Functional characterisation of the T cell mediated anti-tumour response in a melanoma patient : identification of a HLA-DR1*10011 restricted unique antigen

Thesis

FUNCTIONAL CHARACTERISATION OF THE T CELL MEDIATED ANTI-TUMOUR RESPONSE IN A MELANOMA PATIENT: IDENTIFICATION OF A HLA-DRβ1*10011 RESTRICTED UNIQUE ANTIGEN

by

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ABSTRACT

CD4+ T cells are central in regulating most adaptive immune responses and essential for sustaing CD8+ T cell activation and efficient tumour destruction. Therefore, a better understanding of the CD4+ T cell-mediated mechanisms involved in anti-tumour responses will be crucial to improve vaccination strategies. The objective of this thesis was to evaluate the anti-tumour CD4+ T cell response in a metastatic melanoma patient, Pt15392, disease free after 12 years from surgical removal of lymph node metastasis and who was previously shown to display a CD8+ T cell response against epitopes included in the TRP-2 and gp100 antigens.

Several CD4+ T cell clones were isolated from the metastatic lymph nodes by limiting dilution assay. TCR analysis defined five groups of clones which all showed a strong TCR-dependent cytotoxic activity in response to *in vitro* stimulation with the autologous melanoma. Functional analysis demonstrated that all the isolated T cell clones recognised tumour cells in HLA-DR restricted fashion. In addition to exert a cytotoxic activity in response to tumor stimulation, these T cell clones released cytokines compatible with a Th1 profile although they also specifically produced high amounts of IL-10.

An invariant chain (Ii) - cDNA fusion library was constructed in an attempt to identify the melanoma antigen recognised by these CD4+ T cell clones. The screening resulted in the detection of a mutated form of the human *protein tyrosine phosphatase receptor kappa* (*PTPR-κ*) gene, as a DRβ1*10011-restricted tumour antigen. All the T lymphocyte clones isolated *in vitro* were directed against a DRβ1*10011 presented peptide that contained the mutation while none of these T cells were activated by the wild type peptide. Thus, the favourable clinical outcome observed in Pt15392, may be correlated with a strong polyclonal CD4+ anti-tumour response. These data emphasise that continued monitoring of

CD4+ T cell immune responses in cancer patients may allow the identification of new class

II HLA-restricted antigens to be used for designing more effective cancer therapies.

A journey of a thousand miles begins with a single step. – Confucius

FORMAL DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institutes of higher learning, except where due acknowledgment has been made in the text.

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BIOGRAPHIC INFORMATION

The author initiated her academic career by receiving a Certificate in Chemical Engineering from Lund Institute of Technology, Sweden in 1994 after 2 years of full time studies. During this time her interest had turned towards biology and evolved to her graduation as Master of Science in Molecular Biology from Lund University, Sweden in year 1998. She performed her thesis at the department of Tumour Immunology at the Wallenberg Laboratory and since then her interest has remained in the field of cancer immunology.

LIST OF ABBREVIATIONS

Amp	ampicillin
APC	antigen presenting cell
bp	base pair
CIITA	class II trans activator
cDNA	complementary deoxy ribonucleic acid
CLIP	class II associated Ii peptide
CSIF	cytokine synthesis inhibitory factor
C/T	cancer/testis
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
dNTP	deoxyribonucleoside triphosphate
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot
ER	endoplasmatic reticulum
E:T	effector:target ratio
FCS	fetal calf serum
GFP	green fluorescent protein
HLA	human leukocyte antigen
HS	human serum
IC	intracellular compartment
iDC	immature DC
IFN-γ	inteferon - gamma
Ii	invariant chain
IL-10	interleukin - 10
kb	kilobase
LAK	lymphocyte activated killer
LCL	lymphoblastoid cell line
m	mutated
MIIC	MHC class II loading compartment
mAb	monoclonal antibody
MHC	major histocompatibiltiy complex
min	minute
MLTC	mixed lymphocyte tumour cell culture
MTS	melanosomal transport signal
oligo(dT)	oligonucleotide (deoxy-tyrosinase)
OVA	ovalbumin
p	p (probability) -value
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
rpm	rounds per minute
PCR	polymerase chain reaction
PTP	protein tyrosine phosphatase
RT-PCR	reverse transcriptase -PCR
S.D.	standard deviation
SEREX	serological analysis of recombinant cDNA expression libraries

TAA	tumour associated antigen
TAL	tumour associated lymphocytes
TAP	transporter associated with antigen processing
Taq	Thermus aquaticus (DNA polymerase)
TCR	T cell receptor
Th	T helper cell
TIL	tumour infiltrating lymphocytes
TNF-α	tumour necrosis factor - alpha
Tr	T regulatory cells
TRIS	tris[hydroxymethyl] aminomethane
TSTA	tumour specific transplantation antigens
U	units

OUTLINE OF THE THESIS

The overall aim of this thesis was to perform a thoroughly analysis of anti-melanoma specific CD4+ T cell clones involved in the anti-tumour response of a long time surviving melanoma patient (Pt15392). In particular, I aimed at better understanding the T cell mediated anti-tumour effector functions at a molecular level by the evaluation of the cytokine release by CD4+ T cells derived from a metastatic lymph node, and the antigen involved in this tumour recognition. In this context this thesis was aimed at answering the following questions:

- Which is the repertoire of T cells, recognising the metastatic melanoma *in vivo* in this patient?

- Which is the molecular nature of the antigens that presumably contributed to the positive clinical course observed for this patient?

Chapter 1

INTRODUCTION

Chapter 1 summerises the current state of the art in the field of tumour immunology related to the study performed in this thesis. In addition, each of the following result chapters is preceded by a more specific summary focused on addressing the question investigated in that chapter.

1.1 MELANOMA

1.1.1 What is a tumour?

Cancer was first described by the Greek word *oncos* meaning altered, relating to the changes of a normal cell that leads to malignancy (Figure 1.1). Today it is well known that cancer is a multi-step and multi-cause genetic disease which progresses to a metastatic phenotype gradually by accumulating diverse mutations and other genetic changes.

An established tumour is an autonomous unit of heterogeneous cells, which do not respond to the normal cellular signals that control growth and cell division. It respects only its own needs for survival and progression, as it develops from one changed single cell towards a metastatic phenotype. The ancestral cell gradually acquires additional changes causing mutations in key genes directing cellular processes, in particular genes that regulate growth and signalling (Figure 1.1). A solid malignant tumour mass contains many heterogonous cells with individual features: independent cells that are invasive, cells that have lost their antigen expression, cells that require fewer growth factors for survival and cells that are highly metastatic.

1.1.2 What is melanoma?

Malignant cutaneous melanoma is a form of cancer occurring after metastatic transformation of normal skin cells, i.e. melanocytes. These cells are lined in the epidermis (the top layer of the skin) and produce melanin, the brown pigment, in response to ultraviolet radiation. One special characteristic in the development of melanoma is that it
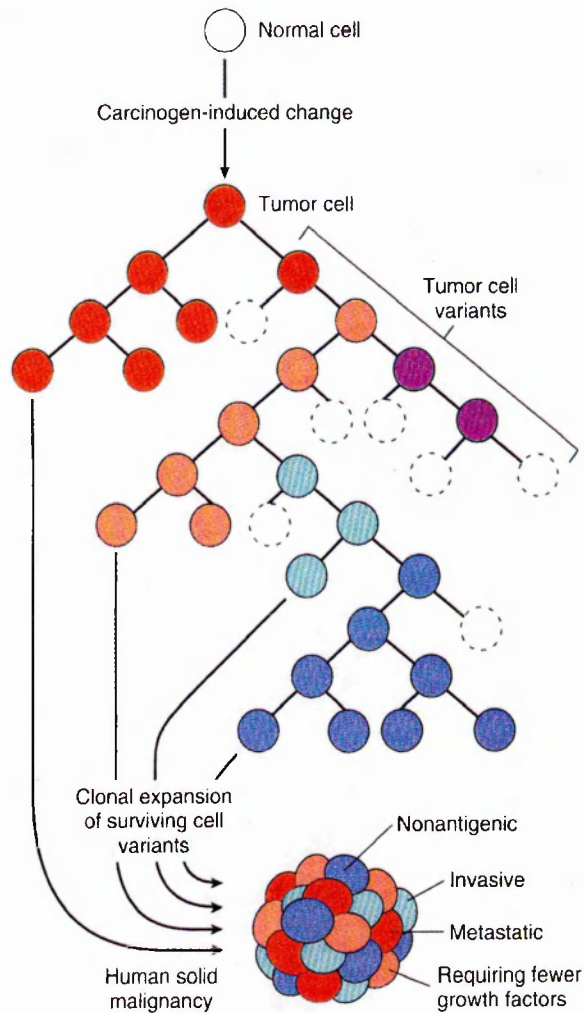
FIGURE 1.1: The multi-step pathway to tumour development. Cancer is a genetic disease that arise through the gradual accumulation mutations. The transformation process starts with a genetic alteration in one cell that become the precursor cell to all the subsequent more aggressive forms that develop by the acquisition of additional mutations in genes critical for growth regulation. The invasive metastatic tumour is composed of a heterogeneous array of cells with diverse features. This figure has been reproduced from Cotran *et al.*, 1997.

has two phases of growth: the initial horizontal growth phase, which can be followed by a vertical (i.e. invasive) growth phase. The second condition may lead to the formation of an autonomous malignant progression and finally the occurrence of metastasis. The main factors that predispose for melanoma are exposure to sun light, severe sunburns (especially during childhood), a fair phenotype with blond hair and light eyes, freckles, family history and atypical (dysplastic) nevi. Melanoma is the most aggressive form of skin cancer and although the death rate of malignant melanoma is only approximately 1%, the incidence of melanoma cases is among the fastest increasing world wide (reviewed in (Bastuji-Garin and Diepgen, 2002; Diepgen and Mahler, 2002)).

Primary melanoma is cured in over 80% of cases by surgery. However, once metastasized to distant organs, the cancer usually kills more than 90% of the patients since the tumour appears to be resistant to all availble anti-tumour drugs. New therapeutic approaches are thus urgently needed to improve such slim chance of survival for metastatic melanoma patients and imunotherapy represents one such possible new approach.

1.2 ANTIGEN PROCESSING

1.2.1 The HLA system

An antigen is defined as any substance that can induce a cellular or humoral immune response, since the immune system has evolved to fight disease agents. Antigens recognized by T cells are generally foreign proteins, derived from pathogens (bacteria, viruses) that have invaded the body. Depending on the physiological routes used to enter the body, antigens will follow separate pathways for the processing and presentation by the host cells: the endogenous or the exogenous pathway. However, independently from their origin, all antigens are presented to T cells as peptides bound to human leukocyte antigen (HLA) molecules.

The HLA system is crucial for the presentation of antigens. It is a highly polymorphic locus of approximately 200 genes located on chromosome 6 (reviewed in (Klein and Sato, 2000)). However, several of these genes are related to immunity and they are divided into a region encoding HLA-A, -B and –C alleles (class I HLA) and a region encompassing HLA-DR, -DP and -DQ alleles (class II HLA). The class I HLA molecules are composed of a heavy chain with three α-domains, α1 and α2 forming the peptide binding cleft and α3, an immunoglobulin-like domain, that is non-covalently bound to a light chain, β2m. Class II HLA molecules are instead heterodimers consisting of one α and one β chain, with the α1 and β1 domain of each chain forming the peptide binding cleft and the immunoglobulin-like α2 and β2 domains located in proximity to the membrane.

HLA class I molecules are expressed on all nucleated cells except spermatocytes (Jassim *et al.*, 1989), whereas the expression of class II HLA is restricted to antigen presenting cells (APCs) like dendritic cells (DCs), macrophages and B cells. The HLA molecules can

present peptides derived from endogenous as well as exogenous proteins: the class I HLA preferentially present endogenous peptides of 8 - 10 amino acids to CD8+ T cells while class II HLA present peptides of an average of 12 - 20 amino acids (mainly derived from exogenous proteins) to CD4+ T cells.

Endogenous antigens are synthesised by ribosomes on the endoplasmatic reticulum (ER) and then, as mature proteins, are released into the cytosol where they are processed to antigenic peptides and targeted to the cytosolic pathway for antigen presentation. In contrast, exogenous antigens are taken up from the extracellular environment and then degraded into peptides inside different intracellular compartments.

1.2.2 The endogenous (cytosolic) pathway for antigen presentation

All endogenously expressed proteins, including tumour antigens derived from normal or mutated self proteins become processed in the cytosolic pathway (Figure 1.2) and then presented on the cell surface in the groove of class I HLA molecules. In addition, RNA or DNA released by viruses can be replicated in the cell nucleus and translated to viral proteins for presentation to CD8+ T cells.

In the first step of this pathway (reviewed in (York and Rock, 1996)), ubiquitinin becomes attached to proteins and targets them for degradation in a barrel-shaped multi-catalytic complex, the proteasome. The resulting peptide fragments are then imported to the ER lumen through a pore complex formed by the transporter associated with antigen processing (TAP) proteins, TAP1 and TAP2, which allow an adenosin triphosphate (ATP)-dependent entrance of peptides (Hammond *et al.*, 1993; Neefjes *et al.*, 1993). Inside the ER, the peptides become modified to a suitable length to fit into the closed peptide binding groove of class I HLA molecules. The association of a peptide with the class I HLA heavy chain and the light beta 2 microglobulin (β2m) chain stabilises the complex, which
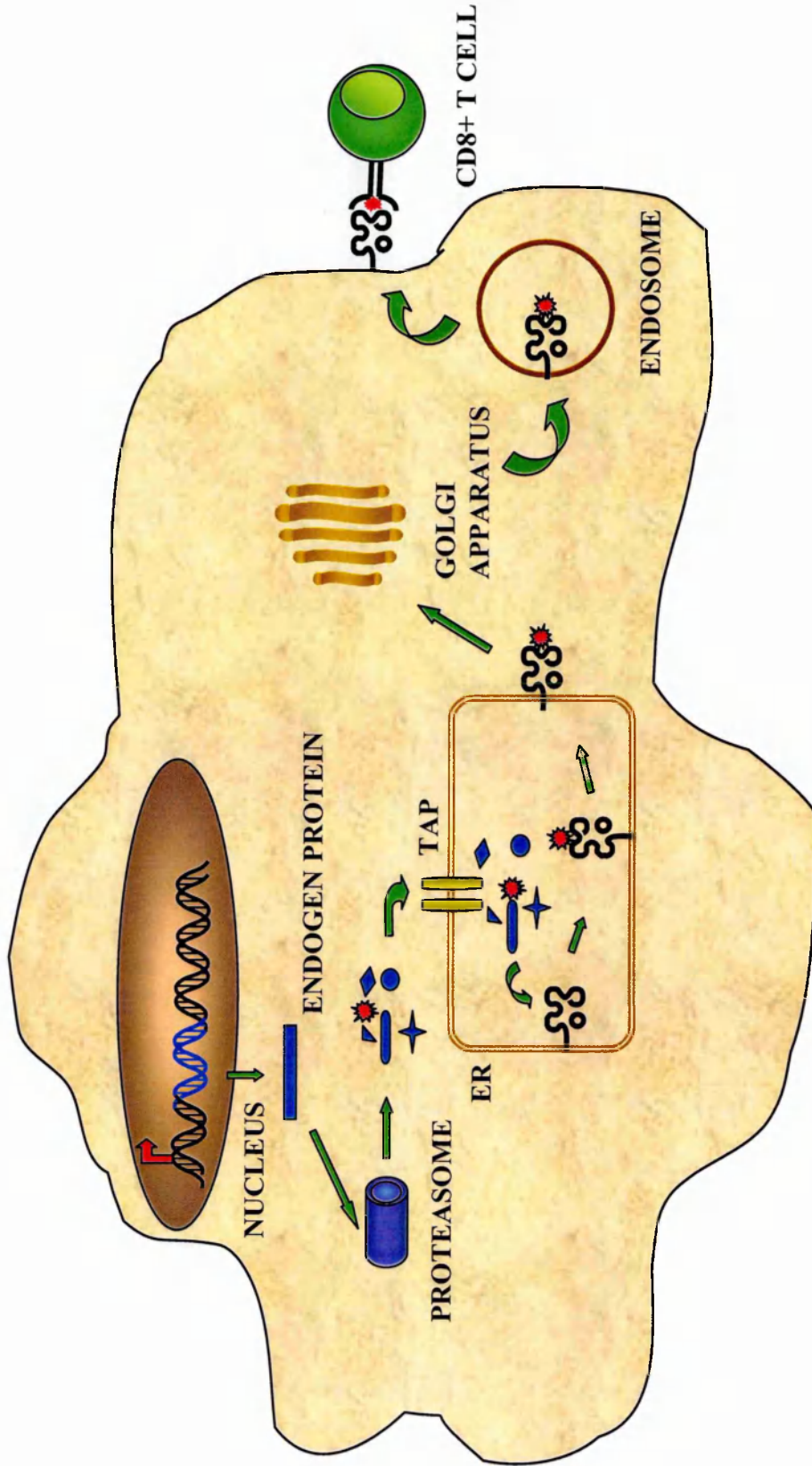
FIGURE 1.2: Class I HLA –dependent antigen processing. Endogenous antigens are constitutively expressed in the cytoplasm or can derive form RNA or DNA released from infective virus. These antigens become processed in a multicatalytic complex, the proteasome, after which the peptides are imported into the endoplasmatic reticulum (ER) through an ATP-dependent mechanism. Peptides become then loaded onto class I HLA molecules, which exit from ER and are transported through the Golgi apparatus before reaching the cell surface where they can be recognised by circulating CD8+ T cells.

becomes exocytosed from the ER and then exported to the cell surface through the Golgi network.

1.2.3 The endocytic pathway

CD4+ T cells are reactive against peptides from processed exogenous antigens in association with class II HLA (reviewed in (Cresswell, 1994; Pieters, 2000)). These exogenous antigens may be either soluble proteins released from inflammatory agents, pathogens or whole bacteria. The process of clearance and elimination of those invaders starts with their internalisation through macropinocytosis, phagocytosis or receptor-mediated endocytosis (reviewed in (Lanzavecchia, 1996)). In this way they have access to the endosomal/lysosomal system for HLA class II-dependent processing and presentation, where they become degraded along a path initiated in lightly acidic early endosomes, that gradually transform into endosomes, then to endolysosomes to finally end up in highly acidic lysosomes (reviewed in (Geuze, 1998)).

Different from the cytosolic pathway for endogenous antigen processing, the endocytic route is characterised by a separation of the degraded antigens and the class II HLA molecules in different intracellular compartments. The class II HLA molecules become synthesised and assembled in the ER, after which they associate with a chaperon protein, the Ii, and form a nonameric complex $(\alpha\beta Ii)_3$ composed of 3 pairs of class II HLA α and β chains, each pair associated with the Ii (reviewed in (Cresswell, 1996)) (Figure 1.3). This non-polymorphic protein has two functions. It prevents loading of endogenous peptides present inside the ER and of unrelated peptides later along the route, as the peptide binding groove of class II HLA molecules is occupied by a special part of the invariant chain, the class II-associated invariant chain peptide (CLIP). Moreover, the cytoplasmic tail of the Ii contains a signal sequence with two di-leucin-like motifs (Bakke and Dobberstein, 1990;
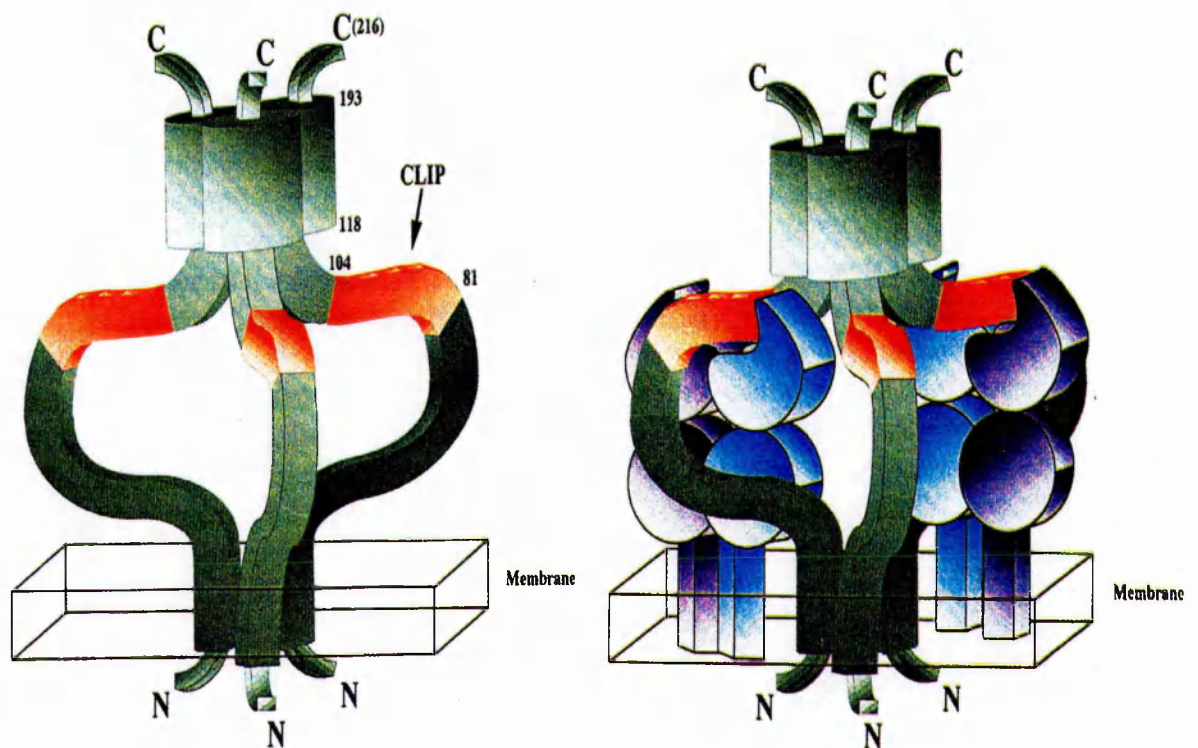
Figure 1.3: A schematic view of the 3-dimensional structure of the invariant chain. The invariant chain (Ii) is a chaperon protein involved the endogenous antigen presentation pathway. It contains a signal sequence in its cytoplasmic region that directs class II HLA molecules to the endoplasmatic reticulum, where they have associated with the Ii to form a nonameric complex $(\alpha\beta Ii)_3$. Another function of the Ii is to fold in such a way to occupy the groove of class II HLA molecules and prevent binding of irrelevant peptides before the complexes have reached the MHC class II peptide loading compartments (MIIC). During the passages through the endosomal/lysosomal system for antigen processing, the Ii gradually become degraded until only the part protecting the groove of class II HLA, the class II invariant chain peptide (CLIP), remains and can be exchanged for an exogenous derived peptide in a last step, before the molecules are routed to the cell surface. This figure has been reproduced from Cresswell *et al.*, 1996.

Odorizzi *et al.*, 1994), which direct the nonameric $(\alpha\beta Ii)_3$ complex to the endocytic pathway for antigen processing and presentation. The complex then enters the Golgi network, from where it becomes released as a vesicle into the endosomal/lysosomal system. This allows the paths for the peptide and $(\alpha\beta Ii)_3$ complex containing endosomes to converge. Thus, the peptides from the processed proteins become available to the class II HLA molecules first in a special intracellular compartments called MIIC (MHC class II containing compartment), where the peptide loading occurs. (Figure 1.4). However, it is still matter of debate if the peptide loading only takes place in the MIIC compartment. In fact, class II HLA have also been observed in intracellular compartments of DCs (Sallusto *et al.*, 1995) and, moreover, HLA-DR molecules can access directly to endosomes by direct internalisation from the cell surface (Roche *et al.*, 1993).

Regardless of where peptide loading occurs, it requires the assistance of the non classical HLA class II molecules, HLA-DM, -DO (Kropshofer *et al.*, 1998). They are involved in the exchange of the CLIP peptide with antigenic peptides. More precisely, the Ii has gradually been trimmed along the endocytic pathway until only the CLIP peptide remains when the complex has reached the MIIC compartment. Having fulfilled its protective role, it is removed with the assistance of HLA-DM as antigenic peptides present inside become loaded onto HLA class II molecules. The presence of a leucine motif in the β-chain of class II HLA directs HLA class II/peptide complexes to the cell surface (Zhong *et al.*, 1997), where they become visible for scanning by CD4+ cells.
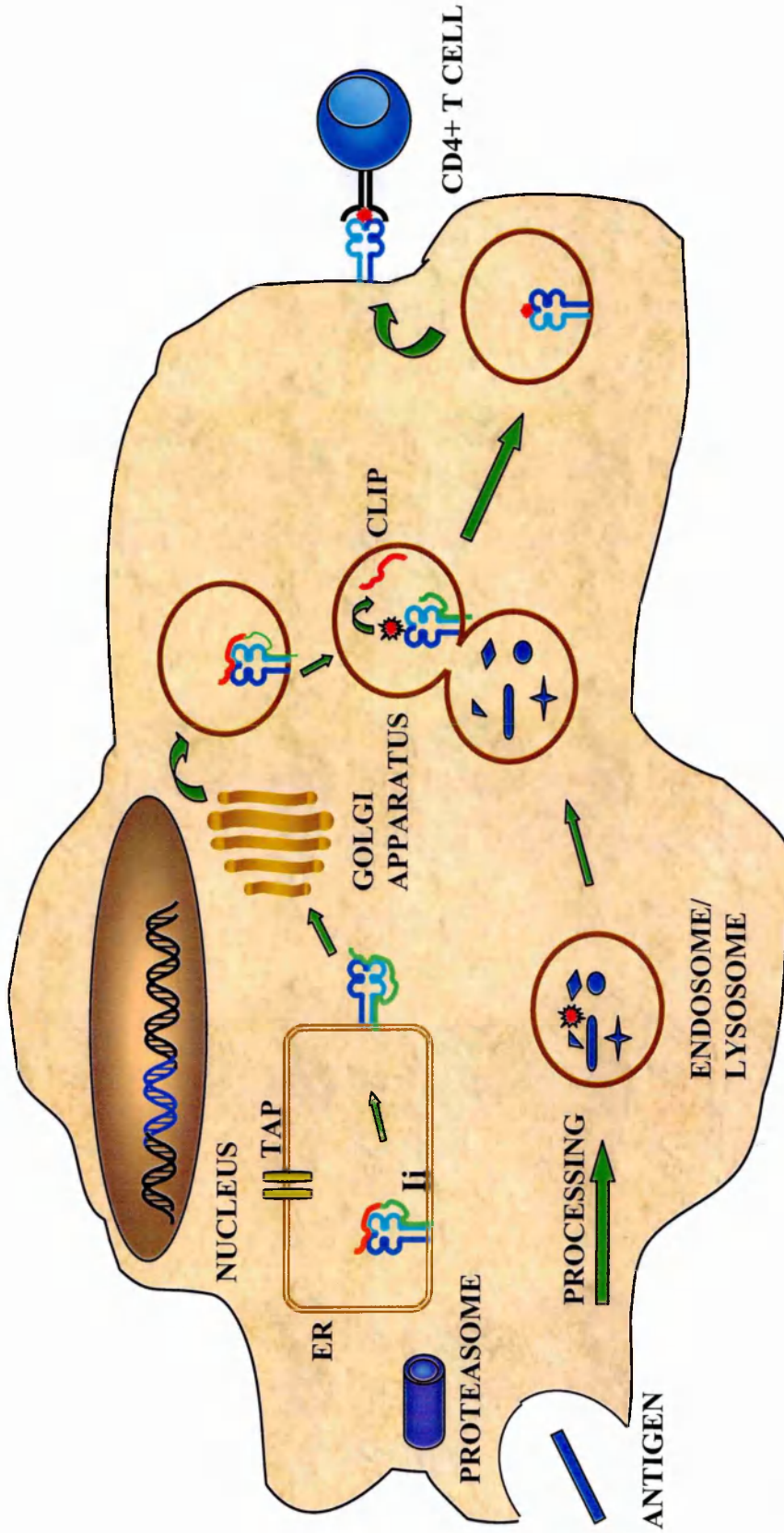
FIGURE 1.4: Class II HLA-dependent antigen processing:exogenous uptake. Exogenous antigens, entered into the cell by either macropinocytosis, phagocytosis or receptor mediated endocytosis, are degraded in a stepwise manner passing from slightly acidic endosomes to lysosomes with high pH. In parallel, the class II HLA complex assembles with the invariant chain (Ii) in the endoplasmatic reticulum (ER) and enroute through the Golgi network before it becomes released into endosomes. These two pathways then merge by the fusion of endosomes and the class II Ii peptide (CLIP) can be exchanged with peptides. The fully HLA/peptide complex can then traffic to the cell surface for then recognition by CD4+ T cells.

1.3 HUMAN TUMOUR ANTIGENS

1.3.1 Tumour antigens as targets of T cells

1.3.1.i   History of T cell defined tumour antigens

The first evidence that tumour could be subjected to immune recognition was obtained in the murine system. Studies performed on chemically induced fibrosarcomas clearly indicated that tumour cells expressed molecules that were perceived by the immune system as foreign proteins and towards which a strong and protective immune response could be generated.

Tumours induced by chemical treatment in mice could be transplanted in a syngeneic host where the tumour was then able to grow with no apparent control by the immune system. However, when tumour-bearing mice were surgically made tumour-free, these mice became resistant to a challenge with the same tumour but susceptible to a challenge by a different tumour (Prehn and Main, 1957). Additional experiments were then performed leading to the conclusion that the mechanism mediating the tumour rejection actively involved the T cell compartment of the host immune system. These data provided the first evidence that mice first encountering the tumours acquire the capacity to recognise tumour antigens uniquely expressed by that particular neoplasm (Basombrio, 1970). The observed characteristics of these antigens led to their designation as tumour-specific transplantation antigens (TSTAs). Thus, tumour antigens were first defined as antigens capable of specifically induce rejection of tumour transplants in syngenic mice. Similar results were obtained in other rodents like rats and guinea pigs.

Evidence for the existence of tumour antigens was also provided for tumours with different etiologies. Virally induced tumours were immunologically studied with an approach similar to the one employed for chemically induced tumours, and in this case, the antigens

mediating the rejection were directly encoded by the viral genome and thus were not intrinsic products of the tumour cell itself.

