D. J. and Gaulton G. N.** The role of the immune response in MULV-induced lymphomagenesis. ***Leukemia*** 1996; 10: 1860-1866.
360. **Gaulton G. N.** Viral pathogenesis and immunity within the thymus. ***Immunol. Res.*** 1998; **17:** 75-82.
361. **Schiavetti F.**, *et al.* A human endogenous retroviral sequence encoding an antigen recognized on melanoma by cytolytic T lymphocytes. ***Cancer Res.*** 2002; **62:** 5510.
362. **Hayashi H.**, *et al.* Molecular cloning and characterization of the gene encoding mouse melanoma antigen by cDNA library transfection. ***J. immunol.*** 1992; **149:** 1223.

363. **Zeh H. J.,** *et al.* High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumour efficacy. ***J. immunol.*** 1999; **162:** 989.

364. **Ossendorp F.,** *et al.* Specific T helper cells requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex II negative tumours. ***J. Exp. Med.*** 1998; **187:** 693-702.

365. **Townsend A. R.,** *et al.* The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. ***Cell*** 1986; **44:** 959-968.

366. **Beatty G. L. and Paterson Y.** IFN-γ can promote tumour evasion of the immune system in vivo by down-regulating cellular levels of an endogenous tumour antigen. ***J. immunol.*** 2000; **165:** 5502-5508.

367. **Grossman Z. and Paul W. E.** Autoreactivity, dynamics tuning and selectivity. ***Curr. Opin. Immunol.*** 2001; **13:** 687-698.

368. **Sambrook J.,** *et al.* Molecular Cloning, 2nd ed., Cold Spring Harbour Laboratory, **Cold Spring Harbour 1989, NY.**, pp. 8.11-8.35.