1.3.1.ii Spontaneously arising tumours

In contrast with the results obtained with chemically or virally induced tumours, spontaneously arising tumours of different histological origins were althogether poorly immunogenic inasmuch as they failed to confer protection against grafts of the parental tumour (Hewitt *et al.*, 1976). These data had such a profound effect in the field of tumour immunology that the existence of tumour antigens in tumours not deliberatley induced with high doses of chemical carcinogens was doubted. However, two different lines of research led to the conclusion that immunogenic and non immunogenic tumours had indeed similar features. In fact, the *in vivo* growth of immunogenic tumours could eventually lead to the *in vivo* selection of growing cells that were no longer immunogenic (Parmiani *et al.*, 1973). On the other hand, starting from spontaneous, non-immunogenic tumours, immunogenic variants, unable to growth in syngeneic hosts were generated by chemical treatment. These tumour lines were named ' tum- ' since they were unable to grown in the syngeneic host (De Plaen *et al.*, 1988). However, these tum- variants were able to induce tumors in irradiated mice indicating that rejection in the normal syngeneic host was mediated by an immune response. An additional indication about their immunogenicity came from experiments in which mice that had been injected with tum⁻ variants were clearly able to reject the parental, tum+ cells.

The main conclusion obtained from these data was that although derived from non-immunogenic tumours, tum- variants and also the parental tumour from which they derived, indeed expressed transplantation antigens, mediating the immunological rejection. On the basis of these results it could be hypothesised that a similar situation could also be

found in humans and that antigens do exist also in human tumours which, however, may be weakly immunogenic (reviewed in (Boon *et al.*, 1989)).

Tumour antigens were first defined on their capability of mediating rejection of tumour transplants in mice; hence they were called tumour specific transplantation antigens. Although available data indicated that T cells were involved in such a rejection, no hints were available concerning the molecular nature of these tumour antigens nor the mechanism allowing their T cell mediated recognition. Initially, the prevailing idea was that T cells recognized a structural part of membrane bound cell surface proteins, as neither the existence of MHC molecules nor the intracellular antigen presentation pathways were elucidated. The interaction between T cells and their nominal antigens was thought to be similar to that occurring between antibodies and antigens. This concept shifted drastically after the discovery of MHC molecules and their function in the 70-ies. The realisation that MHC molecules were working as restriction elements in immune responses, drastically shifted the scientific perspective and highlighted the importance of cell mediated recognition (reviewed in (Zinkernagel and Doherty, 1997)).

Another significant factor that favored antigen identification was advancements in cell culture, which showed that T lymphocytes could be maintained as *in vitro* cultures in the presence of their growth factor, IL-2. Thereby, it became possible to set up *in vitro* models for antigen recognition. However, conclusive data demonstrating that T cells recognise their "nominal" antigen as a MHC molecule-peptide complex was not provided until a decade later in a now milestone paper by Townsend and co-workers. They were able to show that class I MHC-restricted anti-flu T cells killed infected cells through the recognition of a nucleoprotein, implying that nucleoprotein derived peptides produced by the internal protein processing were displayed at the cell surface by MHC (Townsend *et*

al., 1985; Townsend et al., 1986). This observation has formed the present concept of T cell defined tumour antigens, which has become a hallmark for tumour immunology. Then the first gene encoding a mouse tumour antigen was cloned by the group of Thierry Boon (Lurquin et al., 1989). A period of 6 years followed until this result was confirmed in human by the cloning of the first melanoma antigen recognised by T cells (van der Bruggen et al., 1991). Melanoma became the most studied human tumour, as melanoma cells proved to be relatively easy to adapt to in vitro culture in respect to cells from histologically diverse cancers. With the availability of melanoma cell lines and autologous PBMCs present in the blood of cancer patients or tumour-infiltrating (associated) lymphocytes (TILs, TALs) in vitro generation and expansion of tumour-specific T cells was possible; eventually this in vitro stimulation also led to the isolation of tumour-specific T cell lines and T cell clones. Such expanded tumour specific clones have been shown to be the keytools to define tumour antigens recognized by T cells.

In addition to T cell-defined antigens, a recent approach, known as serological analysis of recombinant cDNA expression libraries (SEREX) (reviewed in (Tureci et al., 1997)), that takes advantage of the use of cancer patient's sera led also to the definition of antigens able to induce an antibody mediated response in an autologous setting. The present thesis will focus on T-cell defined tumour antigens.

1.3.2 Classification of T cell defined tumour antigens

1.3.2.i The nature of tumour antigens

In the last decade, an increasing array of T cell defined antigens have been continuously cloned using more sophisticated and innovative molecular techniques. These tumour antigens have been divided into four different groups according to their histological

expression (reviewed in (Renkvist *et al.*, 2001; Van den Eynde and Van der Bruggen, 2001)) (Figure 1.5).

1.3.2.ii   Group 1. Class I HLA-restricted cancer/testis antigens

A milestone in tumour immunology was certainly the cloning of *MAGE-1* (van der Bruggen *et al.*, 1991) and the subsequent characterization of the first T-cell-defined antigenic epitope a year later (Traversari *et al.*, 1992). Those findings were rapidly followed by the identification of new members within this group (Boel *et al.*, 1995; Van den Eynde *et al.*, 1995). The *MAGE, BAGE* and *GAGE* families of genes were added to the group. The antigens belonging to this category, now including also NY-ESO-1, were called cancer/testis (CT) antigens owing to their expression in histologically different human tumours and in normal tissues including spermatocytes and spermatogonia of testis and occasionally in placenta. These antigens now represent one of the main components for anti-tumour vaccine development. CT antigens result from reactivation of genes normally silent in adult tissues (De Plaen *et al.*, 1994), that are transcriptionally activated in some tumors (De Smedt *et al.*, 1997). Their expression in testis does not provide targets for an immune reaction because cells of testis do not express class I HLA on their cell surface (Jassim *et al.*, 1989). Despite the fact that the CT antigens are probably the most characterised tumour antigens, their physiological function remains largely unknown.

Considering that several new genes in the CT group of antigens have been cloned (CT9 (Scanlan *et al.*, 2000), CT10 (Gure *et al.*, 2000), LAGE (Lethe *et al.*, 1998), MAGE-B5, -B6, -C2, -C3 and-D (Lucas *et al.*, 1999; Lucas *et al.*, 2000), HAGE, SAGE (Martelange *et al.*, 2000)) and that no T-cell epitopes have yet been identified from them, the question arises as to how many more genes encoding CT antigens remain to be discovered and how many epitopes exist that could be of use in cancer immunotherapy. Furthermore, a new

FIGURE 1.5: The classification of tumour antigens. Human tumour antigens have been classified into 4 groups according to their tissue expression. Group 1 includes cancer/testis antigen, which have a shared expression among tumours. The MAGE, BAGE and GAGE families belong to this group. Group 2 is characterised by antigens expressed only in normal and neoplastic cells of the same origin, i.e. differentiations antigens. They are best described in melanoma and melanocytes, although some prostate specific antigens also have been identified. Group 3 represents antigens with a wide expression in normal as well as neoplastic tissues, while Group 4 includes antigens that are unique to one patient. Such antigens arise from point mutations. The full list of these antigens can be found at http://www.istitutotumori.mi.it.

MELANOMA ANTIGENS

I) CANCER/TESTIS ANTIGENS

http://www.istitutotumori.mi.it

MAGE, BAGE, GAGE, DAM, NY-ESO-1

CANCER I   CANCER II   CANCER III

Ex: melanoma   breast cancer   lung cancer

II) DIFFERENTIATION ANTIGENS

Melan-A/MART-1, gp100, Tyrosinase,Trp-1, Trp-2

MELANOMA   MELANOCYTES

III) WIDELY EXPRESSED ANTIGENS

CAMEL, HER2/neu, PRAME, P15, RU1, RU2

IV) UNIQUE ANTIGENS

β-catenin, CDK-4/m, GnT-V/m, Myosin/m, MUM-1(-2,-3)

1 PATIENT
1 TUMOR
1 ANTIGEN

MAGE-E1 gene has been found (Sasaki *et al.*, 2001). In fact, numerous members of the MAGE gene family have been identified recently and it now contains 12 subfamilies, MAGE-A to MAGE-L2, including 60 different genes of which most belongs to the MAGE-A, -B and -C groups (Chomez *et al.*, 2001).

1.3.2.iii Group 2. Class I HLA-restricted differentiation antigens

Differentiation antigens are only expressed at a defined stage of development and then they have a shared expression between tumours and the normal tissue from which the tumour arose. Most are found in melanomas and normal melanocytes (Anichini *et al.*, 1993). Many of these melanocyte lineage-related proteins are involved in the biosynthesis of melanin. Epitopes recognized by both CD8+ and CD4+ T cells can be derived from melanosomal proteins (Boon and van der Bruggen, 1996; Topalian *et al.*, 1996; Wang *et al.*, 1999a; Wang *et al.*, 1999b).

1.3.2.iv Group 3. Class I HLA-restricted widely expressed antigens

Genes encoding widely expressed tumour antigens have been detected in many normal tissues as well as in histologically different types of tumours with no particular bias towards a certain type of cancer. It is likely that the many epitopes expressed on normal tissues are below the threshold level for T-cell recognition, while their overexpression in tumour cells can trigger an anticancer response possibly by breaking a previously established tolerance. These widely expressed gene products have revealed a broad spectrum of mechanisms that are involved in generating T-cell-defined epitopes through alterations in gene transcription and translation. To highlight some examples, the epitope of iCE (Ronsin *et al.*, 1999) is derived from a non-AUG-defined alternative open reading frame (ORF) (Aarnoudse *et al.*, 1999), while the RU2 gene creates its epitope by reverse strand transcription (Van Den Eynde *et al.*, 1999).

1.3.2.v   Group 4. Class I HLA-restricted, tumour-specific antigens

Unique tumour antigens arise from point mutations of normal genes (like β-catenin, CDK4) (Robbins *et al.*, 1996; Wolfel *et al.*, 1994), whose molecular changes often accompany neoplastic transformation or progression. These antigens are thus expressed only in the individual tumour where they were identified since it is unlikely that the same mutation may occur in two different neoplasms unless it involves genes (e.g. RAS and p53) whose alteration is an obligatory step in neoplastic transformation.

In mouse models unique antigens have been shown to be more immunogenic than the other groups of shared antigens (Dudley and Roopenian, 1996); since unique antigens are responsible for the rejection of tumour transplants in mice, they have been defined as tumour-specific transplantation antigens (TSTAs). The unique antigens are the most specific targets for immunotherapy, but this potential advantage must be balanced against the difficulty of their clinical use, as they can induce an immune response only against the original tumour in which they were found and their identification is cumbersome.

Only few tumour-specific but shared antigens have been described which are generated by alteration in splicing mechanisms which occur in tumours but not in normal cells, as in the case of TRP-2/INT2 (Lupetti *et al.*, 1998).

1.3.2.vi   Class II HLA-restricted antigens

Stimulation of the CD4+ T helper cells by tumour antigens is considered to be impaired or absent in cancer patients and this may be the reason for an insufficient immune response to tumours. Therefore, the identification of tumour antigen epitopes recognized by such lymphocytes is a crucial step in the long sought improvement of anti-tumour immune responses that may result in clinical efficacy. The first epitope presented by a class II HLA and capable of provoking a CD4+ T-cell response was identified in 1994 in melanoma

tyrosinase (Topalian *et al.*, 1994). Then a gap of 4 years followed during which only one additional epitope was characterized (Topalian *et al.*, 1996), before other genes encoding class II-restricted peptides were discovered. However, as the technical and methodological approaches for identifying CD4+ T-cell epitopes of tumour antigens have become available, an exponential increase in reporting such epitopes has been seen. In fact, since 1998 as many as 27 new class II HLA-restricted epitopes from 14 antigens have been molecularly identified using, among others, Ii-cDNA fusion libraries (Wang *et al.*, 1999a), immunized transgenic mice (Zeng *et al.*, 2000) and biochemical approaches (Pieper *et al.*, 1999).

In conclusion, class II HLA restricted epitopes have been identified from several well known anitgens such as MAGE, Melan-A/MART-1, NY-ESO-1, gp100 and tyrosinase (Table 1.1). Moreover, it is noteworthy that the classification of class I HLA-restricted tumour antigens into 4 groups i.e. CT antigens, differentiation antigens, widely expressed antigens and unique antigens, seems possible also for class II HLA defined antigens. Some antigens, annexin II (Li et al., 1998); HPV-E7 (Hohn et al., 1999); CDC27/m (Wang et al., 1999a); TPI/m (Pieper et al., 1999), EphA3 (Chiari et al., 2000) and fibronectin (Wang et al., 2002) have only been defined by class II HLA epitopes. Considering the wide and heterogeneous array of cloned tumour antigens restricted by class I HLA, it is likely that the group of class II HLA antigens will be extended.

Table 1.1: Class II HLA restricted antigens. An overview of class II HLA restricted antigens classified into the same 4 groups as defined for class I HLA antigens.

GENE	HLA allele	REFERENCE
CANCER/TESTIS ANTIGENS		
MAGE-A1	DR15	(Chaux et al., 2001)
MAGE-A1, -A2, -A6	DRB*1301, DRB*1302	(Chaux et al., 1999a)
MAGE-A3	DRB1*0401, DRB1*0402	(Schultz et al., 2000)
MAGE-A3	DR*1101	(Manici et al., 1999)
MAGE-A3	DRB*1301, DRB*1302	(Chaux et al., 1999b)
NY-ESO-1	DRB4*0101	(Zeng et al., 2000)
NY-ESO-1	DRB4*0101-0103	(Jager et al., 2000b)
DIFFERENTIATION ANTIGENS		
Melan-A/MART-1	DRB1*0401	(Zarour et al., 2000)
Gp100	DRB1*0401	(Li et al., 1998)
Gp100	DR7	(Kobayashi et al., 2001)
Gp100	DR53, DQw6	(Kobayashi et al., 2001)
PSA	DR4	(Corman et al., 1998)
Tyrosinase	DRB1*0401	(Topalian et al., 1994) (Topalian et al., 1996)
Tyrosinase	DRB1*1501	(Kobayashi et al., 1998a)
Tyrosinase	DRB1*0405	(Kobayashi et al., 1998b)
WIDLEY EXPRESSED ANTIGENS		
EphA3	DRB1*1101	(Chiari et al., 2000)
HER2/neu	DR1, DR4, DR52, DR53	(Kobayashi et al., 2000)
MUC1	DR3	(Hiltbold et al., 1998)
UNIQUE ANTIGENS		
HPV-E7	DR*0401, DR*0407	(Hohn et al., 1999)
CDC27/m	DRB1*0401	(Wang et al., 1999a)
Fibronectin/m	DR2	(Wang et al., 2002)

TPI/m	DRB1*0101	(Pieper *et al.*, 1999)
[1]Annexin II	DRB*0401	(Li *et al.*, 1998)

1) As annexin II has been detected in melanoma, while its tissue distribution in normal tissues has not been analysed, there is not enough information to classify this antigen.

The prevailing view of antigen based immunotherapy of cancer, supported by numerous observations during the last decade, has been discussed in (Coulie *et al.*, 2001; Parmiani *et al.*, 2002a; Rosenberg, 2001) and can be summarised as follows:

(i) Cancer antigens can be divided into 4 different groups based on their tissue expression (reviewed in (Renkvist *et al.*, 2001; Van den Eynde and Van der Bruggen, 2001)).

(ii) One antigen can contain several epitopes, sometimes restricted both to class I and class II HLA (e.g. MAGE-3 and tyrosinase).

(iii) In a few cases, one specific HLA allele may show restriction for more than one epitope, as for NY-ESO-1 (Jager *et al.*, 2000b).

(iv) Neoplasms can have a heterogeneous display of multiple tumour antigens (Brasseur *et al.*, 1995; Dalerba *et al.*, 1998; Van den Eynde *et al.*, 1989).

(v) Despite their self origin, tumour antigens can mediate immune responses (reviewed in (Parmiani, 1993)).

1.3.3 Melanoma antigens

Melanoma antigens include the largest number of molecularly characterised TAAs. Among these, the MAGE (Traversari *et al.*, 1992), BAGE (Boel *et al.*, 1995) and GAGE (Van den Eynde *et al.*, 1995) families, are considered as prototype CT antigens. In these groups, 16

antigens, together encoding almost 30 HLA class I epitopes, have been identified, the majority of which occur in the MAGE group, which encodes for no less than 25 epitopes for CTL recognition: of these 11 belong to MAGE-A1 and 8 to MAGE-A3. However, it must be remembered that much attention has been focused on the cloning of MAGE and, therefore, it may be incorrect to assume that they dominate the antigen repertoire since new antigens and epitopes are continuously being described. Nevertheless, they were the first antigens used for *in vivo* vaccination (Marchand *et al.*, 1995).

The differentiation antigens of melanoma are encoded by at least six different genes (Melan-A/MART-1, gp100, tyrosinase, TRP-1/-2 and MC1R), but numerous epitopes have been identified. A good example is given by gp100 with at least 16 reported epitopes mainly restricted by HLA-A2. In fact, approximately 90% of the almost 40 epitopes in this group are presented by HLA-A alleles. One of the reasons why few HLA-B and -C restricted antigens have been identified, may lie in a technical artifact, i.e. the fact that T cells with TCRs recognizing HLA-A alleles are more easily expanded *in vitro* as compared to T cells recognizing epitopes restricted by other alleles.

At present, a similar number of melanoma antigens have been described among widely expressed and unique antigen groups. Also with these TAAs, the marked tendency is the dominant restriction of the HLA-A alleles, preferentially HLA-A2, which is the most widely expressed allele among the Caucasian population (Fleischhauer *et al.*, 1996; Krausa *et al.*, 1995). Thus, the majority of these TAA epitopes are restricted by HLA-A. Whether this reflects a bias caused by the fact that most of the studies have been carried out with HLA-A-restricted T cells or is due to an immunodominant role of the HLA locus in recognition of tumour antigens remains to be established.

1.4 THE IMPORTANCE OF IDENTIFYING NEW TUMOUR ANTIGENS

Remarkably, in the last decade TAAs have been identified from 84 genes together encoding 213 epitopes; of these 160 restricted by class I HLA, 30 by class II HLA and 23 originate from fusion proteins (reviewed in (Renkvist *et al.*, 2001; Van den Eynde and Van der Bruggen, 2001)) with new antigens being continuously cloned in a seemingly infinite number. So, "Why is it necessary to identify new human TAAs?".

First, an endeavour should be made to clone antigens belonging to cancers other than melanoma, not only to gain knowledge on TAAs, but because other tumours such as cancer of breast, lung, liver, stomach, colon and pancreas affect a higher number of subjects than melanoma does. An increased knowledge on these cancers particularly of those known to be quite resistant to standard therapies like lung, colon, pancreatic and ovarian cancers, may lead to the development of clinical immunotherapeutic approaches, from which large groups of patients could benefit.

Second, although anti-tumour activity has been observed against Melan-A/MART-1, gp100 and tyrosinase in *in vitro* studies (D'Souza *et al.*, 1998; Jager *et al.*, 1996a; Marincola *et al.*, 1996b; Rivoltini *et al.*, 1995b), the clinical efficacy of these antigens remain uncertain (reviewed in (Coulie *et al.*, 2001; Parmiani *et al.*, 2002a)). In fact, despite that an increased T cell frequency could be induced in patients vaccinated with epitopes derived from these TAAs, tumour regression was only occasionally observed (Cormier *et al.*, 1997; Jager *et al.*, 2000a; Lee *et al.*, 1999a), reviewed in (Parmiani *et al.*, 2002a).

Another obstacle in tumour immunotherapy is the presence of antigen specific anergic T cells in patients with tumour progression (reviewed in (Nielsen and Marincola, 2000)). One explanation for this "non-responding" state of the T cells, may be that anergy has been

induced, a phenomenon observed for MART-1$_{27-35}$ and Tyr$_{368-76}$ specific CD8+ T cells in melanoma patients (Lee *et al.*, 1999b).

Thus, an increased number of molecularly characterised tumour antigens will probably contribute to an increased knowledge about their role in transformation and tumour progression. In addition, such knowledge will extend the information regarding binding motifs and the number of epitopes that can be made avaibable for multi-vaccine approaches.

A more detailed understanding of the mechanisms involved in an anti-tumour response, may reveal new targets and molecular pathways important for tumour progression. Such information may lead to the development of novel strategies that can counteract tumour formation at an early stage, diverse from the usual approaches aimed at eliciting strong T cell responses against already established tumours.

1.5 FROM ANTIGEN DISCOVERY TO IMMUNOTHERAPY

1.5.1 The present concepts

Given the availability of so many TAAs, we must ask the following question: which antigens and epitopes are most suitable for immunotherapy, being capable of provoking a long lasting and effective anti-tumour immune response *in vivo*?

It has been proposed that antigens involved in driving the oncogenic process should represent immunogenic targets for vaccine development. Unique antigens generated by point mutations in human tumour cells seem to fall into this group, for example CDK-4/m (Wolfel *et al.*, 1995), β-catenin (Robbins *et al.*, 1996) and caspase-8/m (Mandruzzato *et al.*, 1997) have all shown to affect critical cellular processes as cell cycle regulation and apoptosis. In addition, several unique antigens: CDK-4/m (Wolfel *et al.*, 1995), MUM-1

(Coulie *et al.*, 1995), MUM-2 (Chiari *et al.*, 1999) and MUM-3 (Baurain *et al.*, 2000), have been described in patients with favourable clinical outcomes or, as in the case of myosin/m (Zorn and Hercend, 1999b), in a spontaneously regressing melanoma. Collectively, these data may imply that only the mutated antigens are the "true" rejection antigens (TSTAs) known from murine studies and that T cells recognising such TAAs could mediate tumour regression. However, the clinical applicability of these antigens appears limited due to their unique nature, necessitating a single patient tailored immunotherapeutic approach.

A strongly debated issue is the possible use of self-antigens in immunotherapy. Self-antigens, i.e. those encompassing normal proteins (e.g. Melan-A/MART-1 or MAGE), can elicit an immune response with the assistance of appropriate adjuvants. Furthermore, as they are expressed by a large set of tumours, their clinical relevance is considered to be higher than that of unique antigens (reviewed in (Gilboa, 1999b)). Therefore, efforts should be aimed at finding TAAs containing both class I and class II HLA epitopes that are not restricted to only one cancer type and that can provoke an anti-tumour immune response against a wide array of cancers. Telomerase could thus be one possible example of this TAA (Vonderheide *et al.*, 1999).

The time has come to find the second generation of TAAs; antigens identified by novel techniques such as microarray, algorithm studies etc., antigens with relevant role in tumour induction and progression (reviewed in (Schultze and Vonderheide, 2001)), and therefore, that cannot be selected against by the immune system of the host.

1.6 METHODS FOR IDENTIFICATION OF TUMOUR ANTIGENS

1.6.1 Genetic approach

The pioneering technique that allowed the identification of human tumour antigens combined the construction of a tumour-derived cDNA library with the availability of T lymphocyte clones. This has become the classical method for the identification of TAAs and is based on the construction of a cDNA library from tumour derived mRNA (Figure 1.6A). The cDNA is cloned into eukaryotic expression vectors and divided into pools of 100 bacteria carrying statistically 100 independent cDNA vectors (reviewed in (Boon, 1993)). These cDNA pools are co-transfected into recipient cells such as COS-7 or 293 together with the DNA coding for the HLA allele mediating the tumour recognition. The library screening is performed by the addition of tumour reactive T cells to the transfected COS-7 cells, and T cell recognition of a tumour antigen is then measured by evaluating the cytokine content, normally IFN-γ and TNF-α, of the supernatants from the transfectants by enzyme linked immuno sorbent assay (ELISA). The positive cDNA pool or pools are subsequently divided into smaller fractions, for which the screening is repeated until individual cDNAs encoding the antigen have been targeted.

1.6.2 Biochemical approach: Elution from HLA molecules

Tumour epitopes can be also identified by a biochemical approach, where the HLA molecules are stripped of their bound peptides by acid buffer treatment and the extracted peptides are fractionated on reverse phase high pressure liquid chromatography (HPLC) columns (Cox et al., 1994) (Figure 1.6B). The fractionated peptides are then assessed for their ability to sensitise appropriate APC to anti-tumour T cell clone recognition. The fractions that appear positive by ELISA are selected for a second round of chromatography, after which the complexity of peptides contained in the positive fraction
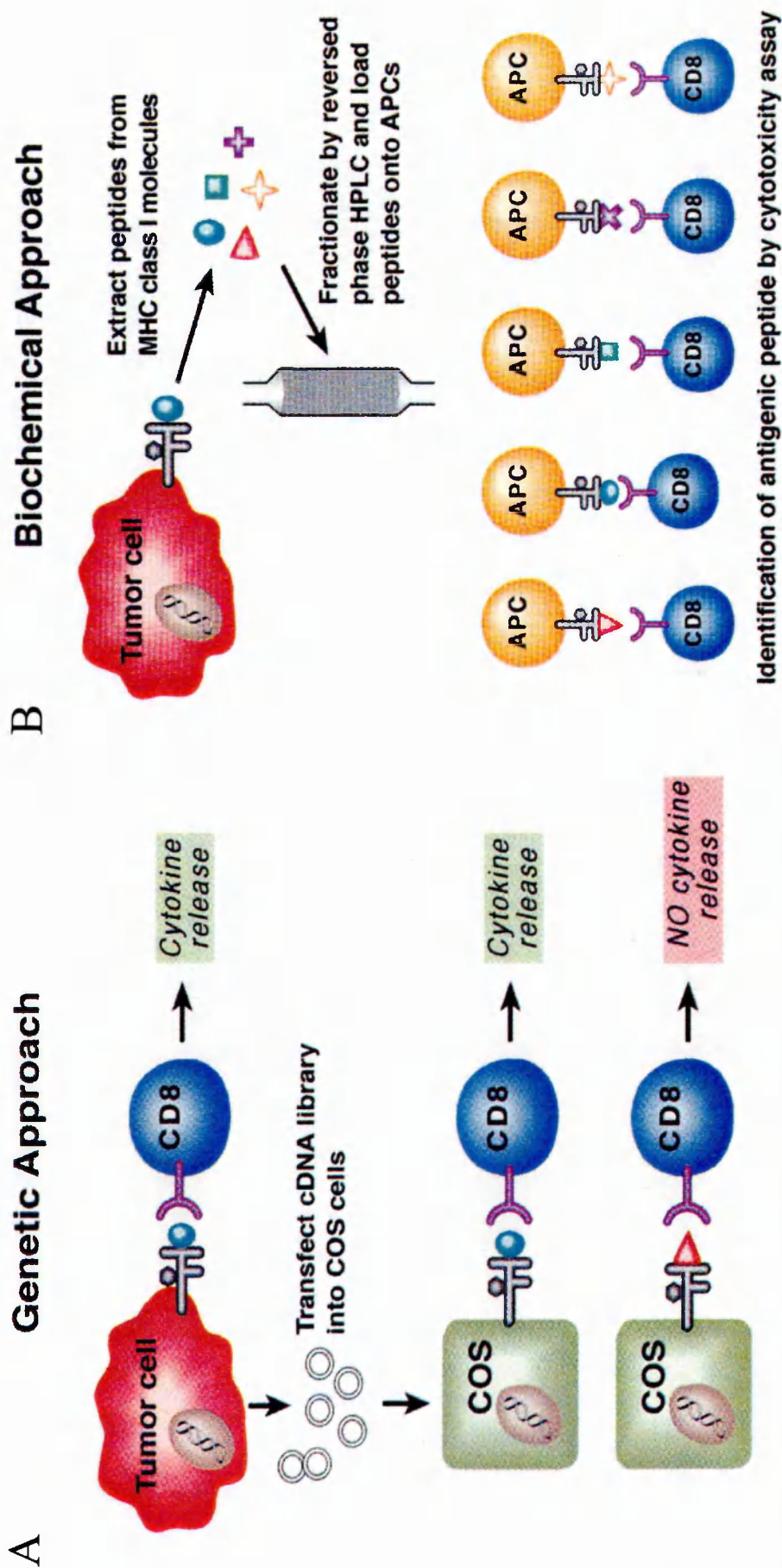
FIGURE 1.6: Methods for antigen cloning (I). (A) Antigens were originally cloned by a genetic approach in which T cells were used to screen for recognition of a tumour derived cDNA library transfected into recipient cells like COS-7. (B) Naturally processed antigens can be derived from acid elution of peptides bound to HLA molecules expressed on the cell surface. The peptide pool then become fractionated by high performance liquid chromatography (HPLC) techniques and the positive peptide peaks evaluated for recognition by T cells. This figure been reproduced from Greten *et al.*, 1999.

can be further dissected by mass spectrometry thereby leading to the identification of single immunogenic peptides. By applying sophisticated mass-spectrometry analysis, the sequence of the immunogenic epitope can be molecularly defined. From the amino acid sequence, the immunogenic protein can be identified from protein data bases.

This method requires that an adequate amount of the peptides must be present on the cell surface to allow identification. Although this method is somewhat cumbersome as it includes the use of HPLC and mass spectrometry, it has the advantage that the defined antigen is naturally expressed on the surface of tumour cells, thus increasing the probability to be recognised by circulating T cells or antibodies.

1.6.3 SEREX

Tumour antigens can be identified using antibodies by a technique called serological analysis of recombinant cDNA expression libraries (SEREX), that has developed from of the classical method for library screening with T cells (reviewed in (Tureci *et al.*, 1997)). Fresh tumour specimens are used to construct a cDNA library, which is then packed into λ-phage expression vectors and transfected into *E. coli* cells. Recombinant proteins are released during the lytic phase of the λ-phage growth and are then blotted onto nitrocellulose membranes, where they can be screened for reactivity against high-titre IgG antibodies present in the antibody repertoire of the autologous cancer patient (Figure 1.7A). Plaques that express reactive proteins are visualised by a staining with a secondary enzyme-conjugated anti-human IgG antibody. In a last step, the single clone encoding the serological identified tumour protein is subcloned and the insert sequenced.

The advantage of this method is that it allows the identification of antigens with strong immunogenicity, as high-titer IgG antibodies are selected from diluted serum and artefacts,
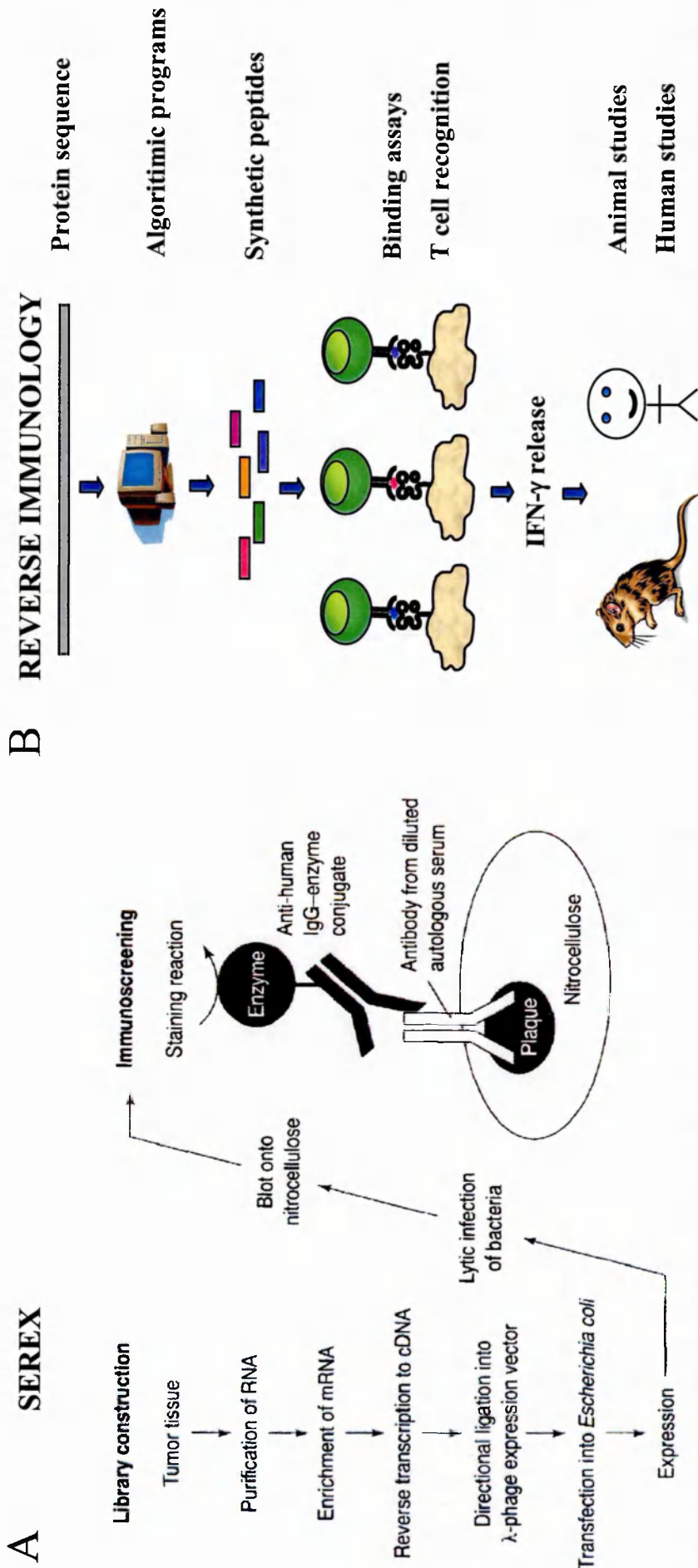
FIGURE 1.7: Methods for antigen cloning (II). (A) Antigens can be cloned by evaluating their humoral immune response in an autologous setting by a method called serological analyses of recombinant cDNA expression libraries (SEREX). Briefly, a tumour derived cDNA library can be inserted into a λ phage expression vector and then transfected into *E.coli* cells. Recombinant proteins can be detected by IgG antibodies derived from the serum of the same patient. (B) A protein with an important function can be screened for the presence of putative T cell defined peptides by the use of computer based algorithm programs. Peptides with high binding affinity for a selected HLA-allele can then become assessed for their ability to induce IFN-g release from tumour specific T cells. This approach is called reverse immunology. Figure 1.7A has been reproduced from Tureci *et al.* 1997.

such as loss of neoexpression of genes from *in vitro* culture of tumour lines, are bypassed. However, one limitation is that only antigens that induce antibody responses in the patient at the time of serum collection can be identified.

1.6.4 Reverse immunology

Candidate peptides with immunogenic potential inside a given protein can be predicted using the theoretical information of binding motifs and cleavage specificities for governing the rules of antigen processing and presentation. (Figure 1.7B). This has been possible with the development of mathematical algorithms that can predict putative peptide targets for T cell recognition based on their HLA binding affinity (Lu and Celis, 2000), for example the SYFPEITHI database (http://www.uni-tuebingen.de/uni/kxi/).

Reverse immunology represents a method that has the advantage of pre-selecting important antigens, which are known to be associated with a particular cancer and then searching them for their potential epitopes. The predicted peptides will finally be evaluated for their capacity to induce *in vitro* peptide-specific and tumour-specific T cells.

1.7 T CELL MEDIATED ANTI-TUMOUR IMMUNITY

1.7.1 Classification of T cells

1.7.1.i CD8+ and CD4+ T cells

The continuous exposure to an infinite number of pathogenic micro-organisms and disease causing agents imposes a potential danger to our body. In order to overcome this challenge our immune system has evolved protective barriers. The first line of defence is non-specific and involves granulocytes, mast cells, macrophages and NK cells (innate immunity), while T lymphocytes play a central role in specific cellular responses, i.e. in acquired or adaptive immunity. CD8+ cytotoxic T lymphocytes (CTLs) directly kill virus infected target cells by recognition of class I HLA presented viral peptides, while CD4+ T helper cells (Th) recognise class II HLA presented peptides thereby releasing different types of cytokines that allow the activation of CTLs (se section 1.7.1.*iii*), but CD4+ Th1 cells also induce macrophages to kill cells infected with intracellular bacteria or parasites. In addition, CD4+ Th2 cells participate in the humoral immune response against helminths and support the maturation of B cells into antibody producing plasma cells.

1.7.1.ii T cell effector killing mechanisms

Two predominant pathways for lymphocyte mediated killing are known (Henkart, 1994; Lowin *et al.*, 1994) that involve perforin-mediated lysis and Fas/FasL induced apoptosis, respectively. CTL mediated killing initiates with the binding of the T lymphocytes to the target cell by accessory receptors and adhesion molecules. When bound to the target cell, T cells secrete lytic granules by exocytosis into the intercellular space, the so called immune synapse. Perforin molecules then form pores in the cell membrane of the target cell leading to its osmotic lysis (reviewed in (Liu *et al.*, 1995)), while released granzymes can induce apoptosis (reviewed in (Smyth and Trapani, 1995)).

1.7.1.iii Th1 and Th2 subpopulations: Polarised cytokine release

The effector functions of CD4+ T cells are associated with various cytokine patterns, which have categorised them into two subgroups; Th1 cells release IL-2, IFN-γ and TNF-β, while IL-4, IL-5, IL-6, IL-10 and IL-13 are characteristic of Th2 cells. IL-3, GM-CSF and TNF-α are overlapping for both subsets. Incubation of uncommitted precursor T cells, Th0 cells, in the presence of IL-12 induces the development of Th1 subsets, while IL-4 directs Th2 polarisation (reviewed in (Glimcher and Murphy, 2000)). Furthermore, a low dose of antigen combined with high affinity for the HLA molecule favours a commitment to the Th1 lineage, whereas the opposite scenario directs the development of Th2 responses (reviewed in (Constant and Bottomly, 1997)). Interestingly, the cytokines of these polarized CD4+ T cell subsets also have strong antagonistic effects on the development of the other subset, most pronounced is the negative influence of IL-10 and IL-4 on Th1 responses, while IFN-γ and IL-12 inhibit Th2 reactions (reviewed in (Constant and Bottomly, 1997)).

The first demonstration of polarised CD4+ T cell responses was observed in studies of murine helper T cells (Cher and Mosmann, 1987; Cherwinski et al., 1987; Mosmann et al., 1986). Additional studies showed that this Th1/Th2 dichotomy was a valid concept also in humans (Del Prete et al., 1991).

1.7.1.iv Tc1 and Tc2 cells

Cytotoxic CD8+ T (Tc) cells can be divided into two subpopulations with polarised cytokine profiles analogue to CD4+ T cells: Tc1 cells secreting IFN-γ and TNF-α and Tc2 cells releasing IL-4, IL-5, IL-10 and IL-13 (Sad et al., 1995). In addition, the differentiation of CD8+ T cell precursors into either Tc1 or Tc2 cells are influenced by IFN-γ and TGF-β respectively IL-4, i.e. the same cytokines that direct the development of

Th1 and Th2 cells (reviewed in (Mosmann and Sad, 1996)). However, the effector functions of CD8+ Tc1 and Tc2 cells are less well characterised, but both subsets have been shown to be cytotoxic and able to produce perforin (Vukmanovic-Stejic *et al.*, 2000). The same study also demonstrated that Tc1 cells can promote Th1 development, while Tc2 cells sustain Th2 responses. Furthermore, Tc1 and Tc2 cells have also been shown to have a role in tumour protection and to induce long-term antigen specific T cell memory when adoptively transferred to mice with pulmonary metastases (Dobrzanski *et al.*, 2000).

1.7.1.v *T regulatory cells*

T regulatory (Tr) cells constitute a novel T cell subset, with a cytokine profile different from the classical Th1 and Th2 cells. They secrete high levels of IL-10, low levels of IL-2 and no IL-4, as demonstrated by repeated stimulation of murine as well as human CD4+ T cells in the presence of IL-10 or both IL-10 and IL-4 (Groux *et al.*, 1997). In addition, when injected into severe combined immunodeficient (SCID) mice, these new CD4+CD25+ T cells, also called Tr1 cells, were able to suppress the development of colitis, a pathological condition caused by autoimmune reactions.

However, several other subpopulations with suppressive functions have been identified (Roncarolo and Levings, 2000): Tr1 cells are suppressive by the secretion of IL-10, while Th3 cells secrete TGF-β. The CD4+CD25+ Tr cells can be phenotypically characterised by the expression of the IL-2 receptor α-chain (IL-2Rα) (Sakaguchi *et al.*, 1995), also known as CD25 (Sakaguchi, 2000). The presence of a minor population of CD4+CD25+ Tr cells with similar properties to murine Tr cells has been demonstrated by analysing cells from human blood (Dieckmann *et al.*, 2001). The human CD4+CD25+ Tr population represented approximately 6% of the CD4+ T cells.

1.7.1.vi   T regulatory cells and IL-10

IL-10 is a key cytokine connected to Tr cells, as it possesses the capacity to regulate their function; thus it could be considered that IL-10 *per se* indirectly controls the peripheral tolerance, as discussed below. Studies in mice revealed several characteristics of Tr cells. They were shown to constitute approximately 5-10 % of the peripheral CD4+ T cells.

These findings stimulated a functional evaluation of the mechanisms used by CD4+CD25+ T cells for suppression of autoimmunity. It was demonstrated that CD4+CD25+ T cells could not be activated by conventional stimuli involving the T cell receptor, but that the combination of anti-CD3 and IL-2 could overcomes their anergic phenotype, while anti-CD28 and IL-2 had no effect (Shevach *et al.*, 2001).

1.7.2   T cells recognition of cancer cells

1.7.2.i   Cancer cells as immunogenic targets

In the early era of tumour immunology, the prevailing idea was that the immune system had developed to naturally eradicate tumour cells, according to the theory of immune surveillance (Burnet, 1970). However, the discovery of tumour antigens as self antigens in addition to the notion that actually T cells have evolved to cope with infectious agents, changed this concept and provided new insights to the understanding of the effector mechanisms involved in tumour recognition and destruction by the immune system.

Recent data suggest that the term "immunoediting" may be more suitable to describe the role of the immune system during tumour development as it not only has a protective function, but also exerts a pressure on tumour cells by selecting those that can escape detection (reviewed in (Ikeda *et al.*, 2002)). This view is based on several findings that together depict IFN-γ as a crucial immuno-modulatory molecule that, secreted by cells of the innate immune system, first participates in the initial attack against the tumour, then

favours the activation of adaptive immune response mediated by T cells but can also allow tumour escape by imposing a selective pressure on tumour cells and enhancing their immunogenicity by up-regulation of class I HLA molecules.

1.7.2.ii CD4+ cells in anti-tumour immunity

CD8+ T cells have been considered as the main effector cells in anti-tumour immunity. This concept originated from the observation that most non-haematopoietic tumours express class I, but not class II HLA molecules (reviewed in (Ferrone and Marincola, 1995)). Furthermore, CD8+ T cells alone were show to be sufficient to eradicate large tumour masses in mice (Kast *et al.*, 1989; Townsend and Allison, 1993). Nonetheless, recent data clearly indicate that CD4+ T cells have a pivotal role in the generation and maintenance of CD8+ T cell responses (reviewed in (Pardoll and Topalian, 1998; Toes *et al.*, 1999; Topalian *et al.*, 1996)). In particular, they are involved in cross-priming of CD8+ T cells (Bennett *et al.*, 1997; Ridge *et al.*, 1998), (reviewed in (Carbone *et al.*, 1998)) a mechanism in which a professional APC, like a DC, acts like a temporary bridge between CD8+ and CD4+ T cells (Figure 1.8).

DCs are central to cross-priming as they are multi-functional. Immature DCs (iDCs) residing in the periphery can sample antigens released from dead tumour cells (Albert *et al.*, 1998) and then migrate to the nearest draining lymph node, where DCs, in their mature state, efficient by cross-present TAAs to CD8+ and CD4+ T cells. The simultaneous presentation of class I and II HLA epitopes by the DCs allow the activation of naive CD8+ and CD4+ T cells in close vicinity to each other. This creates an environment where a local cytokine release, especially IL-2 and IFN-γ, from the Th1 CD4+ T cells efficiently can prime and activate naive CD8+ T cells to become mature effector CTLs capable of tumour killing. Moreover, by interacting with CD40 on the DC, the CD4+ T cells can instruct the
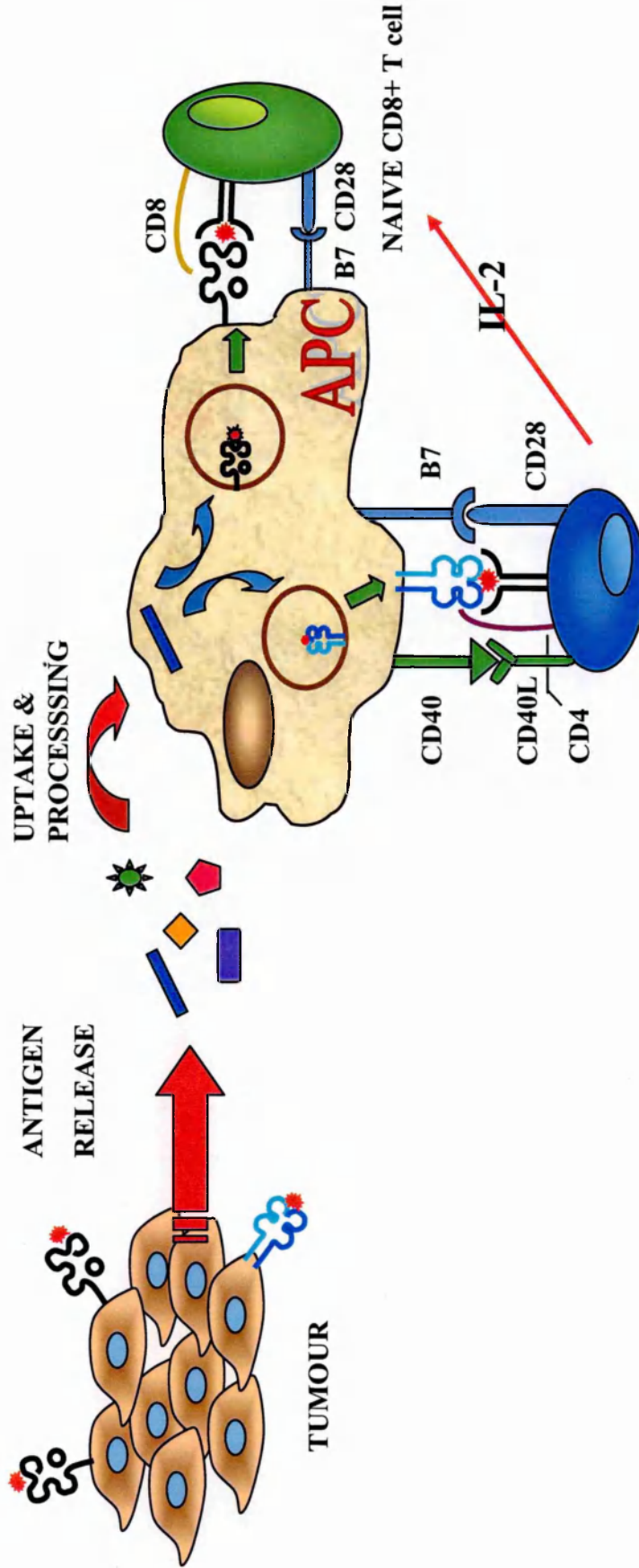
FIGURE 1.8: Cross-priming. Professional antigen presenting cells (APCs), like DCs, macrophages and B cells, can sample antigens released by tumour cells in the periphery and then simultaneously process and present them for CD8+ and CD4+ T cells respectively in the nearest draining lymph node. To be fully activated, naive CD8+ T cells must recognise both the class I HLA/peptide complex and get costimulation through B7-CD28. CD4+ T cells are crucial for the maintenance of CD8+ T cell responses, as they, in addition to release growth inducing cytokines like IL-2, also induce the upregulation of B7 on the APC. This occur s through the interaction between CD40-CD40L.

APC to up-regulate the B7 (CD80) molecule, which provides B7 - CD28 (CD86) co-stimulation for the CD8+ T cells.

In an optimal situation, a tumour cells can be attacked in 3 different ways. Direct cytotoxic killing by CD8+ T cells through the perforin/granzyme mechanism and indirect killing mediated by help of either Th1 or Th2 CD4+ T cells. More precisely, IFN-γ release from Th1 cells can stimulate macrophages to release nitric oxide and reactive oxygen intermediates. Th2 cells recruit eosinophils to the tumour site, where they release the cytotoxic content of their granules. The essential role for Th1 and Th2 cells in mediating tumour killing has been shown in mice vaccinated with a B16 melanoma vaccine, where macrophages and eosinophils were detected at the tumour site *in vivo* (Hung *et al.*, 1998).

T cell mediated immunity is central to tumour eradication, (reviewed in (Pardoll and Topalian, 1998)) and includes both CD8+ CTLs, and CD4+ T helper cells, that participate at different levels in different steps of the anti-tumour response. Whereas CD8+ T cells predominantly lyse target cells by a perforin-granzyme mechanism (reviewed in (Griffiths, 1995)), CD4+ T cells release cytokines that (i) help to maintain and sustain CD8+ T cell responses and (ii) attract phagocytic and inflammatory cells like DCs, macrophages and eosinophils to the affected site (Hung *et al.*, 1998). However, cytotoxic CD4+ T helper cells have also been described (Williams and Engelhard, 1996). Indeed, anti-melanoma CD4+ T cells can kill target cells through a Fas/FasL independent, granule-dependent lytic pathway (Rivoltini *et al.*, 1998) to overcome tumour escape. In addition, another mechanism involving the TNF-related apoptosis-inducing ligand (TRAIL) may be implicated in apoptotic killing of melanoma (Thomas and Hersey, 1998b).

1.7.2.iii Antibody mediated anti-tumour responses

The knowledge about humoral anti-tumour responses is limited, as the serum of most cancer patients do not contain antibodies reactive against TAAs. However, with the development of the SEREX technique (reviewed in (Sahin *et al.*, 1997; Tureci *et al.*, 1997)) it has been possible to serologically characterise antigens. As these antibody-defined TAAs have been demonstrated to induce high-titer IgG responses in cancer patients, this indicates the simultaneous presence of a CD4+ T cell mediated response which is necessary to generate IgG antibodies. However, although approximately 1500 antigens have been identified with the SEREX approach (www.licr.org/SEREX.htm), only MAGE-1 (van der Bruggen *et al.*, 1991), tyrosinase (Brichard *et al.*, 1993) and NY-ESO-1 (Jager *et al.*, 1998; Jager *et al.*, 2000a) have been demonstrated to induce both T cell mediated and antibody responses. The contribution of antibody-defined antigens to clinical tumour regression is thus elusive and current vaccine strategies are aimed at activating T cell mediated anti-tumour responses.

1.8 IL-10 IN IMMUNOLOGY

1.8.1 Cytokines and chemokines

The immune system is a highly regulated network in which signalling molecules such as cytokines and chemokines are critical regulators in directing the outcome of immune reactions. Chemokines recruit leukocytes (mainly DCs and macrophages) to sites of inflammation, while cytokines can have an arry of functions depending on their target cell. However, both chemokines and cytokines have been shown to play a crucial role in different diseases including cancer. For example, (i) tumour cells can express chemokine receptors and thereby locate to specific organs and, (ii) inflammatory cytokines present in the tumour microenvironment can support tumourigenesis (reviewed in (Balkwill and Mantovani, 2001)). Thus, normally cytokines are important factors in regulating different steps of immune reactions whose dysfunction can enhance tumour progression. In particular, IL-10 is a cytokine with several suppressive functions that can affect anti-tumour activity.

1.8.2 Early findings

Endowed with pleiotropic activities, IL-10 has been shown to be a key cytokine in multiple biological systems only a decade after its identification (Fiorentino et al., 1989). IL-10 was initially characterised for its capacity to inhibit the synthesis of IFN-γ, IL-2 and TNF-β by Th1 cells and was therefore first defined as *cytokine synthesis inhibitor factor* (CSIF) (de Waal Malefyt et al., 1991a). However, subsequent studies in mice, showed that IL-10 blocked also the functions of antigen presenting cells like macrophages and thus had an indirect effect on cytokine synthesis (Ding and Shevach, 1992; Fiorentino et al., 1991b). Today, IL-10 is known to be a ubiquitously expressed Th2 cytokine with a complex of biological functions connected both to inhibition and stimulation of the immune response.

1.8.3 The IL-10 gene

The human *IL-10* (*hIL-10*) gene (Acession number U16720) has been mapped to the junction of 1q31 and 1q32 on chromosome 1 (Eskdale *et al.*, 1997a; Kim *et al.*, 1992). It contains 5 exons which covers approximately 8.8 kb. The coding region of the mRNA includes 536 bp, which corresponds to the genomic sequence of the *hIL-10* gene. Moreover, the *hIL-10* gene is highly homologous to the murine *IL-10* gene (Moore *et al.*, 1990; Vieira *et al.*, 1991), and they both show a strong nucleotide homology with an open reading frame of Epstein-Barr virus (EBV), BCRF-1, also known as viral IL-10 (vIL-10). This suggests that the EBV virus may have captured the mammalian *IL-10* gene to favour its own survival.

The *hIL-10* encode for a 160 amino acid protein whose molecular weight is 18 kDa (Vieira *et al.*, 1991), which has 84% identity with the protein expressed by viral IL-10. Moreover, the IL-10 protein is biological active as a non-glycosylated homodimer and binds with high affinity to its dimeric receptor (IL-10R), composed of the IL-10R1 and IL-10R2 chains (Hoover *et al.*, 1999; Tan *et al.*, 1995).

1.8.4 The role of IL-10 in the immune system

The various roles of IL-10 in the immune system are in part related to the fact that IL-10 is produced by a variety of hemopoietic cells (Figure 1.9): CD4+ Th0, Th1 (Yssel *et al.*, 1992) and Th2 (Fiorentino *et al.*, 1989) cells, B lymphocytes, mast cells, monocytes and macrophages (reviewed in (Wakkach *et al.*, 2000)). But even tumour cells have been shown to produce IL-10 (Chen *et al.*, 1994; Dummer *et al.*, 1996; Dummer *et al.*, 1995). Due to its multifunctional activities, IL-10 does affect several different cell types and it has been considered as a powerful immune regulatory cytokine. However, many of the
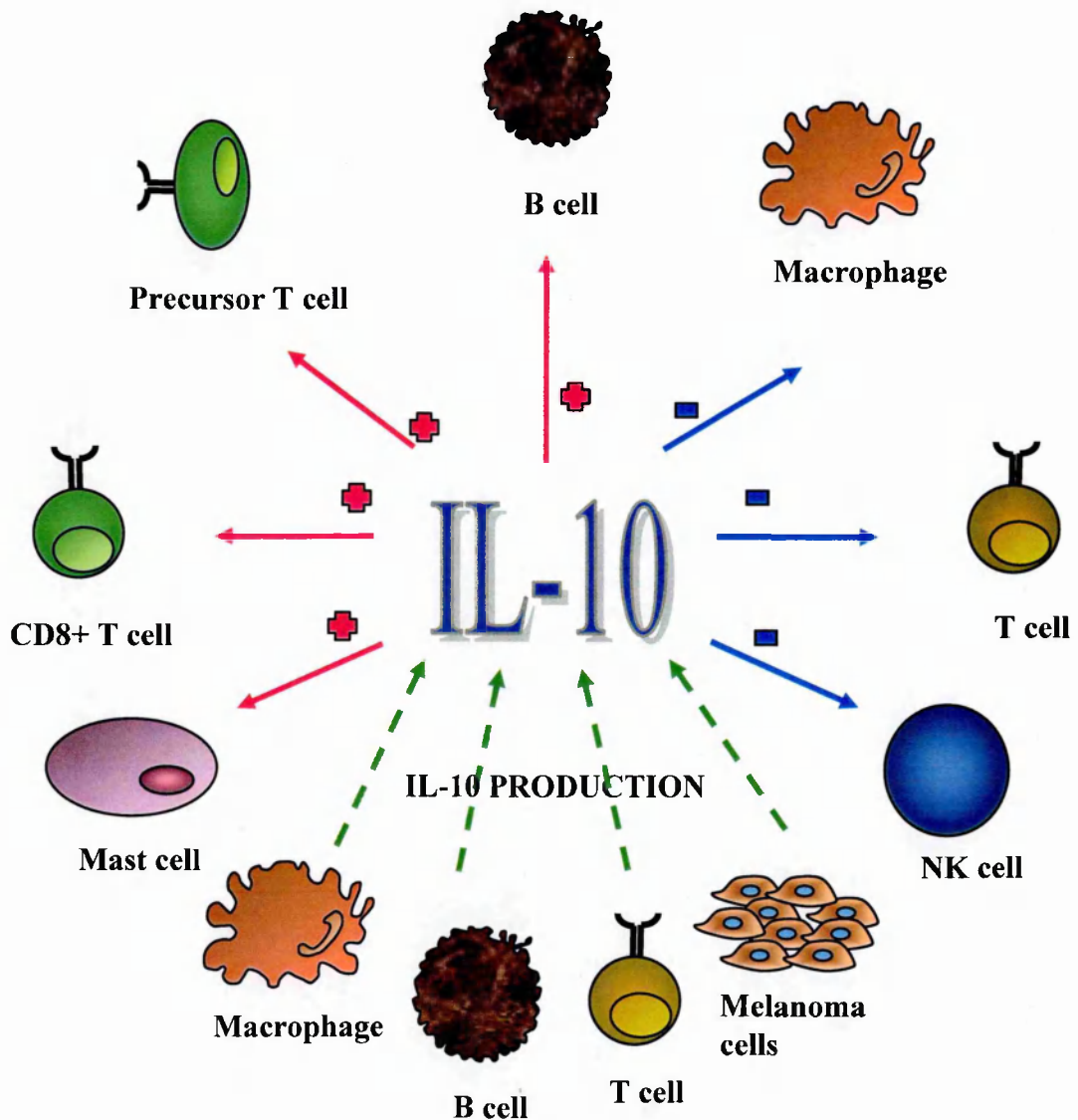
FIGURE 1.9: The pleiotropic activities of IL-10. IL-10 can be produced both by cells in the innate (macrophages) and in the adaptive immune defence (T and B cells). In addition, IL-10 release have been observed from melanoma cells. The multifunctional effects of IL-10 influence the immune response at several different levels. Briefly, B cells are induced to proliferate and secrete IgG antibodies, while proliferative responses are induced for precursor T cells, activated CD8+ T cells and mast cells. However, IL-10 is mainly known as an immunosuppressive cytokine as it can inhibit antigen recognition, proliferation and cytokine production of T cells by interfering with the antigen presenting function of macrophages. Also NK cells can be negatively influenced and produce a decreased level of IFN-g. Positive response are marked with a pink cross and suppressive effects with a blue line. The green dotted lines represents IL-10 production. This figure has been reproduced from Wakkach *et al.*, 2000.

functions of hIL-10 have been assayed in murine systems. This approach is possible, since hIL-10 is active on murine IL-10 receptor, while the opposite combination is not applicable, as the binding of the IL-10 protein to its receptor is species specific in humans, but not in mouse (Ho *et al.*, 1993; Liu *et al.*, 1994).

1.8.5   IL-10 influence on macrophages and monocytes.

The ability of IL-10 to inhibit cytokine production from macrophages and monocytes influence the initiation and maintenance of several other immunological events. The effect of IL-10 is particularly strong on inflammatory responses, as it suppresses TNF and IL-1, two pro-inflammatory cytokines. When activated, monocytes and macrophages can produce a wide array of cytokines: IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF and PAF (de Waal Malefyt *et al.*, 1991a; Fiorentino *et al.*, 1991a; Fiorentino *et al.*, 1991b). The inhibitory effect of the IL-10 on the synthesis of these cytokines makes IL-10 an important suppressor of inflammation.

The blocking of chemokine production from monocytes also inhibits the recruitment of neutrophiles, DCs and T cells to the site of inflammation. Moreover, the inhibitory properties of IL-10 also influence the antigen presenting capacity of macrophages by inducing the retention of assembled HLA class II molecules in intracellular vesicles (Koppelman *et al.*, 1997). In addition, IL-10 impairs proliferation of T lymphocytes stimulated by melanoma cells transduced with the co-stimulatory molecule B7 (Dummer *et al.*, 1998). Thus, IL-10 has the power to modulate a wide range of T cell responses.

1.8.6   IL-10 and B cells

In opposite to its suppressive effects on macrophages, monocytes and DCs, addition of hIL-10 to activated human B cells can enhance their growth and differentiation (Rousset *et al.*, 1992). In addition, activation of B cells through their antigen receptor or via CD40 in

the presence of IL-10 can induce production of immunoglobulin (Ig) IgG, IgM and IgA.

However, during their initial activation also B cells are subjected to the inhibitory effect of

IL-10, while IL-10 promotes stimulatory effects upon activated B cells (Itoh and Hirohata,

1995).

1.8.7 The influence of IL-10 on DCs and T cells

DCs are cells specialized for antigen capture and processing in the periphery then they

migrate to the draining lymph nodes where they release cytokines and up-regulate HLA

and co-stimulatory molecules like B7 to efficiently prime T lymphocytes (reviewed in

(Banchereau and Steinman, 1998)). IL-10 is a cytokine that at various levels can interfere

with the priming functions of DCs (Corinti et al., 2001). This strongly affects T cells, as

they are the main modulators of immune responses and depend upon the right priming

from DCs to become functionally committed. Moreover, in addition to down-regulation of

costimulatory molecules, IL-10 can inhibit the secretion of IL-12 from mature DCs (De

Smedt et al., 1997) and thus suppress the development of inflammatory Th1 responses.

However, IL-10 has also been characterised as a cytokine inducing T cell proliferation and

pre-incubation of human T helper cells with IL-10 induce their cytokine production

(Lelievre et al., 1998). CD8+ T cells show an enhanced proliferation in the presence of IL-

2 and IL-10 (Groux et al., 1998).

However, IL-10 has not only been explored as a Th2 cytokine with cross-regulating

activity on the Th1/Th2 cytokine profiles, but it also has a pivotal role in immune

suppression (reviewed in (Moore et al., 2001)). An array of recent findings show that the

state of the priming DCs is crucial in determining the direction of the subsequent T cell

response (reviewed in (Jonuleit et al., 2001)). More precisley, immature DCs (iDCs)

mediate the maturation of Tr cells, while DCs that have matured in the presence of IL-10

induce anergic T cells. Indeed, it has been observed that IL-10 modulated DCs are capable of suppressing both CD8+ and CD4+ T cells in an antigen specific manner, which can be restored by blocking of CTLA-4 (Steinbrink *et al.*, 2002). Thus, a current hypothesis is that IL-10 somehow influences the conditions of the DCs in a way that impedes their priming capacity.

1.8.8 IL-10 in tumour immunity

IL-10 is an important mediator of the immune response in the tumour microenvironment (reviewed in (Mocellin *et al.*, 2001)). The regulatory role of IL-10 is complex as it not only is expressed as a transcript in several human tumours like breast (Venetsanakos *et al.*, 1997), ovarian (Pisa *et al.*, 1992), lung (Huang *et al.*, 1995) and renal cell carcinoma (Nakagomi *et al.*, 1995) and melanoma (Kruger-Krasagakes *et al.*, 1995), but it is also secreted by CD4+ T helper cells (Fiorentino *et al.*, 1989; Yssel *et al.*, 1992).

In particular, both inhibitory and stimulatory effects have been described for IL-10 in melanoma. For example, elevated IL-10 expression in metastatic lesions (Dummer *et al.*, 1996; Dummer *et al.*, 1995; Sato *et al.*, 1996), may allow melanoma cells to escape detection by down-regulation of HLA (Yue *et al.*, 1997) and induction of anergy in DCs (Enk *et al.*, 1997). In addition, elevated serum levels of IL-10 have been detected in patients with metastatic melanoma (Fortis *et al.*, 1996), a finding that is compatible with the report that melanoma cells use IL-10 as an autocrine growth factor for proliferation (Yue *et al.*, 1997) as a way of escape immune reaction. Thus, tumour cells can also favour their progression by direct secretion of IL-10 in the tumour microenvironment.

Moreover, IL-10 can synergise with TGF-β to suppress anti-tumour responses (D'Orazio and Niederkorn, 1998). However, other data report that IL-10 and TGF-β have been detected at increased levels in regressing versus progressing melanoma (Conrad *et al.*,

1999). In deed, several anti-tumour mediated functions can be attributed to IL-10. Transfer

of the mouse *IL-10* gene into human melanoma cells resulted in inhibition of growth and

reduced metastasis, which could be connected to inhibited tumour blood vessel formation,

i.e. angiogenesis *in vivo* (Huang *et al.*, 1999; Huang *et al.*, 1996). Systemic administration

of IL-10 to melanoma bearing mice inhibited further tumour establishment and led to

complete regression (Berman *et al.*, 1996). In addition, anti-metastatic activity of IL-10 has

been observed through NK cell dependent mechanism in mice models of melanoma

(Zheng *et al.*, 1996). However, positive roles have also been described for IL-10 in

humans. Recent data show that vaccination with IL-10 producing glioma-specific CD4+ T

cells can mediate tumour rejection (Segal *et al.*, 2002).

Chapter 2

MATERIALS AND METHODS

2.1 THE MELANOMA PATIENT 15392

The study of this thesis has been focused on a melanoma patient, who has remained disease free for 12 years, an unusually favourable outcome, after her lymph node metastasis had been resected in 1990 at the Istituto Nazionale dei Tumori of Milan.

In September 1989 a 35 year old lady had a wide excision of a pigmented lesion on the right forearm in another hospital. The patient was referred in October 1990 to the Istituto Nazionale dei Tumori after she had noticed a mass in her right axilla. There were no signs of local recurrence or in transit metastasis. Chest X-ray and liver ultrasonography were uneventful.

In November 1990, the patient underwent a right axillary lymph node dissection. The pigmented lymph node was at the surface of the specimen, with no surgical margin involved. In contrast with the primary tumour, extensive lymphocyte infiltration within the tumour tissue was present. The remaining 22 lymph nodes were free of tumour. Post-operative recovery was uneventful. Up to date, during further routine follow up, including clinical examination, routine chest X ray and liver ultrasonography, the patient remained free of recurrence, metastasis or second primary tumour formation.

2.2 CELLULAR METHODS

2.2.1 Statistics

Statistical analysis was performed using the 2-tailored *"Student-Newman-Keuls test"*. Only data with p values of 0.05 or less were considered as statistically significant.

2.2.2 General principles for eukaryotic cell culture

All eukaryotic cell culture procedures were performed in strictly sterile conditions provided by Bluebeam 4 Class II Type A/B3 Biohazard Laminar Flow Cabinet hood (Arredi Tecnici Villa spa, Varese, Italy). Conventional standard procedures were applied for preparation of cell culture mediums and reagents.

Human serum (HS), obtained from healthy donors at Istituto Nazionale dei Tumori in Milan, or fetal calf serum (FCS) (Biological Industries, Beit Haemek, Israel) were passed through a 0.22 μm Stricup® Millipore Express™ high flow rate filter (Millipore S.A., Bedford, MA) and divided into 50 ml aliquots, which were heat-inactivated at 56°C for 30 minutes. These preparations were stored at 4°C until use or for up to one month.

Culture media, i.e. either RPMI 1640 (BioWhittakerEurope, Vervires, Belgium) or Dulbecco's Modified Eagle's Medium (DMEM) (Euroclone, Europe, TQ4 5ND Devon, UK) were purchased as 500 ml solutions and supplemented with different reagents (described below) depending on the requirements for each cell line or clone. L-Glutamine, HEPES buffer, NaPyruvate solution and phosphate buffered saline (PBS) were all purchased from BioWhittakerEurope (Vervires, Belgium). For practical reasons, pencillin and L-glutamine were prepared as stock solutions. More precisely, 2 g (1×10^6 U) of pencillin (Pharmacia & Upjohn S.p.A., Milan, Italy) was left to dissolve in 10 ml of PBS (BioWhittakerEurope). The dissolved pencillin was then added to L-glutamin together with 10 ml (400 mg) of Gentalyn (Schering-Plough S.p.A., Milan, Italy). This solution was

passed through a 0.22 or 0.45 µm Millex®-GS Duropore® (PVDF) membrane (Millipore S.A., Bedford, MA), distributed as 6 ml stocks, stored at -20°C and rapidly thawed at 37°C just prior to use. One such pre-optimised stock solution was added to each 500 ml batch of culture media providing a final concentration of 200 U/ml pencillin (Pharmacia & Upjohn S.p.A, Milan, Italy), 2 mM L-glutamine and 40 µg/ml Gentalyn.

2.2.3 Cell lines and clones

Cell line or clones used in the present thesis are shown in Table 2.1. All cell lines were mycoplasma negative. 293-Epstein Barr Nuclear Antigen (EBNA) cells (293wt) were purchased from Invitrogene (CA, USA) while 293 cells engineered to constitutively express the class II trans-activator (CIITA) protein, 293/CIITA cells, were kindly provided by Dr. Paul Robbins (National Institute of Health, Bethesda, MA). COS-7 cells and the K562 cell line were purchased from American Type Culture Collection (ATCC), (Rockville, Maryland).

The melanoma cell line (Mel5392) were established *in vitro* from the single cell suspension obtained from the lymph node metastatis of Pt15392 (HLA-A typed as A*0301, B*40012, B*1402, C*0602, C*0802, DRβ1*01021, DRβ1*1001 and DQβ1*0501), while the autologous lymphoblastoid cell line, LCL15392, was established from peripheral mononuclear cells (PBMCs) after EBV infection. Allogenic tumour cell lines, Allo-Mel (HLA-A11, -A28, -B14, -B35, -C4, -C8) and Allo-Me2 (HLA- A3, -A2, -B14, -B44, -C5, -C8), were established in our laboratory.

The Mel5392 and LCL15392 cell lines and T2 cells were maintained in RPMI 1640 supplemented with 10% FCS, 200 U/ml pencillin, 2 mM L-glutamine, 40 µg/ml Gentalyn and 20 mM HEPES buffer. The African green monkey cell line, COS-7, the human

embryonic kidney cell line, 293-EBNA cells, and the NK cell susceptible and class I HLA deficient K562 chronic myelogenous leukemia cell line were maintained in DMEM supplemented with 10% FCS, 200 U/ml pencillin, 2 mM L-glutamine, 40 µg/ml Gentalyn, 20 mM HEPES buffer and 0.5 mM Na Pyruvate solution. Lymphocyte activated killer (LAK) cells were cultured in RPMI 1640 in the presence of 10% HS, 200 U/ml penicillin, 2 mM L-glutamine, 40 µg/ml Gentalyn, 20 mM HEPES buffer and 100 U/ml IL-2.

Established CD8+ and CD4+ T cell clones were cultured in DMEM supplemented with 10% FCS, 200 U/ml penicillin, 2 mM L-glutamine, 40 µg/ml, Gentalyn, 20 mM HEPES buffer, 0.5 mM Na Pyruvate and 50 U/ml of recombinant human IL-2 (EuroCetus, Amsterdam, The Nederlands).

2.2.4 General procedures of cell culture

All cell lines and clones were left to expand at 37°C in a humidified atmosphere of 5% CO_2 in a Forma Scientific CO_2 Jacketed Incubator for 3-4 days before being split and passed on as new subcultures. Recipient cells such as Me15392, COS-7 and 293-EBNA cells (Table 2.1) were normally seeded at a concentration of 1×10^6 cells/flask and grown to 90-95% monolayer confluence in 75 cm^2 Costar Cantec Neck Cell Culture flasks of polystyrene (Corning Incorporated, Corning, New York, USA) sealed with Plug Seal Caps. At this stage, the cells were detached by trypsination: the culture medium was discarded, 8 ml (0.2 mg/ml EDTA, 0.5 mg/ml Tris) of Trypsin-EDTA (BioWhittakerEurope) was added to the cellular monolayer and after 5-10 min of incubation, the trypsin was deactivated with FCS or, alternatively, with HS (1/10 volume of the volume Trypsin-EDTA used). LCL15392 cells were grown in suspension in vertically standing 75 cm^2 Costar Cantec Neck Cell Culture flasks, incubated under the same conditions as adherent cells. All cellular preparations were centrifuged at 1500 rpm and 4°C in a table top centrifuge 5810R

Table 2.1: Cell lines and clones used in this study. All autologous cell lines (Me15392 and LCL15392) were established from melanoma Pt15392. LCL cells express the full HLA haplotype of the patient from whom they were derived. The CD8+ T cell clone, TB686 derived from tumour associated lymphocytes (TALs) of Pt15392 (Castelli *et al.*, 1999). CD4+ T cell clones (TB39, TB48, TB57, TB189 and TB515) were established by mixed lymphocyte tumour culture (MLTC) and limiting dilution approach of TALs. The allogenic melanoma lines (Allo-Me) shared the HLA-B14 and –Cw8 alleles with Pt15392. The LAK cells were derived from PBMCs of a healthy donor.

Name	Origin	Culture Medium
293-EBNA	A human embryonic kidney cell line	DMEM 10% FCS
COS-7	An African green monkey cell line	DMEM 10% FCS
K562	A human chronic myelogenous leukemia cell line	DMEM 10% FCS
T2	A human TAP deficient lymphoma line	RPMI 10% FCS
Me15392	Autologous melanoma cells of Pt15392	RPMI 10% FCS
LCL15392	Autologous LCL cells of Pt15392	RPMI 10% FCS
CD8+ T cell clones	Autologous tumour specific clones	RPMI 10% HS, 50 U/ml IL-2
CD4+ T cell clones	Autologous tumour specific clones	RPMI 10% HS, 50 U/ml IL-2
Allo-Me	Allogenic melanoma cells	RPMI 10% FCS
LAK	Lymphocyte activated killer cells	RPMI 10% HS, 100 U/ml IL-2

(Eppendorf S.r.l., Milan, Italy). Cellular aliquots were stained with trypan blue at a 1:1 volume ratio and applied onto a Burker's chamber glass slide, after which counts were carried out with an Olympus BX41 microscope.

2.2.5 Cryopreservation of cells in liquid nitrogen

Long term storage of cells was performed in liquid nitrogen. Cells were first normally detached by trypsination (see section 2.2.4), centrifuged and counted. Recovered cellular pellets were resuspended to a density of 10^6 or 10^7 cells/ml in pre-chilled RPMI or DMEM supplemented with either 30% FCS or HS together with 10% dimethylsulphoxide (DMSO). Aliquots of 1 ml were then directly added to 1.5 ml Nalgene cyrotubes (Nalgene Nuc International Corporation, Rochester, USA) sealed with screw taps, placed in plastic containers containing 100% isopropanol and then immediately stored at -80°C overnight, before storage at -196°C in liquid nitrogen.

2.2.6 Recovery of frozen cells

Cyrotubes with frozen pellets were thawed in a water bath adjusted to 37°C and rapidly withdrawn upon melting. The cells were then regenerated by the drop-wise addition of 1 ml of RPMI 1640 or DMEM supplemented with either 30% FCS or HS, transferred to a 15 ml Falcon tube and filled up with 10 ml of medium. A minimum of 3 successive rounds of centrifugation at 1500 rpm for 10 min were performed to remove DMSO, before the cells were transferred to the appropriate culture flasks. PBMCs were thawed as described above, but in the presence of 2000 U/ml DNase (for 1×10^6 cells) supplied to the medium.

2.2.7 Generation of tumor-specific T cell clones

TALs were isolated from a lymph node metastasis of Pt15392 by Ficoll-Plaque (Pharmacia Upjohn, Uppsala, Sweden) density-gradient. The purified lymphocytes were subsequently frozen and stored in liquid nitrogen (-120°C) for 7 years. The T lymphocytes were then

thawed and stimulated weekly with irradiated autologous tumour (10,000 rad) at an effector:target (E:T) ratio of 10:1 and maintained in RPMI 1640 supplemented with 10% HS and 300 U/ml of recombinant human IL-2 (EuroCetus, Amsterdam, The Netherlands) in a final volume of 200 μl in U-bottomed 96-well plates. At the end of the 3rd week of *in vitro* culture, the MLTC was either (i) maintained *in vitro* with additional repeated weekly tumour stimulations or (ii) responder T cells cloned by limiting dilution. T lymphocytes were seeded at 1 or 5 cells/well in 2500 U-bottomed wells (for each ratio) in a total volume of 150 μl RPMI 1640 supplemented with 10% HS and 50 U/ml recombinant human IL-2 and in the presence of $5x10^2$ irradiated autologous tumour (10,000 rad) cells and $1x10^5$ irradiated allogeneic pooled lymphocytes (3,000 rad) obtained from healthy donors. The microcultures were weekly re-stimulated with irradiated autologous tumour cells and allogeneic lymphocytes.

The growing T cell clones were screened for the ability to recognize the autologous tumor in HLA-dependent fashion by a cytotoxic assay (see section 2.2.14). The specific clones were further expanded and stored in liquid nitrogen for additional expansion and functional studies. Five of these T cell clones (TB39, TB48, TB57, TB189 and TB515) were then analysed for their capacity to recognise autologous tumour.

2.2.8 Restimulation of T cell clones

The CD4+ T cell clones derived from melanoma Pt15392, were re-stimulated weekly as follows: $2-3x10^6$ CD4+ T cells were stimulated with $1x10^5$ autologous melanoma cells, irradiated at 10,000 rad in a final volume of 2.5 ml RPMI 1640 supplemented with 10% HS and containing 50 U/ml recombinant human IL-2. All T cell clones were added to transfected cells or used for peptide pulsing on their fifth day after re-stimulation, as they

then released the maximum amount of cytokines. CD8+ T cell clones were stimulated in the same way.

2.2.9 FACS analysis

To identify the expression of specific cell surface molecules, cells were labelled with fluorescent antibodies and evaluated by fluorescence-activated cell sorting (FACS). All antibodies were purchased from Becton Dickinson (San Jose, USA) except for W6/32 hybridoma growth in our laboratory and obtained from ATCC (Rockville, Maryland, USA).

Briefly, $3-5 \times 10^5$ cells/sample were centrifuged at 1500 rpm for 10 min, after which the pellet was washed once in serum-free medium (RPMI 1640, DMEM or PBS) and incubated with a primary antibody following the manufacture instructions and then left 20 min on ice. The following monoclonal antibodies (mAbs), were used for the primary staining: W6/32 (anti-HLA-A, -B and –C), OK4 (anti-CD4), OKT8 (anti-CD8) and an anti-human HLA-DP, -DR and -DQ Ab. Cells were then washed once to remove excess of Ab and incubated with the secondary FITC labelled goat anti-mouse Ab in a 1 to 40 dilution in PBS for additional 20 min on ice. Finally, the cells were washed again and then fixed with 1x PBS containing 2% formalin before they were read in the FACScan® machine (Becton Dickinson). Direct immunofluorescence was performed with FITC labled mAbs and required only one incubation step. All washes were performed with serum-free RPMI 1640.

2.2.10 Transfection: the Lipofectamine method

The conditions for transfection of 293 cells were established according to the guidelines in the LipofectAMINE™2000 reagent protocol for transfection (Gibco, Life Technology, Inc. Garthersburg, MD, U.S.A.). In addition to that, the poly-L-lysine (Sigma Chemical Co.,

LTD., Irvine, UK) reagent was used to pre-coat the wells to increase the attachment of the 293 cells during the different steps of transfection. All transfections were performed in duplicate.

Thus, two days before transfection, a flat-bottomed 96-well Costar Cluster plate of polystirene (Corning Incorporated, Corning, New York, USA) was coated with 50 µl poly-L-lysine diluted to 0.1 mg/ml in 1x PBS (BioWhittakerEurope) per well and stored at 4°C overnight. Then, on the day before transfection, the poly-L-lysine was discarded from the wells and the plates coated with $5x10^4$ 293 cells/well in 100 µl DMEM 10% FCS without antibiotics and subsequently incubated in 5% CO_2 at 37°C for 24h. At 90-95% confluence, the 293 cells were double transfected with cDNA pools from the library and the HLA alleles of interest.

More precisely, for each well to be transfected, 150 ng of a cDNA pool (approximately 100 cDNA constructs/pool) was diluted up to 25 µl in OPTI-MEM I medium (Gibco, Life Technology) containing 150 ng of the HLA-DRβ1*01021 or HLA-DRβ1*10011 allele in a V-bottomed 96-well plate. Then, for each well, 0.9 µl of the LF2000 reagent (Gibco, Life Technology, Inc) was diluted up to 25 µl in OPTI-MEM I medium and incubated 15 min in room temperature. 25 µl of the diluted LF2000 reagent was added to each well containing the DNA. After 20 min incubation at room temperature, to allow DNA-LF2000 reagent complexes to form, the DNA-LF2000 reagent mix (50 µl/well) was added to the corresponding wells with 293 cells. The plates were subsequently incubated in 5% CO_2 at 37°C for 24h, before the T cell clones were added.

2.2.11 Transformation: the DEAE-dextran/chloroquine method

Transient transfections were also conducted by the DEAE-dextran/chloroquine method. Briefly, 15×10^3 COS-7 cells were seeded per well in a 96-well flat bottomed tissue culture plate in a final volume of 200µl DMEM 10% FCS. After 24 h incubation in humidified atmosphere 5% CO_2 at 37°C, the medium was discared and replaced by 50 µl/well of DMEM medium containing 1% NuSerum™ (Becton Dickinson, San Jose, USA), 0.4 mg/ml DEAE-dextran (Pharmacia & Upjohn S.p.A., Milan, Italy) and 0.1 mM cloroquine (Sigma Chemical Co., St Louis, USA) admixed with 100 ng of DNA of interest and 200 ng of plasmid DNA. Transfection was performed with plasmids encoding known melanoma-associated antigens (MAGE-1,-2, -3, -4; BAGE-1 and -2; GAGE-1, -2, -3, -4, -5 and -6; Melan-A/MART-1, gp100 and tyrosinase). These vectors were kindly provided by Dr. Thierry Boon, Ludwig Institute for Cancer Research, Brussels, Belgium. The plate was then centrifuged at 900 rpm for 7 min and then incubated for 48 h in 5% CO_2 at 37°C, After 48 h incubation, supernatants were carefully removed and 15×10^4 T cells (on the fifth day after their restimulation) were added to each well. The transfected cells were incubated for a minimum of 18 h with the T cells and then supernatants were collected and directly evaluated for their content of IFN-γ in an ELISA assay.

2.2.12 Cytotoxic assay

Cytotoxic activity of CD4+ T cells was measured in a standard 4 h ^{51}Cr assay. Target cells (2×10^6 cells), Mel5392 and LCL15392 (control), were washed once with RPMI 1640 10% HS and the pellet resuspended in 100 µl (100 µCi) sodium-(^{51}Cr)-chromate, Na(^{51}Cr)O$_4$, (Amersham Biosciences, NJ, USA) and incubated for 1 h at 37°C. The labeled target cells were then washed once in 5 ml RPMI 1640 10% HS and then three times in 10 ml RPMI 1640 10% HS to remove excess of the ^{51}Cr isotope. The target cells were then resuspended in RPMI 1640 10% HS and seeded as triplicates of 1000 cells/well in a U-

bottomed 96-well plate in a volume of 100 μl, after which T cells were added at a serial

effector:target (E:T) ratio of 100:1, 50:1 and 25:1 at a final volume of 150 μl.

The cytotoxic activity was always evaluated in the presence of controls for inhibition of

lysis, i.e. the target cells were incubated for 40 min at 37°C with 2 μg/ml of either W6/32

(anti-HLA-A, -B and –C), B1.23.2 (anti-HLA-B and –C) or L243 (anti-HLA-DR). The

involvement of the T cell receptor (TCR) in the target recognition was evaluated by

incubating the CD4+ effector T cells for 40 min at 37°C with 2.5 μg/ml of OKT3 (anti-

CD3). Spontaneous lysis was measured by incubating target cells with medium alone. The

level of maximum lysis was obtained by incubating target cells with 2% Nonidet P-40

(BDH Limited Pool, England). The samples were incubated for 4 h, after which the plates

were centrifuged for 10 min at 800 rpm. 100 μl of the supernatant was transferred to a

LumaPlate™ - 96 solid scintillation plates (Packard BioScience Company, PerkinElmer,

Meriden, CT), which were let to dry overnight. The radioactivity was measured in an

A9912 Top Count beta counter (Packard Instruments). Percentage of inhibition was

calculated in counts per minute (cpm) by the following equation, i.e. [(experimental release

– spontaneous release) / (maximum release – spontaneous release)].

2.2.13 Stimulation of T cells using OKT3

To achieve an effective T cell stimulation, 2 μg/ml of OKT3, was coated onto a 24-well

plate and incubated for 1 h, after which T cells (5×10^3 cells/well) were added in a final

volume of 0.2 ml RPMI 1640 10% HS. The plates were incubated at 37°C in 5% CO_2

overnight. This allowed the TCRs to be cross-linked by the plate-bound antibodies and

produce an optimal cytokine release. The supernatants were then harvested and assayed for

their content of IFN-γ by ELISA (see section 2.2.17).

2.2.14 Cytokine release assay

Lymphocytes were seeded (5×10^3 in 50 µl) in 96-well U-bottomed plates with 10^4 cells/well of autologus or allogenic melanoma cells in a final volume of 0.2 ml of RPMI 1640 supplemented with 10 % of HS. After overnight incubation at 37°C, supernatants were collected and cytokine content evaluated by ELISA (Endogen, Woburn, MA 01801, USA) (see section 2.2.17).

TCR involvement was evaluated by blocking experiments performed using the OKT3 Ab. Briefly, T cells before being added to the wells containing melanoma cells, were pre-incubated in the presence of 1 µg/ml of the blocking Ab for 45 min at 37°C. The T cells were then added to melanoma cells and supernatants was collected after 18 hour incubation and evaluated for their cytokine content by ELISA (see section 2.2.17).

The involvement of class I and class II MHC molecules in the recognition of tumour cells was evaluated by incubating the target cells with 1 µg/ml of W6/32 (anti-HLA-A, -B and -C), B1.23.2 (anti-HLA-B and –C), L243 (anti-HLA-DR), for 45 minutes at 37°C and then the T cells were added to the wells; supernatants was collected after 18 hour incubation and evaluated for their cytokine content by ELISA (see section 2.2.17).

2.2.15 Peptide synthesis

Peptides were synthesised by conventional solid phase chromatography using Fmoc for transient NH_2-terminal protection and characterised by mass spectrometry. All of the peptides were > 95% pure (Neosystem, Strasbourg, France). The peptides were dissolved at 5 mg/ml in DMSO and stored at 4°C. Prior to use, the peptides were diluted in RPMI 1640 containing 10% HS.

2.2.16 Peptide pulsing

LCL15392 cells were seeded in U-bottomed 96-well micro-plates ($5x10^4$ cells/well) in 100 µl RPMI 1640 10% HS and then pulsed with different concentrations of the synthetic peptides for 2 h at 37°C. To ensure an optimal cytokine release, T cells ($1.2x10^4$ cells/well in 150 µl) were added to the wells (on their fifth days after re-stimulation) with the autologous tumour and incubated overnigth at 37°C. The supernatants were collected after 18 h and evaluated for their IFN-γ content by ELISA (see section 2.2.17).

2.2.17 ELISA

Supernatants collected from cellular experiments were further evaluated for the cytokine content (IFN-γ, TNF-α, GM-CSF, IL2, Il-4, IL-6 and IL-10) by ELISA (Mabtech AB, Stockholm, Sweden) according to the manufactures guidelines.

On the day before the ELISA, a Nunc-Immuno™ Plate with MaxiSorp™ Surface (Nunc™ Brand Products, Nalgene Nuc International, Denmark) was coated with 50 µl of the primary Ab, diluted to 1 µg/ml in 1x PBS (BioWhittakerEurope) and incubated overnight. On the day of the assay, the first Ab was removed by blotting of the plate onto paper towels. The plate was then incubated 1h with 200 µl Assay Buffer (1x PBS with 4% BSA, pH 7.2) per well and washed 3 times with 200 µl/well of a Wash Buffer (50 mM Tris, 0.2% Tween-20, pH 8.0). The samples, supernatants and 1:1-dilutions of cytokine standard (1000 pg/ml), were added as duplicates (50 µl/well) to the plate in parallel and, 50 µl/well of the Biotin-Labled second Ab, diluted to 1 µg/ml in 1x PBS, was added directly to the plate. After 90 min incubation, the plate was washed as described above and then further incubated for 30 min with horse radish peroxidase (HRP)-conjugated Streptavidin, diluted 1:15000 in Assay Buffer (100 µl/well). Finally, the plate was washed as described above, after which the samples were incubated with TMB Substrate Solution for 30 min (100

μl/well), after which 100 μl Stop Solution (0.18 M H$_2$SO$_4$) was added per well. The ELISA plate was subsequently read in a MRX® II microplate photometer (Dynex Technologies, Inc., Chantilly, USA).

The content of TGF-β1 in culture supernatants was evaluated by a special ELISA approach involving an acid pre-treatment which converts its released, latent form to a bioactive, detactable protein. More precisely, according to the guidelines in the TGF-β1 ELISA protocol (BioSource International, Inc, California, USA) each supernatant (50 μl) was admixed with 10 μl Extraction Solution (i.e. a ratio of 5:1), vortexed and incubated for 30 min at 4°C. After this treatment, 50 μl of Standard Dilutent Buffer was added to each sample resulting in a 2.2-fold dilution of the supernatants. The following steps of the TGF-β1 ELISA were performed exactly as described in the manufacture's protocol.

2.3 MOLECULAR METHODS

2.3.1 General principles

All molecular reactions were prepared in Eppendorf tubes and kept on ice. Restriction enzymes were always kept in a thermo block (Stratacooler) when used. Gloves were used for handling of all RNA samples and IsoTip Filter tips (Costar, Corning Incorporated, Corning NY, USA) were used for work with restriction enzymes, PCR reagents and RNA. Centrifugations were carried out in a table-top Biofuge, Heraeus Instruments centrifuge at a maximum speed of 13000 rounds per minute (rpm) or in a SIGMA Laboratories refrigerated table-top centrifuge (B. Braun Biotech International GmbH, Melsungen). All DNA samples were diluted in 10 mM Tris-HCl, pH 8.0, if not otherwise indicated. Chemical solutions were sterilised by filtrated with Stericup™ Millipore Express™ high flow rate filter 0.22 μm microfilter (Millipore S.A., Bedford, MA).

2.3.2 mRNA extraction

RNA was extracted following a modified version of the acid guanidinium thiocyanate-phenol-cloroform method. Briefly, a cellular pellet prepared of a minimum of 1×10^6 cells was thawed in room temperature and then immediately resuspended in 200 μl of RNAzol™ B (Tel-Test, Inc., Friendswood TX, USA); the homogenisation was optimised to the use of 200 μl of RNAzol™ B to achieve an efficient lysis of 1×10^6 cells. The sample was vortexed well to dissolve the RNA pellet and then left for 5 min at room temperature. The lysis reaction was stopped by addition of 200 μl chloroform per 2 ml of homogenate (i.e. 2 μl chloroform in 200 μl homogenate) and agitated vigorously by inverting the tube 4-6 times, after which the sample was incubated on ice for 5 min and then centrifuged for 15 min at 4°C and 12000 g. This treatment resulted in a separation into one upper aqueous phase (transparent) containing the extracted RNA and one lower organic phase (blue) with DNA and proteins. The RNA containing supernatant was carefully transferred to a new

tube and precipitated with the addition of an equal volume of 100% isopropanol. More precisely, the sample was inverted 4-6 times and left for a minimum of 5 min on ice before it was centrifuged for 15 min at 4°C and 12000 g. The recovered pellet was washed once with 1 ml 70% ethanol, vortexed and centrifuged for 10 min at 4°C and 7500 g. The retrieved RNA pellet was dried in a speed vacuum centrifuge, Eppendorf Concentrator 5301 (Eppendorf S.r.l., Milan, Italy), for maximum 10 min and then resuspended in 50 μl diethylpyrocarbonate (DEPEC) RNase-free water. All RNA preparations were stored at -80°C.

2.3.3 cDNA synthesis

The reagents for cDNA synthesis were purchased from Promega (Promega Corporation, Wisconsin, USA) and all steps were performed on ice. The first strand was synthesised from 2 μg of mRNA in a final volume of 21 μl containing 0.01 μg/μl oligo(dT) primer, 0.76 mM dATP, 0.76 mM dTTP, 0.76 mM dGTP, 0.76 mM dCTP, 0.02 M DTT, 19 U/μl of reverse transcriptase (RT) superscript BR2 and 1.9x RT First Strand buffer PCR. More precisely, a volume corresponding to 2 μg of mRNA was resuspended up to 19 μl with sterile water and then incubated with 0.6 μl (0.5 μg/μl) of oligo(dT) for 10 min at 65°C to add a thymidine (T) overhang to the mRNA template. Thereafter, the reaction was stopped for 2 min in ice and centrifuged for 2 min to receive a pellet. A master mixture (dNTPs, DTT and 5x RT First Strand buffer PCR) of 18.4 μl was added to the sample, which was incubated at 45°C for 2 min and then supplied with 2 μl of RT superscript BR2. The reaction was left to proceed for 1 h at 45°C and then stopped on ice. The second cycle was started by denaturing the mRNA/cDNA hybrid at 90°C for 5 min, after which the sample was put on ice and 2 μl of RT superscript BR2 was added. The sample was then incubated at 45°C for 1 h and finally stopped on ice. Prepared cDNA samples were stored at -20°C.

2.3.4 RT-PCR

The reverese transcriptase - polymerase chain reaction (RT-PCR) allows an exponential amplification of complementary cDNA strands in cyclic flow schedule: double stranded denaturation, primer annealing and single strand extension (polymerisation). This is possible due to the action of a thermophilic enzyme, *Taq* polymerase, extracted from a bacterium called *Thermus aquaticus*.

All amplifications were performed on a GeneAmp® PCRSystem 9700 PCR machine (Applied BioSystems, CA, USA) with primers indicated in Table 2.2 with reagents purchased from Perkin Elmer, Roche Diagnostics GmbH, Mannheim, Germany (if not otherwise indicated) and using β–actin as a positive control of the PCR reaction. The PCR for β-actin was performed in a 25 µl reaction mixture containing 2 µl of cDNA sample, all four dNTPs at 1.25 mM, 1x PCR buffer II, 20 µM of each primer, 25 mM $MgCl_2$ and 0.25 µl (5 U/ml) of Ampli*Taq* Gold™ polymerase according to the following PCR program: 94°C, 10 min; 21 cycles (94°C, 30 s; 68°C, 60 s; 72°C, 90 s); 72°C, 14 min and 4°C (hold). The synthesis of the HLA-DRβ1*01021 and HLA-DRβ1*10011 alleles was performed in a 25 µl mixture of 2 µl of cDNA, all four dNTPs at 1.25 mM, 1x PCR buffer II, 20 µM of each primer, and 0.15 µl (5 U/ml) of Ampli*Taq* Gold™ polymerase as follows: 94°C, 5 min; 30 cycles (94°C, 1 min; 57°C, 2 min; 72°C, 2 s); 72°C, 7 min and 4°C (hold). The minigenes were synthesised by amplifying 1 µl (1 ng/µl) cDNA in a total reaction mixture of 25 µl of 1x PCR buffer, all four dNTPs at 1.25 mM, 25 mM $MgCl_2$, 20 µM of each primer, and 0.5 µl (2.5 U/ml) of Platinum® *Pfx* DNA polymerase

Table 2.2 The primers used to amplify β-actin, HLA-DRβ1*01021, HLA-DRβ1*10011 and the minigenes. The primers used to amplify the HLA alleles were specific for conserved 5' and 3' regions of the DRβ1 chain. All primer sequences are written in 5' to 3' direction. F = forward and R = reverse.

NAME	PRIMER SEQUENCE
β–actin-F	GGCATCGTGATGGACTCCG
β–actin-R	GCTGGAAGGTGGACAGCGA
DR-beta-F	CGCGGATCCAGCATGGTGTGTCTG
DR-beta-R	GGAATTCCTCAGCTCAGGAATCCTGTT
Minigene-F	GTGCTCCTATCAGTGCTTAT
Minigene-M1-R	CCGATTGTCACCCACAGTGAA
Minigene-M2-R	GGGCAGGCTCAGGTA
Minigene-M3-R	CTCGGGGGGAGTTCT

(Gibco, LifeTechnologies, Garthersburg, MD) following a denaturation at 94°C for 5 min, then 15 cycles of polymerisation (denaturation for 1 min at 94°C, annealing for 2 min at 50°C and extension for 2 min at 72°C), synthesis completion for 7 min at 72°C and permanent hold at 4°C.

PCR analysis of the TCRAV and TCRBV repertoire was conducted using a panel of described oligonucleotide primers (Genevee *et al.*, 1992). The amplification was

performed in 25 μl of reaction mixture in presence of 1 μl of cDNA, 2.5 μl 10x PCR buffer, 200 μM of each dNTP, 1μM of each primer and 0.625 U of Ampli*Taq*® DNA polymerase (Roche Diagnostics Ltd, UK) on a DNA thermal cycler (Perkin Elmer Cetus Corporation, Emeryville, CA) for 25 cycles of amplification under the following condition: 95°C, 30 s denaturation; 60°C, 30 s annealing; 72°C 60 s extension. Negative controls were included with no cDNA in the mixture.

The RT-PCR preceding the subcloning of IL-10 was performed differently (see section 2.5.1). Moreover, PCR with single colonies has been described as a part of "Library Construction" (see section 2.4.12).

2.3.5 Subcloning of HLA-DRβ1*01021 and DRβ1*10011

The poly(A)+ RNA was extracted from the lymphoblastoid cell line (LCL15392) of melanoma Pt15392 using the Fast Track kit (Invitrogen, NV Leek, The Netherlands) and subsequently converted to cDNA as previously described. RT-PCR was performed using primers specific for the conserved regions of the HLA-DRβ1 chains (see section 2.3.4 and Table 2.2). The amplification reaction was carried out with Platinum® *Pfx* DNA polymerase (Gibco, Life Technology), after which the amplification products were cloned into the pcDNA3.1/V5-His-TOPO TA cloning vector (Invitrogen Corporation, Calsbad, CA). Single colonies were screened for the presence of the insert and the clones bearing the HLA-DRβ1*01021 and -DRβ1*10011 alleles were identify by DNA sequencing.

2.3.6 Electrophoresis

Standard high or low melting agarose gels were prepared from EuroClone® agarose (Euroclone Ltd, UK), while Molecular Biology Certificated agarose (BioRad, CA) was used to separate DNA samples from which fragments were to be purified. All gels were prepared as follow: agarose powder (0.7-1.5% w/v) was added to 1x Tris-acetate-EDTA

buffer, prepared from a 50x stock solution (2 M Tris, 1 M glacial acetic acid, 50 mM EDTA), and was boiled in an microwave until a clear, transparent solution was obtained. The melted agarose was cooled to 50°C, after which it was stained with ethidium bromide (EtBr) to a final concentration of 0.5 µg/ml and casted in a gel tray. 1 µl of gel-loading buffer (Invitrogene™ LifeTechnologies) was added to each 10 µl DNA sample. The samples were analysed on agarose gels run at a constant voltage (90 V) for 1h in a Gibco BRL Horizontal Electrophoresis Apparatus (Invitrogene™ LifeTechnologies) with 1x TAE buffer, after which the DNA fragments in the gel were visualised under UV light. The molecular markers 100 bp DNA Ladder and 1 kb DNA Ladder (Invitrogene™ LifeTechnologies) and MassRuler™ DNA Ladder, High Range (MBI Fermenta, Dasit S.p.a, Italy) were used to size the DNA on the gel.

2.3.7 Purification of DNA bands from gel

DNA fragments were extracted from agarose gels according to the guidelines in the QIAquick Gel Extraction Kit Protocol (Quiagen S.p.A., Milan, Italy). Briefly, an excised gel slice (containing the DNA fragment of interest) was placed in an Eppendorf tube and 3 volumes of QG buffer was added per 1 volume of gel, i.e. 100 µl Buffer QG buffer to 100 mg gel. The sample was then incubated at 50°C with vortexing every 2 min until the gel was completely dissolved (approximately 10 min). The hereafter yellow sample was precipitated with 1 gel volume of 100 % isopropanol, added to a QIAquick column and centrifuged for 1 min at 13000 rpm. Next, 0.5 ml Buffer QG was added to the column that was centrifuged for 1 min at 13000 rpm, a step that removes all traces of agarose. The column was then washed with 0.75 ml Buffer PE and centrifuged for 1 min at 13000 rpm. To remove also residual ethanol, the column was centrifuged one additional time for 1 min at 13000 rpm. The pure DNA sample was eluted with 50 µl of 10 mM Tris-HCl, pH 8.0 by centrifugation for 1 min at 13000 rpm after 1 min rest.

2.3.8 Ligation of DNA insert to an expression vector

All ligations were performed according to the manufacturers' guidelines in the DNA Ligation Kit Version 2 protocol (TaKaRa Biomedicals Inc., CA, USA). A reaction mixture was prepared with the dephosphorylated vector and the purified insert at a molecular insert: vector ratio of 3:1 to a final volume of 10 µl containing 5 µl Solution I (contains T4 DNA ligase). Transformation was performed immediately after the ligation (see section 2.3.21).

2.3.9 Dephosphorylation of vector DNA prior to ligation

To avoid re-ligation of digested vector DNA, calf intestinal alkaline phosphatase (CIP) was used to remove the 5' phosphate group from the vector backbone. Dephosphorylation was performed according to the guidelines provided by the manufacturer (Promega Corporation, Wisconsin , USA). A digestion mixture was prepared as follows: the digested expression vector (0.1 µg/µl) was incubated with CIP (0.01 U/µl), 10x CIP buffer and distilled water up to a volume of 70 µl for 15 min at 37°C. The DNA was then incubated for 15 min at 56°C. 7 µl of CIP (0.01 U/µl) was added to the sample, after which the two incubation steps were repeated. Then the reaction was stopped by the addition of stop solution (a volume corresponding to 1/10 of the original sample volume). Finally, the vector DNA was boiled for 10 min at 68°C.

2.3.10 Precipitation of DNA

DNA samples were precipitated as follow: 0.1 volume of 3M NaAc, pH 5 was added to 1 volume of DNA, after which 3 volumes of 100% ethanol (-20°C) was added. This solution was then left to precipitate on dry ice for 30 min or overnight at -80°C. After precipitation, the sample was centrifuged at 4°C for 20 min and 13000 rpm to recover the DNA. The

pellet was washed 3x with 70% ethanol and then left to evaporate at room temperature for 30 min, before it was resuspended in 10 mM Tris-HCl, pH 8.0.

2.3.11 Preparation of DNA for sequencing

All samples were prepared for sequencing by the same sequential steps: 1) overnight precipitation with 3 NaAc, pH 5; 2) resuspension of the pellet in 10 µl sterile water and 3) preparation of the samples for sequencing. Each sample was prepared as a 15 µl mixture containing 1.3 µg DNA template and 9 pmol of primer. DNA sequencing of IL-10 and the HLA-DRβ1*01021 and DRβ1*10011 alleles were performed with primers specific for the vector regions adjacent to the multiple cloning site, forward primer: 5'-TAATACGACTCACTATAGGGAGA-3', reverse primer 5'-TAGAAGGCACAGTCGAGG-3'.

2.3.12 Digestion

DNA samples (0.5-1 µg) were digested with 3-10 U of enzyme per µg of DNA by incubation for 2-3 h in a 37°C water bath. The enzyme buffer was present as a 0.1 volume of the final reaction volume. Enzymes were purchased either from New England BioLabs, MA, USA or Amersham Pharmacia Biotech, Buckinghamshire, England.

2.3.13 Preparation of antibiotic stocks

All stock solutions of antibiotics were dissolved in distilled water and sterilised by filtration through a 0.22 or 0.45 µm Millex®-GS Duropore® (PVDF) membrane (Millipore S.A., Bedford, MA, USA). Ready solutions were divided as 1 ml aliquots and stored at -20°C for a maximum of 4 months. To avoid inactivation of ampicillin (Sigma Chemical Co., St Louis, USA) due to its sensitivity to light, residual stocks were not re-frozen and used a second time.

2.3.14 Glycerol storage of bacteria

All glycerol stocks were prepared from overnight cultures of bacteria mixed with 20% glycerol (autoclaved), i.e. 800 µl overnight culture and 200 µl glycerol ratio. The bacterial stocks were stored in 2 ml tubes with screw caps at -80°C.

2.3.15 Plating of bacteria

All bacterial host strains were preserved as glycerol stocks at -80°C. Prior to DNA preparation, all bacteria were recovered as follows: a sterile loop stick was used to scrape a layer of bacteria from a frozen glycerol stock and then streak them onto Luria-Bertani (LB) agar plates (see section 2.3.16), which were incubated upside down at 37°C over night and further stored at 8°C for 1-2 weeks.

2.3.16 Preparation of agar plates for bacterial growth

Agar plates were prepared from a 1x LB stock solution, pH 7 (1% w/v NaCl (Fluka, BioChemica, Sigma-Aldrich S.r.l., Milan, Italy), 1% w/v tryptone (Acumedia™, Acumedia Manufacters, Inc., Baltimore, Maryland, USA), 0.5% w/v Bacto Yeast extract (DifcoLaboratories, Detroit, Michigan, USA) and 1.5% w/v Bacto® Nutrient Agar (DIFCO Laboratories)). Sterile Petri dishes were filled to 2/3 with 1x LB medium and left to solidify under a bacterial hood. If selective plates were prepared, the antibiotic (normally ampicillin at 50 mg/ml) was added to the autoclaved medium after it had cooled to approximately 40°C. All LB plates were stored upside down at 8°C for a maximum of 4 months.

2.3.17 Small scale preparation of plasmid DNA: Mini-preparation

Today standard protocols for preparation of plasmid DNA have been adapted from a rapid alkaline lysis method for extraction of plasmid DNA (Birnboim and Doly, 1979) and become optimised by a procedure that mediates adsorption of DNA silica glass surfaces by

high salt treatment (Vogelstein and Gillespie, 1979). All small scale plasmid preparations were performed according to the guidelines in the QIAprep Spin Miniprep Kit (250) protocol (Quiagen S.p.A., Milan, Italy). *Escherichia coli* DH5α (*E.coli*) was used as a host strain, as it is effective in propagating plasmid DNA. All centrifugations were performed in a table-top Biofuge, Heraeus Instruments centrifuge at a maximum speed of 13000 rpm, if not otherwise indicated. Prior to DNA preparation, all bacteria were recovered from glycerol storage (see section 2.3.15) prepared as fresh out streak on LB agar plates, from which single colonies were streaked onto individual selective LB plates to assure a clonal origin for the plasmid DNA.

As a first step of the mini-preparation, one single bacterial colony was picked from such a fresh outstreak of *E.coli* DH5α, inoculated with 3 ml of 1x LB medium containing 50 mg/ml of ampicillin and incubated overnight at 37°C in Orbital Incubator, Model 4520 (Forma Scientific, Inc.) with vigorous shaking, i.e. 200 rpm. A volume of 0.8 ml overnight culture was prepared as glycerol stock (see section 2.3.14), while 2.2 ml was used further for plasmid DNA extraction. The cellular pellet was recovered by centrifugation for 10 min at a maximum speed of 13000 rpm in a table-top centrifuge, carefully resuspended in 250 µl of P1 Buffer and transferred to 1.5 ml Eppendorf tubes. The cells were lysed by the addition of 250 µl of P2 Buffer and the tube gently inverted 4-6 times. The lysis reaction was stopped after exactly 5 min by the addition of 350 µl of N3 buffer, the tube was then immediately inverted 4-6 times. This treatment caused precipitation of denatured proteins, chromosomal DNA, cellular debris and sodium dodecyl sulphate (SDS), while the plasmid DNA was released in the supernatant. This solution was centrifuged for 1 min, the supernatant was transferred to a QIAprep Spin column placed in 2 ml collection tube and centrifugated for additional 1 min. Residual wash buffer was removed by centrifugation of the empty column for 1 min and left the purified plasmid DNA bound to the silica-gel

membrane. In the last step, the QIAprep Spin column was left for 1 min with 100 μl of 10 mM Tris-HCl, pH 8.0, before the pure plasmid DNA was eluted by centrifugation for 1 min. The collected DNA was stored at -20°C.

2.3.18 Large scale plasmid preparation of plasmid DNA: Maxi-preparation

All large scale plasmid preparations were performed according to the guidelines for low copy-plasmids described in the QIAGEN Plasmid Maxi Kit (25) protocol (Quiagen S.p.A., Milan, Italy). *E.coli* DH5α was used as a host strain. A Sorvall® RC-5C Plus Superspeed Centrifuge (Kendro Laboratory Products, Germany) was used for all centrifugations.

A single colony was picked from a fresh out streak of a glycerol stock (see section 2.3.15), inoculated with 500 ml 1x LB medium, 50 μl (50 mg/ml) of ampicillin (Sigma Chemical Co., St Louis, USA) and 200 μl (1%) glucose (prepared from 18% w/v glucose in deionized water) and left to incubate overnight at 37°C with vigorous shaking, i.e. 250 rpm. This pre-culture was then inoculated with 500 ml LB medium containing 500 μl (50 mg/ml) of ampicillin and incubated overnight at 37°C and with shaking at 250 rpm. The bacterial pellet was harvested by centrifugation for 15 min at 6000 rpm and carefully resuspended in 10 ml of Buffer P1. 10 ml of Buffer P2 was added to the cells, which were left to lyse at room temperature for maximum 5 min to avoid irreversible denaturation of the plasmid DNA. The reaction was neutralised by the addition of 10 ml pre-chilled Buffer P3 and left on ice for 15 min to allow full precipitation of genomic DNA, proteins, cellular debris and SDS. The sample was then centrifuged for 30 min at 12000 rpm, after which the plasmid containing supernatant was applied to a pre-equilibrated QUIAGEN-tip 500 (10 ml Buffer QBT) and allowed to enter the resin by gravity flow. The plasmid DNA was washed twice with 30 ml of Buffer QC to remove contaminants as RNA, carbohydrates and proteins. The DNA was eluted with 15 ml of Buffer QF, precipitated with 10.5 ml of

isopropanol and collected by centrifugation for 30 min at 4°C and 9500 rpm. The DNA

pellet was washed once with 70% ethanol, air dried (5-10 min) and resuspended in 250 μl

10 mM Tris-HCl, pH 8.0.

2.3.19 Spectrophotometric measurement of DNA concentration

The absorbance of all DNA and RNA samples were measured at 260 nm and 280 nm in an

Ultrospec® 3000 Spectrophotometer (Amersham Pharmacia Biotech UK Limited,

England). The concentration was automatically calculated using the convention that an

absorbance unit at 260 nm corresponds to 50 μg/ml for double stranded DNA and 40

μg/ml for RNA samples. An optical density ratio (OD_{260}/OD_{280}) of 1.8-indicated that a

pure DNA sample free from protein or phenol, only such samples were used further.

2.3.20 Preparation of competent cells

A smear of colonies (see section 2.3.15) was taken from a freshly streaked *E.coli* DH5α

cells and resuspended in 2 ml of 1x SOC medium (1% w/v tryptone (Acumedia™,

Acumedia Manufacters, Inc.), 0.5% w/v Bacto Yeast extract (DifcoLaboratories) and

0.05% w/v NaCl (Fluka, BioChemica, Sigma-Aldrich S.r.l.), 10 mM MgSO4 and 0.4% w/v

glucose). This 2 ml suspension was then used to inoculate 100 ml SOC medium. The cells

were then incubated at 37°C and 250 rpm for approximately 2-3 h, i.e. until an optical

density (OD) of 0.3 - 0.6 at 600 nm was reached. The culture was then divided into two

pre-chilled centrifuge tube, left on ice for 20 min and centrifugated at 4°C at 3000 rpm for

15 min. All steps hereafter were performed on ice. The pellet was carefully resuspended, in

30 ml prechilled TBI buffer (30mM KoAc, 50mM $MnCl_2*4H_2O$, 100 mM RbCl, 10 mM

$CaCl_2*2H_2O$ pH5.8 and 15% glycerol,). The cells were left on ice for 15 min and then

centrifuged as described above before the pellet was resuspended in 4 ml prechilled TBII

buffer (10 mM MOPS, 75mM $CaCl_2*2H_2O$,10 mM RbCl pH6.8 and 15 % glycerol). The

cells were left for additional 15 in on ice and then divided as 220 µl aliquots of competent cells into Eppendorf tubes that had been prechilled for 2 min on dry ice and ethanol. The competent cells was then stored immediately at -80°C and kept for a maximum of 4 months.

2.3.21 Transformation with competent cells

To preserve the transformation efficiency of the competent cells (see section 2.3.20), *E. coli* DH5α, all steps were performed on ice with pre-chilled tubes. The competent cells were thawed on ice and immediately upon thawing, 100 µl of cells were added to 15 ml Falcon tubes. In a subsequent step, the cells were treated with β-mercaptoethanol (prepared as a stock solution of 14.4 mM in water) to a final concentration of 25 mM. The cells were gently swirled every 2 min for a period of 10 min, after 2 µl (1 ng/µl) DNA was added and the samples left for 30 min to allow the DNA to adhere to the cell surface. The samples were then heat pulsed for 45 seconds in a 42°C water bath and placed on ice for 2 min. The physically stressed cells were subsequently recovered by the addition of 900 µl of pre-heated (42°C) SOC medium (2% w/v tryptone (Acumedia™, Acumedia Manufacters, Inc.), 0.5% w/v Bacto Yeast extract (DifcoLaboratories) and 0.05% w/v NaCl (Fluka, BioChemica, Sigma-Aldrich S.r.l.), 10 mM $MgSO_4$ and 0.4% w/v glucose) incubated for 1 h at 37°C and shaken at 250 rpm. The cells were plated as 50 µl, 100 µl and 150 µl aliquots onto LB/ampicillin plates (see section 2.3.16) and incubated overnight at 37°C. Plates with colonies of interest were stored at 4°C for up to 1 month. Each transformation was performed with 1 ng of the highly transfectable pUC19 (NewEngland BioLabs, MA, USA) as positive control of the reaction and as a reference for the transformation efficiency (i.e. colonies/µl) of the samples.

2.4 CONSTRUCTION OF THE MELANOMA CDNA LIBRARY

2.4.1 Extraction of polyA-mRNA

In order to isolate mRNA from melanoma cells, the guidelines reported in the FastTrack™ 2.0 Kit for Isolation of mRNA (Invitrogen) were followed. Briefly, 40×10^6 *in vitro* cultured melanoma cells, derived from melanoma Pt15932, were lysed, filtered 4 times through a plastic syringe (0.22 µm) and then incubated for 40 min at 45°C to digest residual proteins and ribonucleases. To further ensure an isolation of cleaner mRNA, the sample was adjusted to a final NaCl concentration of 0.5 M and filtered 4 additional times through a plastic syringe to shear any remaining DNA. The mRNA sample was gently rocked for 60 min at room temperature together with oligo(dT) cellulose to increase its binding to the oligo(dT) cellulose. Additionally, the oligo(dT) cellulose was centrifuged and after removal of the supernatant, the resin bed containing the mRNA, was submitted to various washing steps. The resin bed was resuspended in Binding Buffer, centrifuged and then repeatedly washed and centrifuged 4 times with Low Salt Wash Buffer to remove SDS and contaminating rRNA. The oligo(dT) cellulose was then transferred to the spin-column. Finally, the column was filled with Low Salt Wash Buffer and centrifuged. This wash procedure was repeated until the OD_{260} of the flow-through was < 0.05. In a final step the mRNA was eluted with 2 washes using the Elution Buffer and then centrifuged. The two eluates were pooled together in one tube and then precipitated with 3 M NaAc and 100% EtOH, stored at -80°C overnight and then centrifuged at 13000 rpm for 20 minutes. The mRNA pellet was resuspended in 20 µl Elution Buffer and its concentration determined by Ultrospec 3000 UV/Visible Spectrophotometer (Amersham Pharmacia Biotech).

In order to obtain a more pure mRNA, a second round of washing was performed. More precisely, the previously obtained pellet was dissolved in 1.5 ml Elution Buffer and then

adjusted with 150 µl 5 M NaCl to a NaCl concentration of 0.5 M (corresponding to the composition of the Binding buffer) to favour the binding of the mRNA to the resin.

All the subsequent steps were performed as described for the first purification. The final mRNA was then resuspended in 400 µl and the content of RNA determined using a Ultrospec 3000 UV/Visible Spectrophotometer (Amersham Pharmacia Biotech). mRNA was then stored at −80°C.

2.4.2 cDNA synthesis

With the aim to construct a cDNA library, the SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning (Gibco, Life Technology, Inc.) was used as a guideline.

However, in order to obtain the Ii-cDNA fusion library corresponding to our demands, another adapter and an expression vector different from the ones indicated in the protocol were used (see section 2.4.5 and 2.4.8 respectively); otherwise the suggested conditions were followed. Furthermore, no radioactive labelling with $[\alpha^{32}P]dCTP$ was used neither in the first or the second cDNA strand synthesis to calculate the yield.

2.4.3 First strand cDNA synthesis

6.6 µg of polyadenylate (polyA) mRNA were use for cDNA construction. mRNA was thawed and centrifuged at 4°C at 16000xg for 30 min, washed once in 70% ethanol. Then, in order to perform the first strand synthesis, the protocol recommendations were carefully followed. The starting amount of 6.6 µg of mRNA was dissolved in 6 µl DEPC-treated water, incubated for 10 min at 70°C with 2 µl an oligo(dT) primer [5'-pGACTAGT<u>TCTAGA</u>TCGCGAGCGGCCGCCC(T)$_{15}$-3'] containing a *Xba* I site (underlined) and quickly chilled on ice. The additional components for the reaction, 4 µl 5x First Strand Buffer, 2 µl 0.1 M DTT and 1 µl 10 mM dNTP Mix, were added and

following 2 min incubation at 37°C the SuperScript II reverse transcriptase (RT) was added. The quantity of enzyme was related to the starting amount of mRNA used. More precisely, 5 µl of SuperScript II RT (1000 U) was used to convert the 6.6 µg of polyA-mRNA into first strand cDNA during 1 h incubation at 37°C. The reaction was terminated on ice.

2.4.4 Second strand cDNA synthesis

In order to synthesise the second cDNA strand, the following components were added to the 20 µl of first strand cDNA: DEPC-treated water (91 µl), 5x Second Strand Buffer (30 µl), 10 mM dNTP Mix (3µl), *E. Coli* DNA Ligase (10 units/µl), *E. Coli* DNA Polymerase I (10 units/µl) and *E. Coli* RNase H (2 units/µl) to a final reaction volume of 150 µl. The second strand synthesis has been conducted for 2 h at 16°C. Then 2 µl (10 units) of T4 DNA polymerase was added, and the tube was left for additional 16 min incubation at 16°C. The reaction was stopped on ice and 10 µl of 0.5 M EDTA was added. The new synthesised cDNA was purified from contaminating proteins by the usage of QuickClean™Enzyme Removal Resin (Clontech Laboratories, Paolo Alto, USA). According to this protocol, a volume of QuickClean Resin corresponding to 1/10th volume of the cDNA sample should be used for purification; in our case 16 µl QuickClean Resin was added to the synthesised cDNA (162 µl). The tube was centrifuged at 14000 rpm for 1 min and the procedure was repeated once with the supernatant, now using 18 µl of QuickClean Resin to a total volume of 180 µl cDNA.

Finally, the purified cDNA was precipitated with 98 µl 7.5 M NH$_4$OAc and 0.7 ml 100% ethanol and left overnight at -80°C. The next day, the cDNA was centrifuged at 4°C and 16000xg for 30 min, washed twice with 70% ethanol with equal centrifugations and finally

dried in a speed vacuum centrifuge. The cDNA pellet was dissolved in 25 µl DEPC-treated water and stored at -20°C until the adapter ligation reaction was performed.

2.4.5 BstXI adapter ligation

With the purpose to ligate the *Bst* XI adapter to the synthesized cDNA, the following components were added to the cDNA pellet: 25 µl DEPC-treated water, 10 µl 5x T4 DNA Ligase Buffer, 10 µl *Bst* XI Adapters (1 µg/µl) and 5 µl T4 DNA Ligase (1 unit/µl) to a final volume of 50 µl. The reaction was left to proceed at 16°C overnight (i.e. for a minimum of 16 h). The next day, the cDNA was deproteinised as previously described using the QuickClean™Enzyme Removal Resin protocol (Clontech Laboratories, Palo Alto, CA, USA) and then precipitated with 30.2 µl 7.5 M NH_4OAc and 18.5 ml 100% ethanol and left at -80°C overnight.

2.4.6 Digestion with Xba I

cDNA was centrifuged at 4°C and 13000 rpm for 30 min, washed twice with 70% ethanol and the cDNA pellet resuspended in 36 µl DEPC-treated water; 5 µl 10x NEB 2 buffer, 5 µl 10x BSA and 4 µl *Xba* I (16 U/µl) was then added and the mixture incubated at 37°C for 5 h. In an additional step, the digested sample was heat inactivated at 65°C for 20 min. Moreover, the sample was further purified from contaminating proteins and precipitated and washed as described above.

2.4.7 Column chromatography

With the aim to obtain a high quality library with large inserts, the *Xba* I digested-cDNA was size fractionised by liquid chromatography. This procedure optimises the size of the cDNA fractions in a way such that unwanted components like smaller cDNAs (< 500 bp),

residual adapters and primer-adapter fragments released by the *Xba* I digestion can be separated from the larger inserts.

Briefly, cDNA Fractionation Column was equilibrated exactly as described in the SuperScript protocol by 4 complete washes with 0.8 ml TEN buffer each. The pellet of the *Xba* I digested cDNA was diluted in 100 μl TEN buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA, 25 mM NaCl) and then left to hydrate on ice. After this time, DNA was loaded onto column, which was left to drain completely, and the eluted buffer collected in tube 1. Next, 100 μl TEN buffer was added to the top of the column and collect in the sample n 2. These two first fractions just contained the flow through of the TEN buffer and were thus not further considered. A second aliqout of 100 μl TEN buffer was added to the column. A total volume of 550 μl was collected in separate fractions of 35μl each. The fractions # 3 to #12 were further analysed for their content of large cDNA inserts, while the remaining ones were not considered.

2.4.8 Library construction

With the aim of a constructing a special cDNA library, in which the invariant chain (Ii) was fused in frame with the cDNA, the pEAK vector containing the first amino acid of Ii (pEAK8/Ii) was used. This vector was kindly provided by Dr. Paul Robbins, National Institute of Health, Bethesda, MA.

2.4.9 Digestion of the pEAK8/Ii vector

The pEAK8/Ii vector was submitted to double digestion as follows: 2.8 μg pEAK8/Ii vector, 30 U *Bst* XI, 6μl BSA (10x) and 6 μl NEB 2 buffer (10x) was diluted with water to a final volume of 58 μl, incubated at 55°C for 7 h and then heat inactivated for 20 min at 65°C. The second digestion proceeded immediately with the addition of 2 μl *Xba* I (16

U/µl) and an incubation for 7 h at 37°C. The double digested vector was heat inactivated and stored at -20°C. The *Bst* XI - *Xba* I digested vector was later on deproteinised twice using the QuickClean™Enzyme Removal Resin protocol (Clontech Laboratories), as previously described. The vector was then resuspended in 30 µl TRIS 10 mM, pH8. Its concentration was determined equal to 47 ng/µl by an Ultrospec® 3000 UV/Visible Spectrophotometer (Amersham Pharmacia Biotech). The *Bst* XI – *Xba* I digested pEAK8/Ii vector was used for cDNA ligation

2.4.10 Ligation of cDNA to the pEAK8/Ii vector

For each ligation a 2:1.molar ratio of cDNA:vector was used. The ligation was performed as follows: 4 µl 5X T4 DNA ligase buffer, 80 ng of *Bst* XI - *Xba* I digested pEAK8/Ii vector (47 ng/µl), 25 ng cDNA and 1 µl T4 DNA ligase (1 U/µl) were diluted with DEPC-treated water to a final volume of 20 µl per ligation and incubated for 6 h at room temperature. cDNA was obtained from fractions #7 and #8 (pooled together; 1.14 ng/µl), #9 (3 ng/µl) and #10 (4.5 ng/µl).

2.4.11 Electroporation

The ligation samples from above (after 6 h incubation) were precipitated with 1 µg/µl tRNA, 750 mM ammonium acetate and 2.5 volumes of 100% ethanol and left at -20°C overnight. The following day, the precipitated cDNA washed with 70% ethanol, dried for 10 min at 37°C in a speed vacuum centrifuge. Finally, the DNA pellet (totally 105 ng) was dissolved in 4 µl DEPC-treated water resulting in a maximum DNA concentration 100 ng/µl, as advised in the protocol. Thus, for each fraction (# 7 + # 8, # 9 and # 10 now ligated to the pEAK8/Ii vector) 2 µl purified DNA was added to 40 µl ElectroMAX™ DH10B™ Competent Cells (Gibco, Life Technology) in a pre-chilled microcentrifuge tube

(the samples were prepared in duplicate) and then directly electroporated. Immediately after, room temperate SOC medium (2% w/v tryptone (Acumedia™, Acumedia Manufacters, Inc., Baltimore, Maryland, USA), 0.5% w/v Bacto Yeast extract (DifcoLaboratories, Detroit, Michigan, USA) and 0.05% w/v NaCl (Fluka, BioChemica, Sigma-Aldrich S.r.l.), 10 mM $MgSO_4$ and 0.4% w/v glucose) was added (up to 1ml) to the electroporated cells that were left to shake for 1 h at 37°C . The transformed cells from each fraction (each ligation) were pooled together and diluted for subsequent plating onto LB / ampicillin plates (see section 2.3.16).

2.4.12 Evaluation of colonies by PCR

In order to evaluate the Ii-cDNA fusion library in a quantitative and qualitative way, individual colonies were picked from the LB/Amp plates and further analysed by PCR for their content of cDNA inserts with the 5'-ACCTCGATTAGTTCTCGAGCTT-3' forward primer and 5'-ATTAGGACAAGGCTGGTGGGCACT-3' reverse primer, specific for the pEAK8 vector. The PCR was performed as follows: 5 min of denaturation at 95°C, 25 repeated cycles with 30 sec denaturation at 95°C, 1 min annealing at 54°C and 2 min extension at 72°C and then 7 min final extension at 72°C. The PCR cycle ended with the samples were kept at constant temperature of 4°C.

2.4.13 Multi-screen Minipreparations

The electroporated bacteria from fraction #9 and #10 of the Ii-cDNA library were divided in pools of about 100 bacteria and seeded in 96 deep wells in 2x LB (double strength LB without NaCl) containing 50 mg/ml of ampicillin (Sigma Chemical Co., St Louis, USA) Bacteria , cultured for 20 h, were then collected by centrifugation for 15 min at 2500 rpm and the cellular pellet resuspended in 80 μl of Solution I (30 mM glucose; 15 mM Tris-HCl, pH 8; 30mM Na_2EDTA; 60 μg/ml RNase A) . 80 μl of Solution II (0.2 N NaOH; 1%

SDS) was added to the deep well blocks, and then incubated for 2 min at room temperature. Then, 80 µl of Solution III (3.6 M potassium; 6 M acetate) was added and the cultures mixed vigorously for 2 min.

Approximately 240 µl of lysate was removed from each well and applied to a MultiScreen-NA lysate clearing plate (Millipore S.A., Bedford, MA, USA), that then was placed above the MultiScreen-FB plates in a special designed vacuum manifold equipment. The two different plates were then fixed onto the vacuum mainfold by applying the vacuum to maximum 8 inches Hg vacuum for 3 min. The supplied vacuum mediates a rapid transfer of the cell lysate from the wells in the MultiScreen-NA lysate clearing plate to the MultiScreen-FB plates filled with Binding Solution and their absorbance was read in an Ultrospec® 3000 Spectrophotometer (Amersham Pharmacia Biotech).

2.5 SUBCLONING AND SEQUENCING OF IL-10 PRODUCED BY MELANOMA SPECIFIC CD4+ T CELL CLONES

2.5.1 Subcloning of IL-10

To subclone the coding region of the *IL-10* gene, a pair of primers (Table 2.3) was designed with the forward initiating at the start codon for the IL-10 gene and the reverse ending with the stop codon, i.e. these primers lie in the exon regions contained in the complete coding sequence of the human *IL-10* gene (Accession number U16720 in the GenBank). They were used to generate the full length *IL-10*, using as template the RNA purified from the stimulated CD4+ T cell clones (Vieira *et al.*, 1991).

Thus, mRNA was extracted from anti-CD3 Ab (OKT3 used at 100 ng/ml) stimulated CD4+ T cell clone and then converted to cDNA (see sections 2.3.2 and 2.3.3). The RT-PCR for IL-10 was performed in a 50 µl reaction mixture containing 2 µl of cDNA template, all four dNTPs at 1.25 mM, 10x PCR buffer, 20 µM of each primer, and 0.4 µl (5 U/ml) of *Taq* polymerase according to the following PCR program: 94°C, 3 min; 30 cycles (94°C, 60 s; 60°C, 45 s; 72°C, 120s); 72°C, 7 min and 4°C (hold). The obtained full length (536 bp) PCR product of the IL-10 gene was subcloned into the pcDNA3.1/V5-His-TOPO TA cloning vector (Invitrogen Corporation).

The amplified full length *IL-10* gene, from two individual amplifications, was prepared for subcloning by the addition of 3'A overhangs to both ends using the TOPO cloning kit (Invitrogen). These modified IL-10 fragments were subcloned into the pcDNA3.1/ V5-His-TOPO TA cloning vector using TOPO cloning kit (Invitrogen) and then transformed into *E.coli* DH5α cells. After 24h incubation of the transformed cells, plated onto LB/Amp-plates, single colonies were picked up and analysed directly in RT-PCR for the presence insert using external primers mapping in the polylinker region of the vector. The cloning

procedures gave several positive colonies that were all found to contain the IL-10 cDNA (not shown).

Table 2.3: The primer sequences used to amplify the full length sequence of the IL-10 gene. The forward (F) primer cover the ATG start site of the genomic *IL-10* gene (Accession number U16720 in the GenBank) and the reverse (R) primer cover the stop site.

Name of primer	Primer sequence
IL-10-F	5'-ATG CAC AGC TCA GCA CTG CTC TGT TGC CTG-3'
IL-10-R	5'-TCA GTT TCG TAT CTT CAT TGT CAT-3'

2.5.2 Preparation of genomic DNA (for IL-10)

Genomic DNA was prepared from $10\text{-}15 \times 10^6$ cells by centrifugation for 10 min at 1800 rpm. The pellet was washed with 1.5 ml 1x PBS (BioWhittakerEurope), diluted in 2 ml 1x PBS and transferred to a 2 ml Eppendorf tube and centrifuged for 45 s at 13000 rpm. The pellet was resuspended with 500 µl of lysis buffer (50 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 100 mM NaCl and 1% SDS), after which 10 µl each of freshly prepared proteinase K (10 mg/ml) and pronase (10 mg/ml) were added. The sample tubes were placed horizontally and left to shake at 55°C overnight, which caused the break down of cellular membranes and components leaving only nucleic acids intact. On day 2, 30 µl of

RNase (10 ng/ml) was added and the tubes were incubated for 2 h at 37°C and then centrifuged for 8 min at 14000 rpm to separate the DNA from the debris of digested RNA. The DNA-containing supernatant was transferred to a tube containing 1 ml cold 100% ethanol and then mixed gently until chromosomal DNA was visible. The DNA was winded around a 5 μl glass micropipette and transferred to a new tube containing 750 μl cold 70% ethanol. The micropipette with the attached DNA was then inverted and the DNA left to air dry in this position for at least 10 min. Hereafter, the DNA was placed into a tube containing 150 μl 1x Tris EDTA (TE) buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). The micropipette was broken and the tip with the attached DNA was left to shake at 55°C overnight to dissolve the DNA. On the third day, the tubes were vortexed for 5 min at 14000 rpm and the pellet dissolved in 150 μl of 10mM Tris-HCl, pH 8.0. The genomic DNA was stored at -20°C.

2.5.3 Cytokine genotyping

The cytokine genotyping tray (One Lambda Inc) is a PCR based assay that uses primers specific for polymorphism detectable in the 5' promoter region of cytokine genes. The kit includes TNF-α, TGF-β1, IL-10, IL-6 and IFN-γ promoter-specific primers. These genetic polymorphisms strongly influence the capacity of IL-10 secretion and each genotype is associated with a low, intermediate or high phenotypes. Reagents were provided in pre-optimized quantities and used according to manufacturer's instructions. In addition, as the CD4 + T cell clones were too precious to use for this analysis, the genomic DNA was obtained from LCL of Pt15392.

The PCR-single strand polymorphism (SSP) analysis was performed in a 96-well 0.2 ml thin-walled tube tray for PCR divided into 6 sections with dried, pre-optimized primers for TNF-α (2 wells), TGF-β1 (4 wells), IL-10 (5 wells), IL-6 (2 wells) and IFN-γ (2 wells)

present in a detailed pattern related to each specific polymorphism (see Figure 5.4). More precisely, 1 μl of *Taq* DNA Polymerase (5 U/μl) was added to 180 μl of a D-mix (i.e. a pre-set dNTP-buffer mix), which was vortexed for 5 s and then pulsed-spinned to collect the whole mixture of which 9 μl was added to the negative control reaction tube containing 1 μl of deionised water. Then, 19 μl of genomic DNA (30 ng/μl) derived from LCL cells of Pt15392 (see section 2.5.2) was added to the reamining D-mix. The tube was vortexed for 5 s and then pulsed-spinned, after which 10 μl was added to each reaction tube except the negative control tube. Amplification was run on a GeneAmp® PCRSystem 9700 PCR machine (Applied BioSystems, CA, USA) according to the following protocol: 1 cycle (96°C, 130s; 63°C, 60 s), then 9 cycles (96°C, 10s; 63°C, 60s), followed by 20 cycles (96°C, 10s; 59°C, 50s; 72°C, 30s) and finally 1 cycle at 4°C (hold). The samples were run on a 2.5% low melting EuroClone® agarose gel (0.5 μg EtBr/ml) (Euroclone Ltd, Whetherby West Yorkshire, UK) with 0.5% TAE running buffer containing 0.5 μg/ml of EtBr at 50 V for approximatley 2 h. The DNA bands were then visualised with UV ligth.

2.5.4 Transwell experiments

Co-culture experiments were performed using polycarbonate transwells (Costar Inc., Acton, MA, USA) with 0.4 μm pores to allow passage of the cytokine containing medium but not of cells. PMBCs (5×10^5 cells/ml) from Pt15392 were added to the lower chamber and restimulated with the irradiated autologous melanoma (Me15392) (5×10^5 cells/ml) in a total volume of 2 ml RPMI 1640 10% HS. The transwell (upper chamber) contained an activated CD4+ T cell clone (5×10^4 cells/well), added to the well on its fifth day after restimulation with irradiated Me15392 (1×10^6 cells) at an effector:target (E:T) ratio of 4:1. After 2 weeks of co-culture PBMCs were tested for the presence of generated T cell populations by incubation with autologous melanoma (Me15392) or LCL (LCL15392) cells in the presence or absence of W6/32 (anti-HLA-A, -B and –C) or L243 (anti-HLA-

DR) mAbs. The supernatants were evaluated for their IFN-γ content by ELISA (see section 2.2.17). In a second setting, PMBCs (5×10^5 cells/ml) from Pt15392 were added to the lower chamber and pulsed with 1 μg/ml of the MART-1$_{27-35}$ peptide in the presence of an activated CD4+ T cell clone (5×10^4 cells/well) in the upper transwell. This clone was added to the well on its fifth day after restimulation with 1 μg/ml of the MART-1$_{27-35}$ peptide. Generated T cell populations were evaluated as described above.

RESULTS

Chapter 3

HLA-Cw8 RESTRICTED RECOGNITION OF A GP100 EPITOPE BY CD8+ T LYMPHOCYTES

3.1 INTRODUCTION

Several studies have been conducted on the immune biology of melanoma and this tumour has been found to express a variety of antigens as targets for T cell mediated recognition (as reviewed in (Robbins and Kawakami, 1996; Van den Eynde and van der Bruggen, 1997)). The majority of these antigens are self antigens, derived from transcription of normal, non-mutated genes (reviewed in (Kawakami and Rosenberg, 1996)), which, despite this feature, have been shown, under ceartain circumstances, to break self tolerance and generate cytotoxic T lymphocytes (CTLs) *in vitro* (Anichini *et al.*, 1993; Anichini *et al.*, 1996; Bakker *et al.*, 1994). In addition, the differentiation antigens MART-1 and gp100 have been reported to be recognised by tumour infiltrating lymphocytes (TILs) derived from melanoma patients (Kawakami *et al.*, 1995; Robbins *et al.*, 1994) and adoptive transfer of CTLs reactive against gp100 can mediate tumour regression (Kawakami *et al.*, 1995). Melanoma patients vaccinated with such antigens have occasionally been shown to deelop vitiligo as a side effect, especially in clinical responsive subjects (Rosenberg and White, 1996). This demonstrates that immune recognition of differentiation antigens can mediate destruction of melanocytes and therefore, immunotherapy of melanoma should be considered carefully for the risk of autoimmunity. In addition, recent studies have reported the first evidence of skin and eye damage in melanoma patients as a direct consequence of adoptive transfer of T cells directed to differentiation antigens (Dudley *et al.*, 2002; Yee *et al.*, 2000).

The differentiation antigen gp100 has been associated with tumour regression (Kawakami *et al.*, 1994b) and several of its T cell epitopes, presented by different HLA alleles, induce *in vitro* reactivity against melanoma (Kawakami *et al.*, 1995; Salgaller *et al.*, 1995). Moreover, CTLs directed against an epitope of gp100 (gp100 209-2M), modified to have stronger HLA-A2 binding capacities, have been detected at the metastatic site (Kammula

et al., 1999). Thus, peptide-based vaccine is a promising approach for cancer therapy, but peptide-specific immunity is not always associated with clinical response and tumour regression has only been observed in 10 – 30% of treated patients (reviewed in (Parmiani *et al.*, 2002a)) with the exception of one study in which 42% of subjects showed a clinical response (Rosenberg *et al.*, 1998). Reasons for this partial failure can be found in all the mechanisms activated by tumor cells to evade immune recognition and destruction. Many of these mechanisms have been identified by studies performed in melanoma patients (reviewed in (Marincola *et al.*, 2000)). One well documented strategy used by tumour cells to escape T cell control is represented by the down-regulation of HLA and/or of the target antigens (reviewed in (Garrido *et al.*, 1997)). The latter phenomenon has been well documented for Melan-A/MART-1 and gp100 in melanoma (Marincola *et al.*, 1996a).

Moreover, the fact that several of the most common tumour antigens (MAGE-3, tyrosinase, gp100 and Melan-A/MART-1) are presented by HLA-A2 (Kawakami *et al.*, 1995; Kawakami *et al.*, 1994c; van der Bruggen *et al.*, 1994; Wolfel *et al.*, 1994) that is frequently down modulated in melanoma (reviewed in (Ferrone and Marincola, 1995)), highlights the importance of finding other antigens and/or epitopes restricted by alternative HLA-A, -B and -C alleles. A combination of epitope loss and down regulation of HLA alleles facilitates tumour escape. Therefore, to be effective, a tumour vaccine should induce and sustain a broad CTL anti-melanoma response directed against multiple antigens restricted by alternative HLA alleles (Thomson *et al.*, 1998).

In this context, the study presented in this chapter was focused on the identification of antigens that can be presented on non-HLA-A2 alleles. A melanoma patient (Pt15392), still disease-free 12 years after surgical resection of lymph node metastases, was selected for the study. A previous analysis of the anti-tumor CD8+ T cell mediated response in this

patient revealed an immune response directed against gp100 and TRP-2 derived epitopes presented by HLA-A3 and -Cw8 alleles, respectively (Castelli *et al.*, 1998; Castelli *et al.*, 1999). However, additional tumor-specific CD8+ T cell clones were available and their nominal epitopes remained to be defined. One of these clones, not previously characterised, was used for my study. The results presented in this chapter indicate that this selected CD8+ T lymphocyte clone, namely TB686, is directed against a new gp100 derived epitope presented by the HLA-Cw8 allele.

3.2 AIM OF THE CHAPTER

• To functionally characterise an anti-melanoma CD8+ T cell clone and define its nominal epitope.

3.3 RESULTS

3.3.1 The selected CD8+ T cell clone TB686 recognises the autologous tumour in the context of HLA-B or -C.

The immune response directed against the autologous tumour in the melanoma Pt15392 has been analysed at the clonal level. Previous studies on this patient documented the presence at the tumour site of CD8+ T lymphocytes directed against peptides derived from melanosomal proteins including gp100 and TRP-2 (Castelli *et al.*, 1998; Castelli *et al.*, 1999; Mazzocchi *et al.*, 1996). Additional clones were available that, bearing different T cell receptor alfa (TCRAV) and beta variable regions (TCRBV), were potentially able to recognise different epitopes. With the aim of identifying such additional epitopes, a previously uncharacterised CD8+ T cell clone, namely clone TB686, was selected.

In order to define whether clone TB686 was involved in an anti-tumour response directed against a unique or shared antigen, TB686 lymphocytes were tested for the ability to lyse the autologous and allogeneic, partially HLA-matched melanoma lines (Figure 3.1). From
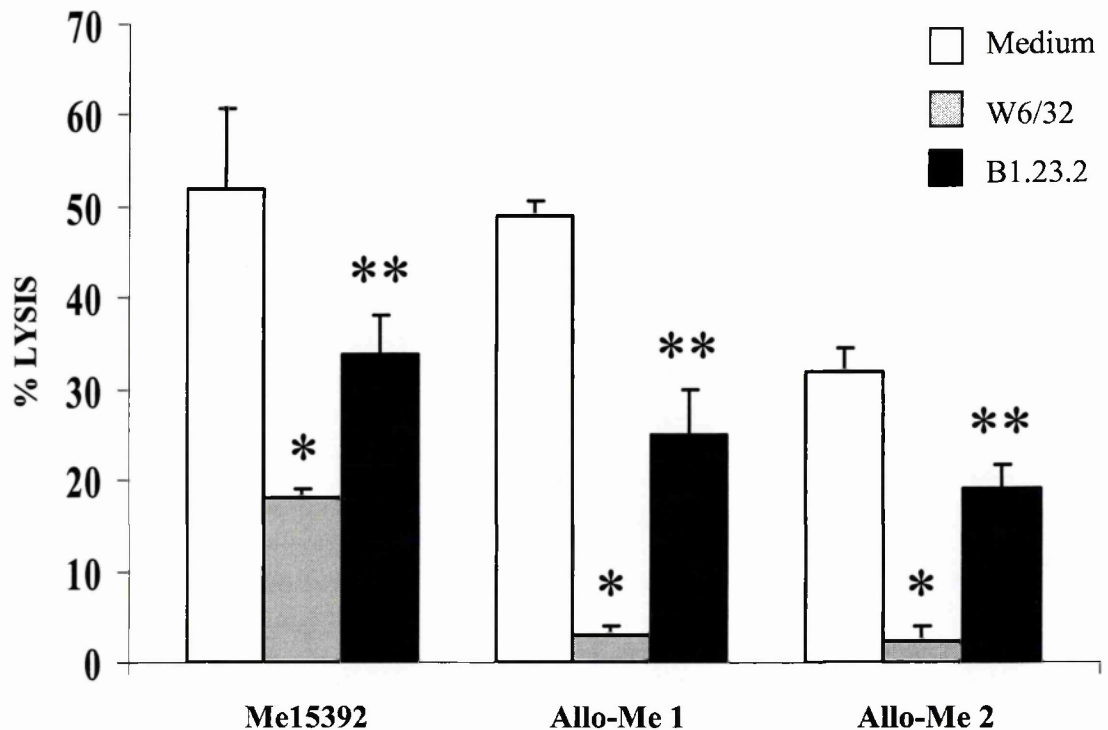
FIGURE 3.1: Cytotoxic activity against a HLA-B or –C restricted antigen expressed on both the autologous tumour and allogenic melanoma cells sharing the class I HLA haplotype with Pt15392. ^{51}Cr-labled autologous tumour cells (Me15392) and allogeneic HLA matched (HLA-B14 and –Cw8) melanoma lines (Allo-Me) (1000 cells/well), were co-incubated with the CD8+ T cell clone TB686 for 4 h at an E:T ratio of 20:1. There was a significant difference (*$p \leq 0.001$) between target cells blocked with the mAb W6/32 compared to unblocked controls. Also incubation with B1.23.2 mAb significantly blocked the cytotoxicity in a HLA-B and –C restricted way (**$p < 0.05$) compared to the unblocked control. W6/32 = anti-HLA-A, -B and -C mAb; B1.23.2 = anti-HLA-B and -C mAb. Results are expressed as mean ± S.D. for one representative experiments of two.

these data, it was concluded that TB686 T cells have a strong lytic activity directed against the autologous tumor. Tumor lysis was markedly inhibited by the addition of a mAb recognising a common HLA-A, -B and –C determinant, as well as the mAb B1.23.2 directed against a common determinant shared only by the HLA-B and –C alleles. Moreover, TB686 was not only activated by autologous tumor cells but also by other melanoma cell lines sharing the HLA-B and –C alleles. This pattern of recognition indicated that TB686 was directed against a common shared antigen. Unfortunately, normal melanocytes positive for the expression of the indicated alleles were not available and, therefore it was not possible to conclude whether or not the shared antigen was a differentiation antigen. The pattern of specificity of clone TB686 in the ^{51}Cr-release assay was fully consistent with the data obtained from parallel cytokine release experiments where clone TB686 produced a high amount of IFN-γ when incubated with the autologous melanoma, and with HLA-B14 and HLA-Cw8 matched allogenic melanoma lines (Figure 3.2). As expected from the cytotoxic profile, the release of IFN-γ was inhibited in the presence of an antibody specific for HLA-B and-C alleles.

However, while the use of W6/32 did not completely inhibit the cytotoxic release, a complete block could be observed after overnight incubation in the IFN-γ release assay. Although it is possible that W6/32 has a somewhat higher affinity for its determinant than B1.23.2, this partial effect may be due to the short incubation time.

3.3.2   Identification of the gene encoding the TB686 epitope

In order to identify the antigen recognised by TB686, transfection experiments were performed. COS-7 cells were double-transfected with either HLA-B14 or -Cw8 together with different known melanoma-associated antigens as shown in Figure 3.3. The screening had to be done individually with these alleles as it was not possible to define the restriction
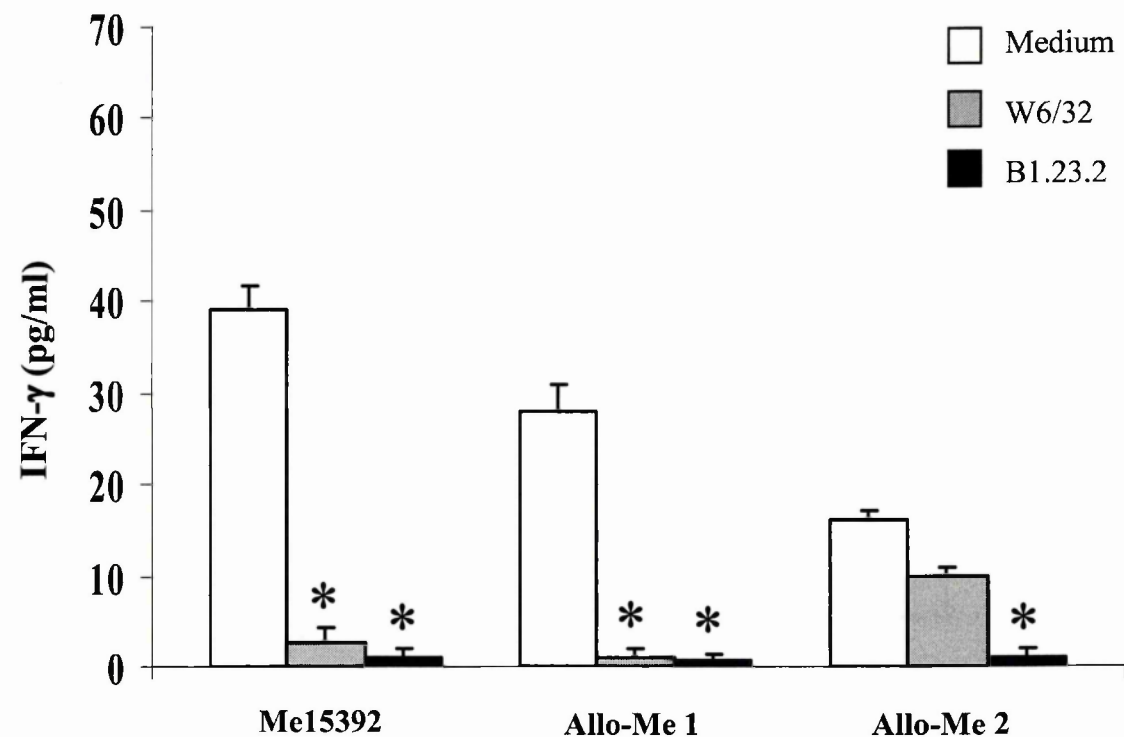
FIGURE 3.2: HLA-B or –C restricted recognition of an antigen expressed on both the autologous tumour and allogenic melanoma cells sharing the class I HLA haplotype with Pt15392. The CD8+ T cell clone TB686 (5×10^4 cells/well) was incubated overnight with the autologous melanoma cells (Me15392) or HLA matched (HLA-B14 and –Cw8) allogenic melanoma lines (Allo-Me) at an E:T ratio of 1:1 alone or in the presence of 1µg/ml of W6/32 or B1.23.2. A significant difference (*$p \leq$ 0.001) was observed for both W6/32 and B1.23.2 antibodies compared to the unblocked control, except for W6/32 in blocking of Allo-Me2. The supernatants were assayed for their IFN-γ content by ELISA. W6/32 = anti-HLA-A, -B and –C mAb; B1.23.2 = anti-HLA-B and –C mAb. These results are representative of the mean ± S.D. of 2 experiments.

FIGURE 3.3: HLA-Cw8 restricted recognition of the gp100 antigen. COS-7 cells (15×10^3 cells/well) were double transfected with 100 ng of plasmid cDNA encoding known tumour antigens and the pcDNA3.1 vector encoding the HLA-C*0802 (Cw8) restriction allele respectively. After over night incubation, the reactivity of TB686 against these antigens was evaluated by the addition of 5×10^3 TB686 cells/well. After an additional overnight incubation, the supernatants were tested for their IFN-γ release by ELISA. Transfection of gp100 significantly increased the IFN-γ release (*p<0.001) compared to control COS-7 cells transfected only with the HLA-Cw8 allele. However, a non-statistically significant increase over the background values of IFN-γ release was observed for MAGE-12 and Trp-1. The DEAE-dextran-cloroquine method was used for transfection. The dotted line indicates the background value of the test as determined by the IFN-γ release from COS-7 cells transfected only with the HLA-Cw8 allele. These data represent the mean ± S.D. values for one representative experiment out of two.
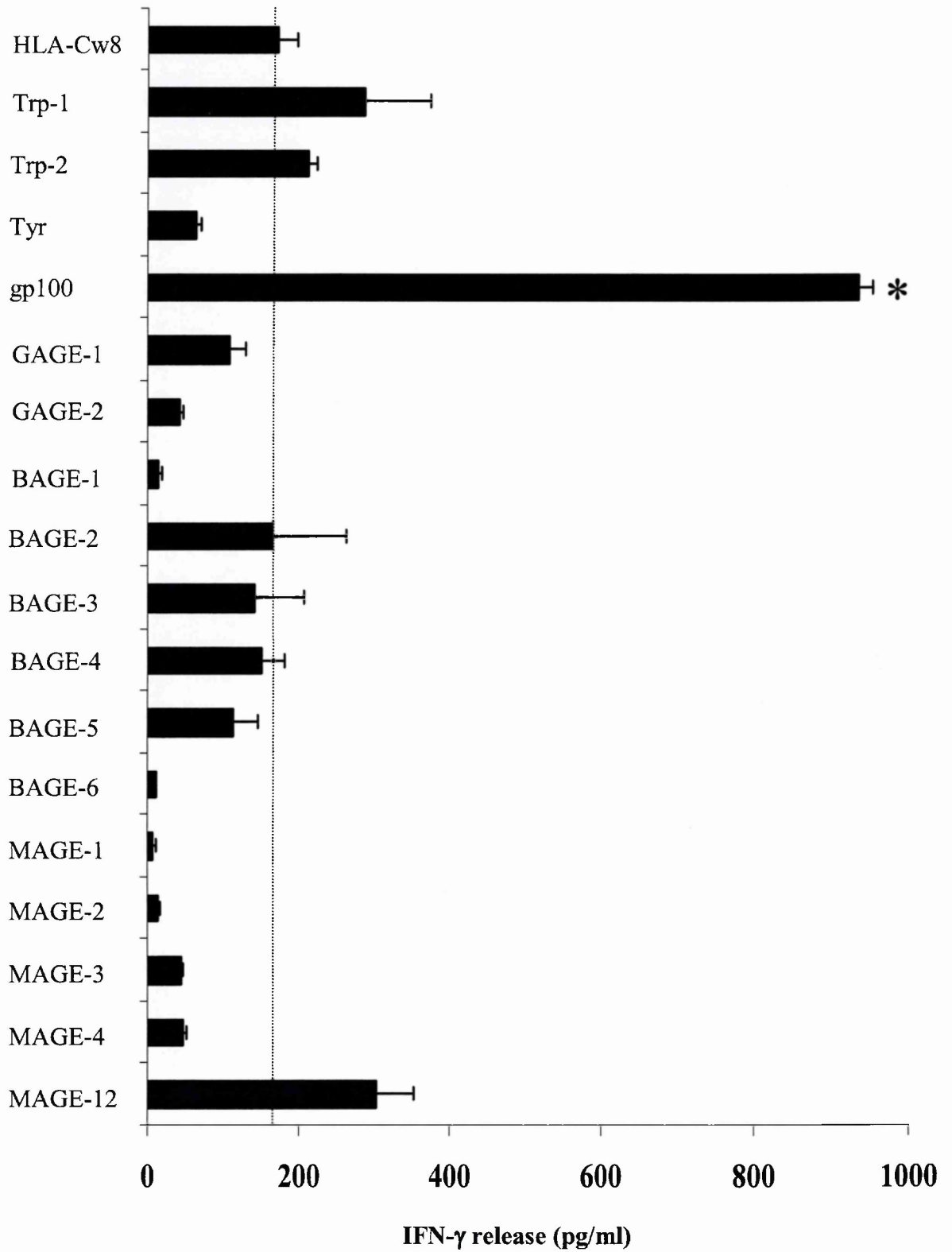
element for the TB686 clone, as the Ab B1.23.2 can not discriminate between the HLA-B and -C alleles and no melanoma cell lines expressing either B14 or Cw8 alone were available.

However, each transfectant was then evaluated for the capacity to specifically stimulate clone TB686 by a cytokine IFN-γ release assay. No positive response was observed upon transfection with the HLA-B14 (data not shown), whereas clone TB686 demonstrated an HLA-Cw8 restricted recognition of the gp100 antigen (937 pg released IFN-γ per ml) (Figure 3.3). Although some variation of the background values could be observed, especially for TRP-1 and MAGE-12, these data were not reproducible in subsequent tests and therefore disregarded. In addition, a gp100 peptide was shown to reconstitute the nominal epitope of TB686, as demonstrated by peptide pulsing of autologous LCLs in a IFN-γ-release assay (see Figure 3.4).

3.3.3 TB686 and TB254 recognise the same epitope of gp100

An HLA-Cw8 restricted clone, TB254, recognising the SNDGPTLI epitope of gp100, had previously been identified from the patient used in this study (Castelli *et al.*, 1999). Therefore, given that clone TB686, derived from the same patient and shown to be restricted in HLA-B or –C, also recognised gp100, TB686 was evaluated for its reactivity against SNDGPTLI. Autologous lymphoblastoid cells (LCL15392) were pulsed with the 3 previously described (Castelli *et al.*, 1999) overlapping peptides; gp100$_{71\text{-}78}$ (SNDGPTLI), gp100$_{70\text{-}78}$ (VSNDGPTLI) and gp100$_{69\text{-}78}$ (KVSNDGPTLI), and then incubated overnight with TB686 lymphocytes. As demonstrated in Figure 3.4, all 3 peptides were well recognised by TB686, with the decamer gp100$_{69\text{-}78}$ stimulating a somewhat higher IFN-γ release. However, this result shows that although expressing a different TCR than clone TB254
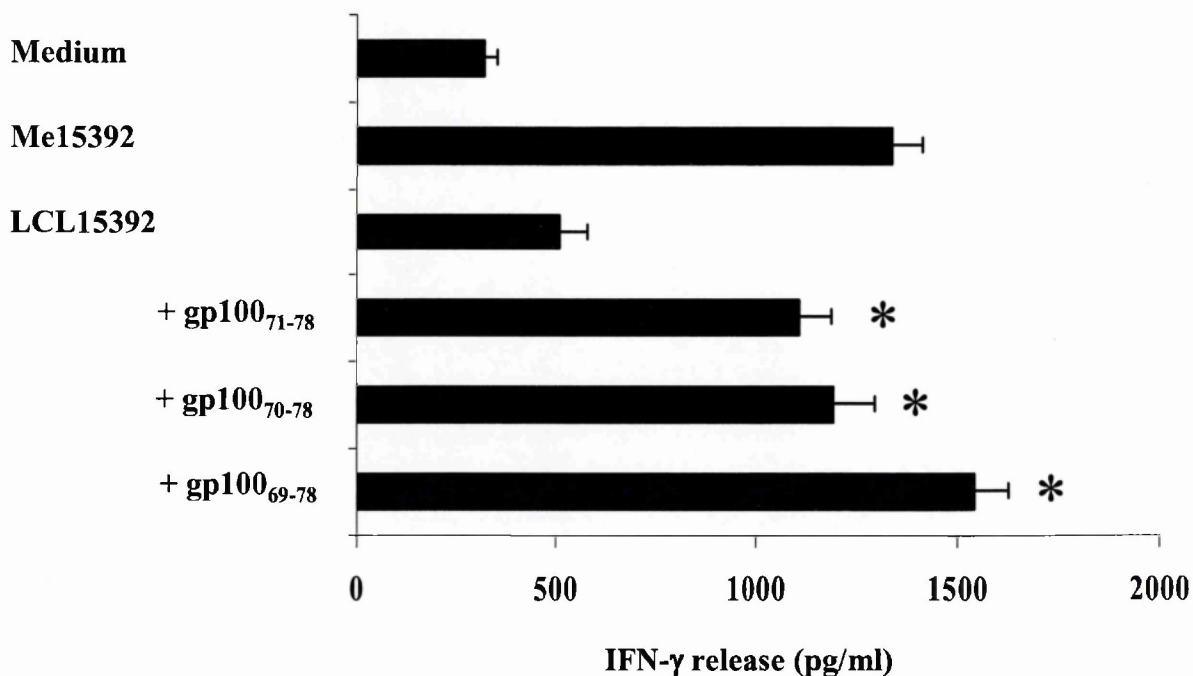
FIGURE 3.4: Reconstitution of SNDGPTLI as the immunogenic epitope for clone TB686. Autologous LCL cells (LCL15392) from Pt15392 were pulsed with 3 overlapping peptides of gp100, i.e. $gp100_{71-78}$ (SNDGPTLI), $gp100_{70-78}$ (VSNDGPTLI) and $gp100_{69-78}$ (KVSNDGPTLI), at a concentration of 1 µg/ml for 2 h at room temperature. Following over night incubation with CD8+ T cell clone TB686 (5000 cell/well) at an E:T ratio of 1:1; the supernatants were evaluated for their IFN-γ content by ELISA. Autologous melanoma cells (Me15392) were used as positive control. Transfection of the 3 peptides of gp100 all induced a significant IFN-γ release from clone TB686 (*$p \leq 0.005$) compared to unpulsed LCL15392 cells. Medium = lymphocytes without peptide. Data are expressed as mean ± S.D. of three experiments.

(Table 3.1) for which gp100$_{71-78}$ was identified, clone TB686 was specific for this same

peptide.

Table 3.1: The TCR expression of CTL Clones TB254 and TB686, which both recognise the
gp100$_{71-78}$ SNDGPTLI epitope. V = variable; J = joining region of the TCR. S = indicates members that
belong to the same variable-gene subfamily.

T cell clone	TCRVA	TCRVB	Reference
TB254	V8S1J50	V21S1J2S7	(Castelli *et al.*, 1999)
TB686	V7S2J24	V14S1J2S2C2	[1]Castelli

1)   Personal communication.

3.4 DISCUSSION

The data presented in this chapter show that a long surviving melanoma patient developed

at the tumor site a CTL response directed against a gp100-derived epitope. As illustrated in

Figure 3.3, clone TB686, derived from T lymphocytes purified from a lymph node

metastasis, recognised the differentiation antigen gp100. These data are consistent with

previous results reporting that other differentiation antigens such as Melan-A/MART-1,

tyrosinase, TRP-1,-2 and gp100, can be targets for a specific recognition by TILs

(Kawakami *et al.*, 1994a; Kawakami *et al.*, 1994b; Robbins *et al.*, 1994; Wang *et al.*, 1996; Wang *et al.*, 1995). Moreover, since this anti-gp100 response is associated with a good clinical prognosis in Pt15392, the results presented in this chapter further support the conclusion, based on previous studies, that melanosomal antigens, despite their self nature, are promising reagents for developing more efficient cancer vaccines (reviewed in (Kawakami *et al.*, 1998)).

TB686 is not the only CTL clone found in the tumor derived lymphocytes of Pt15392 directed against a gp100 derived epitope. The herein analysed CD8+ T cell clone, TB686, was shown to be specific for the gp100$_{71-78}$ SNDGPTLI epitope and this same peptide also reconstituted the nominal epitope for a previously defined CD8+ T cell clone, TB254, from this patient (Castelli *et al.*, 1999). The TCR sequences for these clones are presented in Table 3.1. Although these two clones recognise the same epitope, no obvious constraint in their TCR variable chain usage could be observed. The induction of multiple CTL clones directed against gp100 in the same patients has been described before (Kawakami *et al.*, 1995). In addition, similar results have been obtained for other tumour-specific antigens, and a polyclonal response in term of TCR composition of epitope specific CD8+ T cells has been observed for the Melan-A/MART-1. Nine AAGIGILTV (Kawakami *et al.*, 1994c) and three EAAGIGILTV specific T cell clones (Schneider *et al.*, 1998) expressed TCRs with different VA and VB families. Moreover, as previously shown (Castelli *et al.*, 1998; Castelli *et al.*, 1999), the gp100 response in this patient also included an additional gp100 derived epitope presented by the HLA-A3 allele. Thus, previously published data and the results reported in this chapter suggest that the anti-gp100 response was a crucial component in the overall immune response of Pt15392 to the autologous tumour. In fact, the anti-gp100 response in Pt15392 is polyclonal and two different gp100 derived epitopes

are presented by HLA-A3 and HLA-Cw8 respectively. Moreover, the HLA-Cw8-restricted anti-gp100 immunity involved independent CTLs expressing different TCRs.

The SNDGPTLI epitope presented in this chapter differs from other melanosomal antigens since it is presented by an HLA-C allele. This finding may have implications for vaccine development. In fact, more than half of the 15 previously identified epitopes of gp100 are restricted by the HLA-A2 allele, which is also the main restriction element for all other differentiation antigens of melanoma defined up to now. In addition, the predominance of the HLA-A allele has been exemplified by a recent study where TILs derived from 123 patients with metastatic melanoma were found to be specific for the HLA-A1, -A2, -A3, -A24 or -A31 alleles (Kawakami et al., 2000). HLA-B restriction has been reported in one case only for both MART-1/Melan-A and tyrosinase (Brichard et al., 1996; Schneider et al., 1998).

Although the HLA-A2 is expressed by a large proportion of the Caucasian population (40 - 45%) making the HLA-A2 restricted epitopes valuable tools for cancer vaccine, it is also true that this allele is frequently lost in metastatic tumors presumably as result of an in vivo T cell-driven immune selection (Kageshita et al., 1993; Maeurer et al., 1996; Natali et al., 1989). Therefore, it has become clear that to induce an immune response potentially able to control tumour growth, a vaccine should be polyvalent and include multiple CD8+ epitopes derived from different antigenic proteins and presented by different HLA alleles. By expressing HLA-B and HLA-C alleles on the cell surface after down regulation of the HLA-A allele, a tumour may still be susceptible to recognition by anti-melanoma CTLs restricted by those alleles. That the level of expression of a given HLA allele could be a crucial factor limiting the efficacy of T cell recognition has been demonstrated in a study where the percentage of melanoma cell lysis by an antigen specific HLA-A2 restricted

CD8+ T cell clone was directly correlated to the expression level of the HLA-A2 at the cell surface (Rivoltini *et al.*, 1995a).

Thus, this newly identified HLA-Cw8 restricted $gp100_{71-78}$ epitope can be useful, in conjunction with the previous defined HLA-A restricted epitopes of TRP-2 and gp100, for designing a polyvalent vaccine. Taken together, gp100 is an antigen which holds promise for future immunotherapy and with the identification of epitopes restricted also on HLA class II alleles (Kobayashi *et al.*, 2001), it may be possible to develop polyvalent vaccines that can also induce CD4+ T cell responses.

In conclusion, the TB686 anti-melanoma CTL was characterised as HLA-Cw8 restricted by showing that its recognition of the $gp100_{71-78}$ epitope results in an IFN-γ release that is 5 times higher than the background (Figure 3.3). These data suggest that epitopes presented on the HLA-B and HLA-C alleles can provoke an immune response similar to HLA-A restricted epitopes, despite some indications that those alleles and therefore their corresponding MHC–peptide complexes, may have a lower cell surface expression compared to that of HLA-A. Thus, this epitope can be considered a candidate for a peptide-based vaccine in melanoma patients. However, to induce an effective immune response it is of importance to also activate CD4+ T cells (Topalian *et al.*, 1996), reviewed in (Pardoll and Topalian, 1998). Thus my next experiments were aimed at characterising anti-tumour CD4+ T cells of Pt15392 (Chapter 4) in an attempt to identify genes encoding HLA class II-restricted melanoma antigens (reported in Chapter 6 and 7).

Chapter 4

CHARACTERISATION OF THE CD4+ T CELL RESPONSE IN A LONG TERM SURVIVING MELANOMA PATIENT: IDENTIFICATION OF TUMOUR SPECIFIC CD4+ T CELLS WITH A Th1-Th0 CYTOKINE PROFILE

4.1 INTRODUCTION

T lymphocytes are the main effector cells in the immune system that actively participate in anti-tumour responses (as reviewed in (Boon and van der Bruggen, 1996; Kawakami and Rosenberg, 1997; Pardoll and Topalian, 1998)). To optimise vaccination strategies for cancer immunotherapy, it is essential to understand the characteristics of the T cell response against the tumour and to elucidate the T cell mediated effector mechanisms involved in tumour regression. Several studies clearly documented the involvement of CD8+ T cells in the anti-tumor response in vaccinated (Jager *et al.*, 2000a; Jager *et al.*, 1996b; Pass *et al.*, 1998) and even in non vaccinated patients (Anichini *et al.*, 1999), or in subjects with melanoma undergoing spontaneous regressions (Zorn and Hercend, 1999a; Zorn and Hercend, 1999b). Thus, although CD8+ effector T cells, are actively involved in controlling tumour growth, CD4+ T cells are crucial for an optimal anti-tumour response, as demonstrated in mice (Faiola *et al.*, 2002) and humans (reviewed in (Pardoll and Topalian, 1998)). In fact, by providing co-stimulation, cytokine release and cross-priming, CD4+ T cells are essential for the induction and maintenance of specific CD8+ T cells (Bennett *et al.*, 1998; Schoenberger *et al.*, 1998).

Moreover, CD4+ T cells may also participate in the final anti-tumour effector phases (Hung *et al.*, 1998) by using different mechanisms that not only include tumour cell cytotoxicity mediated by perforin (Rivoltini *et al.*, 1998), but also the induction of apoptotic death in a calcium-independent fashion (Thomas and Hersey, 1998a). Although initial evidence for a role of CD4+ T cells in promoting the generation of tumour specific CD8+ T cells *in vitro* throughout DC activation have been recently provided, (Baxevanis *et al.*, 2000), it is still not completely clarified to which extent CD4+ T cells do actively participate in the anti-tumour T cell- mediated response in human cancer (reviewed in (Fischer *et al.*, 1999)).

Tumour-specific T cells recognise TAAs on target cells. Genetic methods have been developed that, taking advantage of established T cells clones generated from TILs or TALs, have led to the identification of these antigens (as reviewed in (Boon, 1993; Coulie, 1997; Rosenberg, 1997)).

The majority of TAAs identified so far are recognised by CD8+ T cells. Only recently efforts have been made to characterise class II HLA-restricted TAAs (as reviewed in (Wang, 1999; Wang, 2001); examples of class II HLA-restricted TAAs have been reported for melanoma, (Chaux et al., 1999b; Kobayashi et al., 1998a; Topalian et al., 1996; Zarour et al., 2000), and even for breast, colon and pancreatic cancer (Perez et al., 2002), detailed reviewed in (Renkvist et al., 2001).

Several technological advances now allow a more in dept analysis of the T cell response against molecularly defined TAAs both *in vitro* and *in vivo*. In fact, tumour-specific epitopes in the form of chemically synthesised peptides can easily be prepared and represent a crucial tool for dissecting and monitoring the natural tumour-specific immunity in cancer patients or to evaluate the immunisation capacity of specific cancer vaccines (Chaux et al., 1998; Hu et al., 1996; Valmori et al., 1997). The evaluation of the frequency of antigen specific T cells in the blood from cancer patients benefits also from the new tetramer technology (Dunbar et al., 1999), and (reviewed in (Klenerman et al., 2002)), only recently applied for HLA class II presented epitopes (Nepom et al., 2002). Moreover, innovative techniques for enumeration and quantification of antigen reactive T cells allow also live sorting (Brosterhus et al., 1999), making it possible to perform additional functional studies on a selected antigen-specific T cell population.

However, instrumental for these methods is the knowledge of the molecular nature of the T cell defined TAAs. In this perspective the analysis of CD4+ T cell anti-tumour response in

cancer patients with conventional techniques including MLTC and limiting dilution cloning may be a first step in dissecting the potential role of these cells in the anti-tumour response in cancer patient. The subsequent identification of CD4+ T cell epitopes will provide new tools for additional fine analysis that may potentially include tetramer technologies.

In order to assess an anti-tumour response in an appropriate and quantitative way, it is important to consider several parameters (as reviewed in (Romero *et al.*, 1998; Whiteside, 2000)):

(i) The characteristics of anti-tumour reactive T lymphocytes, especially their cytotoxic function and clonal distribution.

(ii) The molecular nature of the antigens involved in this response.

(iii) The frequency of memory T cells recognising the TAA and which are present in the blood or tumour tissue, as estimated by techniques as ELISPOT and HLA/peptide tetramer staining.

The study presented in this chapter used several of these parameters to assess the nature of the CD4+ T cell mediated response developed at tumour site in a melanoma patient remaining disease free 12 years after the surgical resection of a lymph node metastasis. In this chapter, the CD4+ T cell response has been analysed at the clonal level, while the identification of the tumour antigens evoking this response will be the topic of Chapter 6 and 7.

Some of the work with the establishment of the CD4+ T cell clones has been performed in collaboration with Dr. Arabella Mazzocchi (now at the Unit of Blood Transfusion, Istituto Nazionale dei Tumori, Milan).

4.2 AIM OF THE CHAPTER

- To eveluate the CD4+ T cell mediated response at tumour site in a metastatic melanoma patient and isolate tumour-specific CD4+ T cell clones for further antigen screening procedures.

4.3 RESULTS

4.3.1 Characterisation of anti-melanoma CD4+ effector T cells

4.3.1.i Phenotypic evaluation of TAL and PBL derived from Pt15392 by FACS

The anti-tumour CD8+ T cell repertoire from Pt15392 has been well characterised (as described in Chapter 3), while the reactivity against HLA class II restricted TAAs remained to be elucidated. Therefore, a clonal analysis of T cells infiltrating the metastatic tumour was planned, in order to address the question as to whether CD4+ T cells may have played a role in the anti-tumour response of this patient.

The cell surface phenotype of TALs obtained at the day of surgical resection of the metastatic tumour (Day 0) was analysed by indirect immunofluorescence and FACS. As control, PBMCs also obtained at the day of operation were included. Antibodies directed against CD3, CD4 and CD8 molecules were used in order to evaluate the percentage of T cells and their composition in terms of CD4+ or CD8+ T cells. As shown in Table 4.1, CD3+T cells account for the 70% of all the lymphocytes isolated from the metastatic tumour. CD4+ T cells constituted roughly 50% of the total cell number both in the TALs and the PBMCs. CD8+ T cells form a minor population of approximately 30% in these two groups. The intensity of the overall staining for the markers is consistent, which correlates with the expected phenotypic distribution of lymphocyte populations not yet manipulated by *in vitro* culture.

Table 4.1: Cell surface phenotype of lymphocytes derived from the blood and a lymph node melanoma metastasis. Tumour associated lymphocytes (TALs) and peripheral blood mononuclear cells (PBMCs) were obtained from Pt15392 at the day of surgery (Day 0) and then analysed for their expression of cell surface markers by indirect immunofluorescence followed by FACS analysis. W6/32 (anti-HLA-A,-B and –C), OKT3 (anti-CD3), OKT4 (anti- CD4) and OKT8 (anti-CD8) mAbs.

		W6/32	OKT3	OKT4	OKT8
TALs	% positive cells	90	71	44	34
	Intensity	192	128	95	136
PBMCs	% positive cells	98	90	58	26
	Intensity	174	131	99	147

4.3.1.ii Cytotoxic responses of the TAL and PBL populations

With the aim of characterising the anti-tumour response of TALs, these T cells were stimulated *in vitro* with the autologous tumour. After 10 days of *in vitro* culture, the functional activity of growing T cells was evaluated in a 4 h ^{51}Cr-release cytotoxic assay. The involvement of the TCR in the cytotoxicity of these populations was assessed by the usage of the OKT3 Ab. Figure 4.1 clearly shows inhibitions of the tumour lysis when the TAL derived T lymphocytes were pre-incubated with the anti-TCR antibody OKT3 before being added to the autologous melanoma. These data demonstrated that the T cell population expanded *in vitro* displayed a cytotoxic activity directed against the autologous tumour and that this activity involved TCR triggering. Only a very low lytic activity directed against the autologous tumour was found with T cells obtained from the *in vitro*
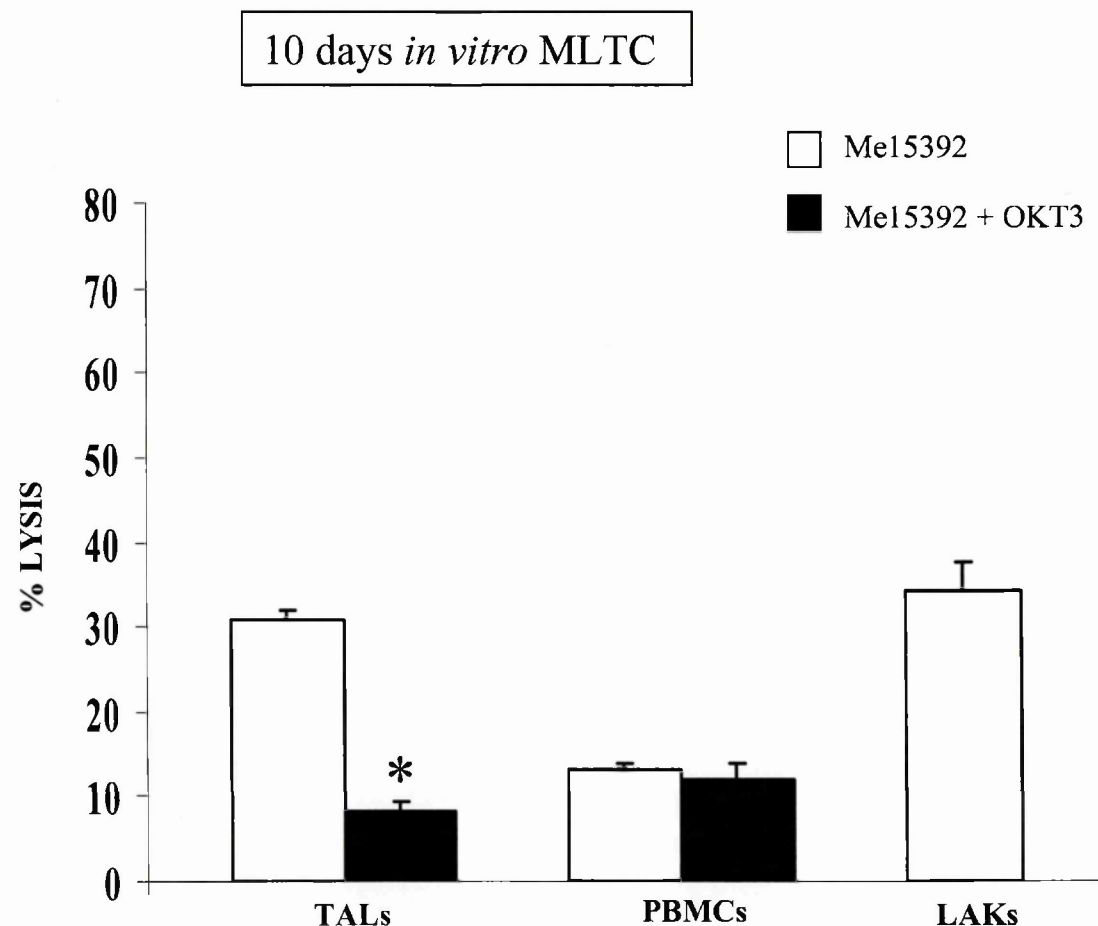
FIGURE 4.1: TCR-dependent lysis of the autologous tumour by T cell derived from TAL. TALs and PBMCs autologous to tumour Me15392 were re-stimulated weekly in a MLTC. After 10 days of *in vitro* culture T cells were tested for their cytotoxic activity against the autologous melanoma in standard 4 h in ^{51}Cr - release assay at E:T ratio of 100:1 in the presence or absence of an anti-CD3 mAb (OKT3) (1 μg/ml). Me15392 was lysed by TALs by a TCR-dependent mechanism since lysis in the presence of OKT3 was significantly reduced in respect to the lysis achieved in the absence of mAb (*p<0.001). Lymphocyte activated killer cells (LAKs) were used as positive control as they are known to kill by a TCR-independent mechanism. TAL = tumour associated lymphocytes; PBMCs = peripheral blood mononuclear cells. Data are representative of one experiment performed in duplicate.

stimulated PBMCs. Moreover, this activity was not affected by the presence of OKT3 in the test, indicating that no tumour-specific reactive T cells were detected in that population. Lymphocyte activated killer (LAK) cells were used as an internal positive control of the cytotoxic assay, as LAKs are known to exert their lytic activity through a TCR-independent mechanism. As further control, the lysis of the NK sensitive and HLA-deficient target cell line K562 was not affected by the presence of OKT3 (Figure 4.2) and no differences were seen in K562 cytotoxicity for either the TALs or PBMCs derived T cell effectors.

These data showed that as early as at 10 day of *in vitro* culture, TAL-derived T lymphocytes already displayed a tumour-specific recognition, suggesting that tumour-specific T cell precursors were already present *in vivo* at tumour site. These T cells were then further expanded *in vitro* by stimulation with autologous tumour cells.

4.3.1.iii The in vitro cultured T cells released IFN-γ in response to tumour stimulation

After the 3rd week of *in vitro* culture with autologous irradiated Me15392 cells, TALs were analysed for the capacity to produce IFN-γ in response to stimulation with autologous tumour. In addition, to evaluate the TCR involvement in the recognition of the tumour, the stimulation assay was performed using T cells pre-incubated for 45 min at 37°C alone without or with OKT3.

The data shown in Table 4.2 clearly indicate that T cells derived from TALs were able to produce IFN-γ in response to the autologous tumour and that cytokine production was largely dependent upon TCR triggering since pre-incubation of T cells with an anti-CD3 mAb resulted in a marked decrease of the IFN-γ release.
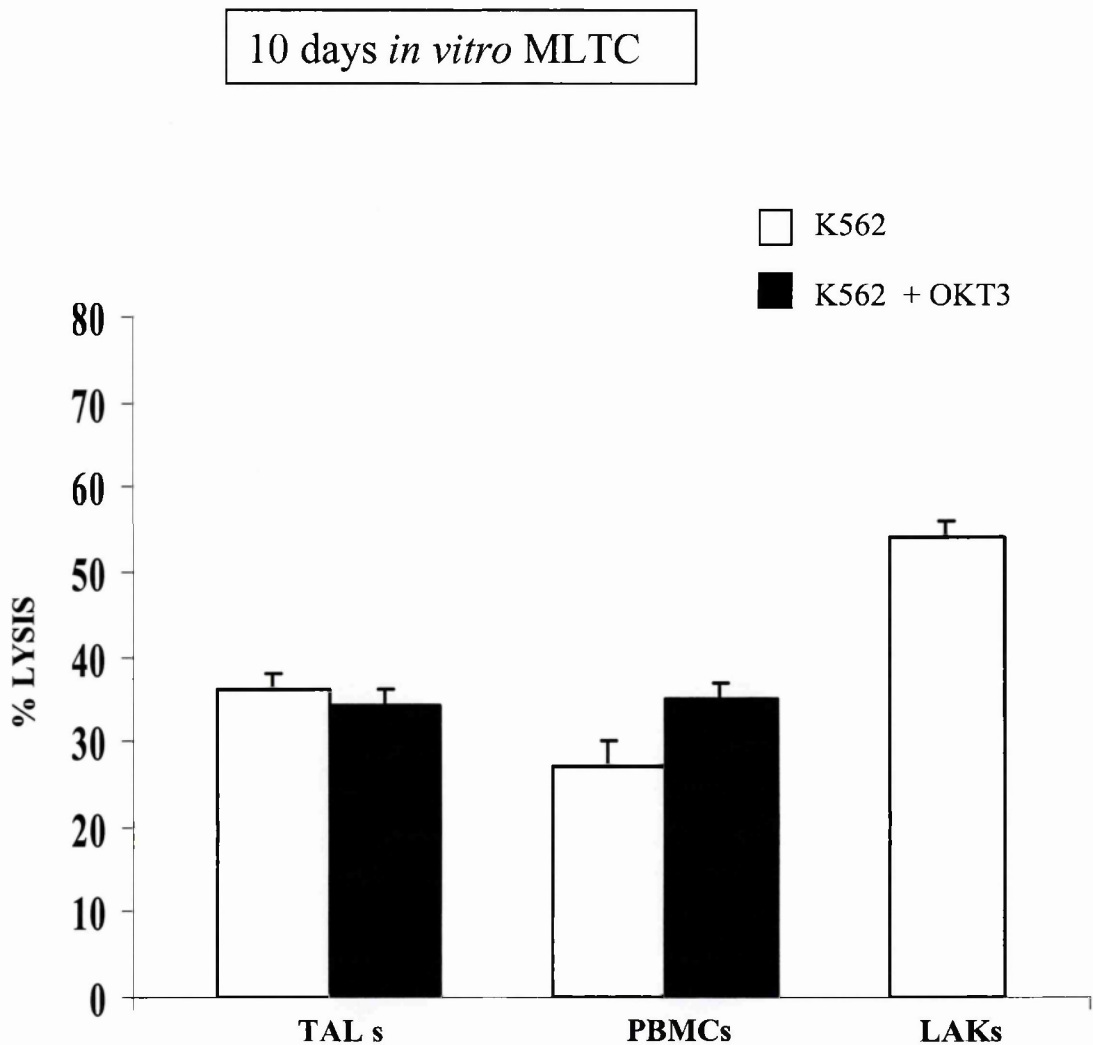
FIGURE 4.2: NK-like activity of TALs and PBMCs. Tumour associated lymphocytes (TALs) and peripheral blood mononuclear cells (PBMCs) were stimulated *in vitro* with autologous tumour. After 10 days of *in vitro* culture the lymphocytes were tested for their cytotoxic activity against the NK susceptible and HLA-deficient cell line K562 in a standard 4 h ^{51}Cr - release assay in the presence or absence of an anti-CD3 mAb (OKT3, 1 μg/ml). The K562 cells were lysed by TALs, PBMCs and lymphocyte activated killer cells (LAKs). As expected no significant inhibition was observed in the presence of mAb OKT3. LAK cells were used as controls. Data are representative of mean ± S.D. of one experiment performed in duplicate.

Table 4.2. IFN-γ release from TALs in response to different stimuli. After 3 weekly restimulations in MLTC, tumour associated lymphocytes (TALs) (1×10^4 cells/well) and melanoma cells (Me15392) (1×10^4 cells/well) of Pt15392 were incubated alone or in the presence of an anti-CD3 mAb (1 μg/ml) for 45 min. T cell recognition of Me15392 was measured by analysing the IFN-γ release by ELISA.

	IFN-γ release (pg/ml)
TALs + medium	15
TALs + Me15392	1900
TALs + OKT3 + Me15392	800

4.3.1.iv Characterisation of the CD4+ T cell subset

In order to characterise the anti-tumour response in these TALs at the end of the 3rd week of *in vitro* culture, T lymphocytes were cloned by limiting dilution. Cloning was performed in two parallel settings; 96 well plates were coated with one and 5 T cells/well, respectively, for a number of 2500 wells each and cultured in RPMI 1640 supplemented with 10% pooled HS, 50 U/ml rIL-2 in the presence of 5×10^2 autologous tumour cells and 1×10^5 allogeneic pooled lymphocytes obtained from healthy donors. The T lymphocytes were re-stimulated after one week with irradiated autologous melanoma cells and allogeneic lymphocytes and screened at day 14 for the presence of growing clones. 120 growing clones were selected and transferred to 24 well plates. Their capacity to recognise the autologous melanoma was assessed by a 4 h ^{51}Cr-release cytotoxic assay. 40 clones showing specific tumour recognition were selected, further expanded and then frozen in

liquid nitrogen. The functional characterisation of some of these clones is reported in Figure 4.3.

The T cell clones isolated from the *in vitro* expanded TALs displayed different patterns of reactivity. The cytotoxic assay was performed in the presence or in the absence of the class I HLA (W6.32) and class II HLA-DR (L243) mAbs. The data clearly show that some of the clones recognised tumour cells in a class I HLA restricted fashion (TB164, TB311, TB361, TB670), while for other lytic T cell clones tumour recognition was independent from both class I and HLA-DR molecules (TB120, TB335). However, several clones (TB39, TB48, TB89, TB426, and TB515) showed a strong anti-tumour lytic activity that was HLA-DR-restricted.

4.3.1.v TCR expression of established CD4+ clones

T cell clones displaying HLA-DR restricted tumour-specific activity were selected for further analysis. In order to group all the sister clones, i.e. clones derived from the expansion of a single common precursor, and with the aim of evaluating the repertoire of the restricted melanoma- specific response in TALs, the TCR expression of each of the HLA-DR restricted T cells was molecularly defined by RT-PCR. cDNA, prepared from each of these clones, was submitted to amplification with primers specific for the different families of the variable (V) alpha and beta regions paired with reverse primers specific for the constant (C) alpha and beta regions.

Each of the 5 identified CD4+ T cell clones expressed different TCR specificities (Table 4.3). Comparing their TCR composition, the results showed a dominant representation of the TCRAV13 and TCRBV21 chains among the analysed HLA-DR-restricted clones. The clones TB515, TB39 and TB426 all expressed TCRAV13, while TB48 and TB89 expressed TCRAV3 and TCRAV17.
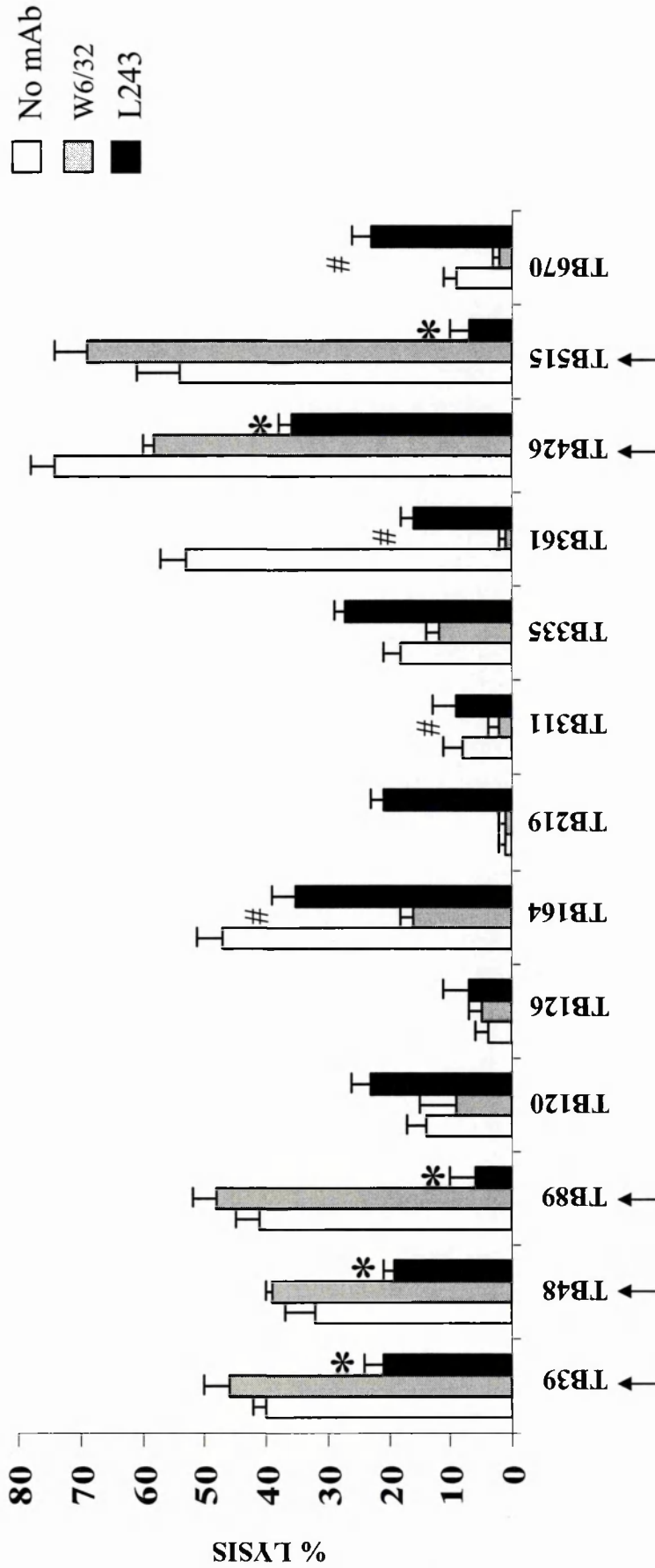
FIGURE 4.3: Reactivity of T cell clones derived from TAL of the long time surviving melanoma patient (Pt15392). Tumour associated lymphocytes (TALs) were stimulated with autologous melanoma cells (Me15392) at an E:T ratio of 25:1 for 3 weeks and then cloned by limiting dilution. After 2 weeks the growing clones were tested for cytotoxic activity against the autologous tumour in a standard 4 h ^{51}Cr - release assay in the absence or in the presence of 1 μg/ml of W6/32 (anti-HLA-A, -B and –C) or L243 (anti-HLA-DR) mAbs. Incubation with L243 showed a significant blocking for 5 clones (TB39, TB48, TB89, TB426 and TB515) (*p≤0.001) compared to the lysis with no mAb. The lytic activity was also significantly blocked by W6/32 for 4 CD8+ T cell clones (TB164, TB311, TB361 and TB670) (#p≤0.05) compared to the control. The CD4+ T cell clones (TB39, TB48, TB89, TB426 and TB515) selected for further characterisation are indicated with an arrow. Data are representative of mean ± S.D. of one experiment performed in duplicate.

Table 4.3: The TCR repertoire of class II-HLA restricted anti-melanoma T cell clones from Pt15392.

cDNAs derived from various well proliferating clones were analysed by RT-PCR using primers specific for the variable (V) and constant (C) regions of the T cell receptors (TCR).

CLONE	TB515	TB48	TB39	TB89	TB426
TCRAV	13	3	13	17	3
TCRBV	21	21	13	21	13

4.3.1.vi Functional characterisation of established CD4+ clones

With the aim of identifying which T cell subset these anti-melanoma T cell effectors belonged to, clones TB515, TB39, TB48, TB89 and TB426 representing different TCR specificity were selected and then analysed for their capacity to recognise the autologous melanoma. Cytokine release assays were performed to define the cytokine expression pattern. The data obtained for TB39, TB48 and TB515 are reported in Table 4.4. All the clones tested were able to release IFN-γ, GM-CSF and IL-2, characteristic of a Th1 subset. However, upon tumour stimulation, IL-10, the hallmark cytokine for Th2 subsets (Kelso, 1995), was also detectable in the supernatant, whereas neither IL-4 nor TGF-β could be found. Thus, this stimulation assay implies the existence of anti-melanoma CD4+ T cells able to release cytokines not only belonging to the Th1 subset, but also the typical Th2 cytokine IL-10.

Table 4.4: Functional characterisation of anti-melanoma CD4+ T cell clones derived from TALs of Pt15392. Cytokine release from TB515, TB48 and TB39 (upper, intermediate and lower panels, respectively) cells in response to different stimuli. The OKT3 Ab was used as a positive control as it induced an optimal release of cytokines by cross-binding of the TCRs, whereas the LCL cells were used as a negative control. LCL = lymphoblastoid cell line that express all the HLA molecules of the patient, from whom LCL cells were derived; Auto-Me = autologous melanoma; Auto-LCL = autologous LCL cells; OKT3 = anti-T3 mAb.

Cytokine release (pg/ml)							
Stimulus	IFN-γ	GM-CSF	TNF-α	IL-2	IL-10	IL-4	TGF-β
Auto-LCL	250	355	24	5	15	0	0
Auto-Me	2800	2890	850	252	2490	0	0
OKT3	3350	3250	410	198	2480	0	0
Auto-LCL	155	412	45	11	22	0	0
Auto-Me	1950	3360	1250	369	2215	0	0
OKT3	2155	3120	955	355	1850	0	0
Auto-LCL	214	122	25	15	12	0	0
Auto-Me	3855	2750	980	232	1560	0	0
OKT3	2520	2845	1040	245	1890	0	0

4.4 DISCUSSION

Due to the key role of CD4+ T cell in viral and tumour immune response (reviewed in (Pardoll and Topalian, 1998; Wang, 2001)), it was interesting to address the question as to whether a CD4+ T cell response could have been developed in the long time surviving melanoma Pt15392. This analysis started by considering T cell composition at tumour site. Comparing the phenotype of lymph node derived TALs with those of peripheral blood obtained simultaneously, i.e. at the day of operation, no major differences in the percentage of CD4+ versus CD8+ T cells were observed. Nevertheless, with the aim of identifying a possible CD4+ T cell mediated tumour-specific immune response at tumour site, conventional MLTC was set up using T cells derived from TALs. A tumour specific, CD3-dependent killing could be observed at the second week of *in vitro* culture suggesting the presence of a relative high number of tumour-specific precursors in the original T cell population *in vivo*. In order to better characterise this response and with the precise aim of evaluating the presence of CD4+ tumour-specific T cells, we resorted to a clonal analysis. 120 individual T cell clones, growth out in the 96-well plate, were tested for their tumour recognition in a ^{51}Cr-release cytotoxic assay. Among these T cell clones, 40 were tumour-specific and HLA-DR restricted. By TCR analysis, performed using RT-PCR, the HLA-DR-restricted T cell clones could be classified in 5 different groups according their TCR specificity. One clone representative for each TCR specificity and demonstrating a good proliferative response *in vitro* to IL-2 and tumour stimulation, was used for further functional characterisation.

T cells involved in anti-tumour responses can be characterised by their profile of cytokine production, which has been extensively studied in melanoma (Goedegebuure *et al.*, 1994; Maccalli *et al.*, 1994; Markus *et al.*, 1995; Salmeron *et al.*, 1992). Taken together, these published studies showed that tumour reactive CD4+ T cells secrete the classical pattern of

Th1 and Th2 cytokines (Mosmann *et al.*, 1986), but that these profiles can be modulated depending on the source of stimulus. In agreement with these data and besides being cytotoxic, the CD4+ clones of Pt15392 were able to release cytokines in response to tumour stimulation. However, their cytokine pattern was surprising, since, in addition to release cytokines belonging to the Th1 subset, they also released high amount of IL-10 while no IL-4 or TGF-β were detected. These findings are in accordance with the continuously increasing complexity observed for CD4+ T cells (as first reviewed in (Kelso, 1995) and then in (Mosmann and Sad, 1996)). Nevertheless, the high levels of IL-10 in the absence of IL-4 are consistent with a cytokine profile described for murine and human CD4+ T cells upon repeated stimulation with ovalbumine (OVA) peptide (Groux *et al.*, 1997). These previously described CD4+ T cell subsets did not produce IL-2, but released high amounts of IL-5, which rendered them different from the CD4+ cells identified in Pt15392. A strong release of IFN-γ by T cells upon recognition of antigenic epitopes is highly advantageous for anti-tumour response, as IFN-γ mediates up-regulation of HLA class I molecules on tumour cells (as reviewed in (Anichini *et al.*, 1993; Garbe and Krasagakis, 1993)) and thus render them susceptible for T cell killing.

A study evaluating cytokine profiles of CD8+ and CD4+ T cell subsets in melanoma patients by stimulation with anti-CD3 mAb OKT3 and with autologous tumour cells revealed different patterns of cytokine secretion (Maccalli *et al.*, 1994). Both IL-4 and IFN-γ could be released from some anti-melanoma CD4+ T cell clones induced by soluble anti-CD3 mAb, while activation of another panel of CD4+ T cell clones with autologous tumour did not result in production of IL-4. Different findings have been obtained with the CD4+ T cell subsets of Pt15392, as they secreted comparable amount of IFN-γ, GM-CSF, TNF-α, IL-2 and IL-10, but not of IL-4 or TGF-β in response to activation by the anti-CD3 mAb OKT3 or mediated by the autologous tumour. Indirectly these findings indicate that

the TAA presented on melanoma cells could induce a full activation of its nominal T cells. This may suggest (i) that the TCR expressed by the examined T cells clones have a good affinity for the peptide/MHC complexes and/or (ii) that their nominal antigen(s) is/are highly expressed by the tumour cells. These T cells are therefore fully activated by the encounter with their nominal TAA. Such complete T cell activation by tumour cells is not always observed; some anti-melanoma CD8+ T cells may be suboptimally activated as a result of low expression of HLA/peptide complexes or low avidity interactions (Gervois *et al.*, 1996).

Furthermore, it is of note that CD4+ T cells from Pt15392 did express TCR that shared some common features. In fact, the CD4+ T cell clones of Pt15392 do share TCRBV21 or TRCAV3 and TCRVA13 indicating that a selection of TCR expression could have been occurred. These data suggest that these TCRs indeed are directed against the same antigenic determinant and, moreover, that they could have been selected for having a better affinity/avidity for the nominal MHC/peptide complexes. With the molecular identification of the nominal peptide I will be able to partially address these questions.

Taken together, the study presented in this chapter led to the identification of melanoma-specific CD4+ T cells with an atypical cytokine profile; they can be considered mainly Th1 cells, releasing high amount of IL-10. No other CD4+ T cell subsets, reactive against tumour cells or viral and bacterial determinants, have been defined that displays a cytokine profile compatible with the pattern released by the anti-melanoma CD4+ T lymphocyte clones of Pt15392.

The question that will be addressed in the next chapter is whether the CD4+ T cells, owing to the release of IL-10 in their cytokine cocktail, may exert a suppressive role in the anti-melanoma response. IL-10 has actually been reported as mediating the immune-suppression activity of the CD25+ CD4+ T cells (Brady *et al.*, 1999); moreover, IL-10 plays a central role in the regulation of immune network (reviewed in (Lalani *et al.*, 1997; Moore *et al.*, 2001)). However, since different molecules have been described having high homology with IL-10 and since also for cellular and viral IL-10, differences in the primary sequence may account for different functional activities, the question was also asked whether the IL-10 produced by CD4+ T cells clones from Pt15392 was indeed a wild type IL-10 or if some mutations could have been present in the mRNA transcripts of our CD4+ T cell clones.

Chapter 5

ANTI-MELANOMA CD4+ T CELL CLONES RELEASING HIGH

AMOUNTS OF NON-INHIBITORY IL-10

5.1 INTRODUCTION

5.1.1 Functions of IL-10

The hallmark of a T helper response is the specific pattern of cytokines secreted upon recognition of antigenic peptides. This has been clearly demonstrated both in mice (Cherwinski *et al.*, 1987; Mosmann and Sad, 1996) and humans (Del Prete *et al.*, 1991). Among these cytokines, IL-10 has a major role, as it regulates a complex pattern of both immune suppressive and stimulatory activities (as reviewed in (Wakkach *et al.*, 2000)). Released by Th2 cells and associated with inflammatory responses (reviewed in (Lalani *et al.*, 1997)), produced by cancer cells to evade immune recognition, (reviewed in (Salazar-Onfray, 1999)) and recently found to be specifically expressed by regulatory T cells (Tr) (reviewed in (Roncarolo and Levings, 2000), IL-10 has a central role as a regulator of CD4+ T cell mediated responses

Indeed, the opposing functions of IL-10 in the immune system may be symbolised with the Chinese metaphor of Yin and Yang; a theory representing two opposite aspects, which are, at the same time, both interdependent and in conflict (Kuby, 1997). For example, it is proposed that IL-10 can be secreted by tumour cells as an escape mechanism to promote their own progression (Chen *et al.*, 1994; Dummer *et al.*, 1996; Dummer *et al.*, 1995), a concept compatible with recent observations of tumour progression in B6 mice treated with IL-10 transduced B16-melanoma cells (Garcia-Hernandez *et al.*, 2002). In contrast to these findings, human melanoma cells that have been transduced with the murine IL-10 gene show a lower metastatic ability in nude mice (Huang *et al.*, 1999). The anti-metastatic activity was linked to the inhibition of angiogenesis.

An additional controversial function of IL-10 lies in the ability of this cytokine to influence the expression of MHC molecules. For example, IL-10 by down-regulating class I and

class II HLA expression in melanoma cells promotes immune escape of tumour cells (Matsuda *et al.*, 1994; Yue *et al.*, 1997). However, the lack of HLA molecules also renders tumour cells sensitive to lysis by natural killer (NK) cells which have also been detected at the tumour site in mice injected with IL-10 (Zheng *et al.*, 1996). IL-10 strongly impairs antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down regulation of class II MHC expression (de Waal Malefyt *et al.*, 1991b).

IL-10 has been demonstrated to have a protective anti-tumour effect either when systemically administered to tumour-bearing mice or when expressed locally at very high levels by the engineered tumor cells (Berman *et al.*, 1996; Dorsey *et al.*, 2002; Kundu and Fulton, 1997). The mechanisms involved in such a protection are not completely clarified, but they include anti-angiogenic activities (reviewed in (Huang *et al.*, 1999)) and possibly direct stimulatory action of IL-10 on tumour specific T cells (Fujii *et al.*, 2001; Santin *et al.*, 2000). On the other hand, IL-10 has been reported to impair the ability of T cells to release cytokines. Indeed, IL-10 mainly inhibits the functional activities of T cells by affecting different types of antigen presenting cells (APCs). IL-10 has been reported to suppress cytokine production by modulating the functions of monocytes (de Waal Malefyt *et al.*, 1991a), macrophages (Fiorentino *et al.*, 1991a Ding, 1992 #251) and DC (Caux *et al.*, 1994; Macatonia *et al.*, 1993).

Other findings support a stimulatory role of IL-10 for B lymphocytes. First, IL-10 enhances the *in vitro* viability of B cells and mediates up-regulation of their class II HLA (Go *et al.*, 1990; Thomssen *et al.*, 1995). In addition, IL-10 augments both proliferation and differentiation of human B cells into antigen secreting plasma cells (Defrance *et al.*, 1992; Rousset *et al.*, 1992). Moreover, IL-10 has been shown to be a differentiation factor

for CD8+ T cells and to stimulate their growth when cultured together with IL-2 (Chen and

Zlotnik, 1991).

Table 5.1: An overview of the major inhibitory and stimulatory functions of IL-10.

INHIBITORY FUNCTIONS	STIMULATORY FUNCTIONS
Inhibits production of IFN-γ, IL-2 and TNF-α by Th1 cells.	Enhances some NK functions.
Inhibits production of IL-1α, IL-1β, IL-6, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF and PAF from activated monocytes and macrophages; i.e. anti-inflammatory properties.	Enhances proliferation of B cells and supports their differentiation into antibody producing plasma cells (IgM, IgG and IgA). Upregulates class I HLA on B cells.
Down-regulates the expression of class II HLA molecules on monocytes, dendritic and Langerhans cells.	Shows anti-tumour activities by inhibition of angiogenesis.
Inhibits proliferation of Th0, Th1 and Th2 cells.	Increases proliferation of activated of CD8+ T cells.
Decreases IFN-γ production from NK cells.	
Inhibits antigen dependent proliferation of T cells.	

5.1.2 Polymorphism of the IL-10 gene promoter

The importance of IL-10 as a regulatory cytokine with possible therapeutic implications (Volk *et al.*, 2001), has generated interest in how IL-10 is regulated at the genetic level. In particular, IL-10 has been associated with several auto-immune diseases (reviewed in (Moore *et al.*, 2001)) as well as with several types of cancer (Fortis *et al.*, 1996). Analysis of constitutive IL-10 expression in Epstain Barr virus (EBV)-positive Burkitt's lymphoma cells resulted first in the identification of the hIL-10 promoter (Kube *et al.*, 1995) and then in the mapping of the *hIL-10* gene (Eskdale *et al.*, 1997a). It was found that the 5'-flanking region of the *hIL-10* gene contains positive and negative regulatory sequences important for the *IL-10* gene. More precisely, the promoter region of *IL-10* is heterogeneous with three polymorphic sites present at positions -1082, -819 and -592. This polymorphism has been linked to the level of IL-10 production *in vitro* by stimulated peripheral blood lymphocytes (PBLs) (Hurme *et al.*, 1998; Turner *et al.*, 1997). In addition, the *hIL-10* gene encodes two microsatellites (IL-10.R and IL-10.G), associated with polymorphism (Eskdale and Gallagher, 1995; Eskdale *et al.*, 1996).

In this context, the gene sequence associated with an IL-10 phenotype was proposed to constitute a prognostic marker for diseases associated to IL-10. However, the IL-10 promoter sequences have been analysed in several patients with various auto-immune diseases and, although some correlations do exist between polymorphic sites of IL-10 and IL-10 production, no general conclusions could be drawn (Eskdale *et al.*, 1998; Eskdale *et al.*, 1997b; Hurme *et al.*, 1998; Kube *et al.*, 1995; Turner *et al.*, 1997).

As described in Chapter 4, the CD4+ T cell anti-tumour immune response was studied in a melanoma patient (Pt15392), who experienced a favourable prognosis. Several anti-melanoma specific CD4+ T cell clones were isolated from metastatic lymph nodes

obtained at the time of surgery. As further demonstrated in Chapter 4, these CD4+ clones showed a cytotoxic activity against the autologous tumour and, after stimulation by tumour cells, released high levels of IL-10, IFN-γ, GM-CSF, TNF-α and IL-2, but not IL-4 and TGF-β (Chapter 4/Table 4). This cytokine pattern does not correlate with the classical Th1, Th2 (Mosmann *et al.*, 1986) or to the newly reported Tr profiles (reviewed in (Mosmann and Sad, 1996)). Thus, it is important to further evaluate the role of such a CD4+ T cell population.

To explain the role of IL-10 in the atypical cytokine profile observed for the anti-melanoma CD4+ T cell clones described in the previous chapter, experiments were designed to assess the immune-suppressive role mediated by the cytokine cocktail released by these T cells in response to tumour stimulation.

5.2 AIMS OF THE CHAPTER

- To evaluate a possible inhibitory activity of the cytokines released by CD4+ T cells upon tumour stimulation.

- To perform a molecular analysis of the *IL-10* gene with the objective of finding possible changes in the nucleotide sequence of the promoter and coding region of IL-10 released by the CD4+ T cells of Pt15392.

5.3 RESULTS

5.3.1 Analyses of CD4+ T cells releasing high amounts of IL-10

5.3.1.i IL-10 present in the cytokine cocktail did not result in T cell inhibitory activity

To assess whether the IL-10 containined in the cytokine mixture released from the anti-melanoma CD4+ T cells of Pt15392 could exert an inhibitory effect on the generation of anti-tumour T cells, two different *in vitro* experiments were performed. In these

experiments, the activated high IL-10 releasing CD4+ T cells of Pt15392 were added to semi-permeable upper wells and their ability to affect the induction of tumour or MART-1_{27-35} specific T cells in the lower wells was evaluated, as depicted in Figure 5.1. Generated T cells were further tested for recognition of the same targets, used to stimulate the T cells.

In the first experiment (Figure 5.1, left panel A and B) PBMCs obtained from an HLA-A2 metastatic melanoma patient were cultured with the autologous tumour (Me2) in the lower wells. Normal medium or stimulated CD4+ T cells of Pt15392 were added to the upper wells. At the end of the second week of *in vitro* culture, the T cells generated in the lower well were tested for their tumour specificity. They were incubated with medium, autologous LCL cells (LCL2) and melanoma cells (Me2) in the absence or in the presence of the mAbs W6/32, a mAb directed against class I HLA or of mAb L243 directed against class II-HLA. The supernatants were collected and T cell activation evaluated by monitoring the amount of IFN-γ released. The results of this experiment are shown in Figure 5.2 and demonstrate that the cytokine mixture released by the CD4+ T cell clones of Pt15392 did not reveal any suppressive effect on the generation of tumor-specific T cells. In fact, the amount of IFN-γ obtained in the presence of the cytokine cocktail was similar, or even higher, than that achieved when T cells were generated in the presence of medium alone. Furthermore, the IFN-γ release was significantly blocked by W6/32 mAb ($p<0.001$), a finding suggesting that class I MHC-restricted CD8+ T cells had been generated in the co-culture setting.

A second experiment was similarly conducted but, in this case, patient's PBMCs were stimulated directly with the MART-1_{27-35} peptide in the presence of either stimulated CD4+ T cells or of medium alone (Figure 5.1, right panel A and B). At the end of the second week, the T cells generated in the lower well were then tested for peptide and
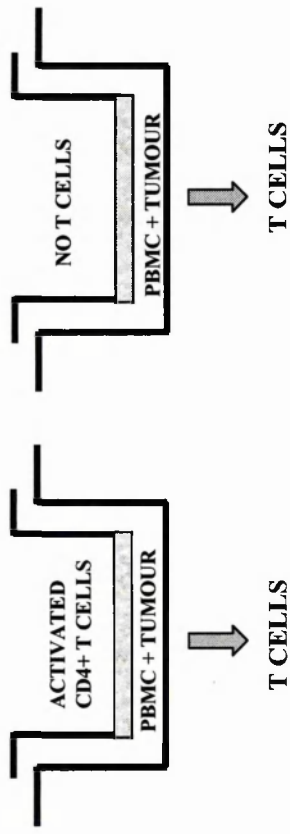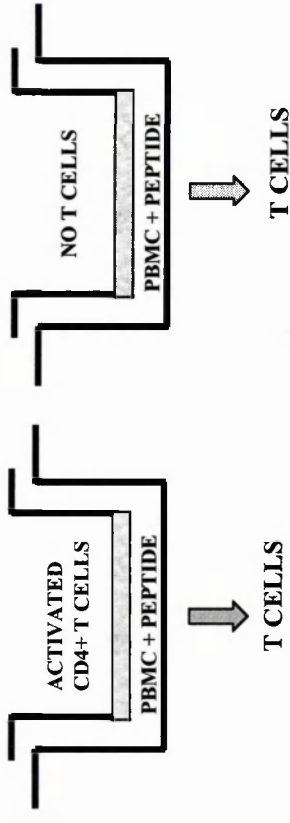
Figure 5.1: Overview of the transwell setting. (A) CD4+ T cells of 15392 were stimulated weekly with autologous melanoma cells for 2 weeks, after which they were added to the transwells on the 5th day after activation. The effect of the cytokines released from these activated CD4+ T cells on the generation of tumour specific T cells in an MLTC (lower well) was evaluated. The lower well contained an MLTC with PBMCs and the autologous melanoma (Me2). This transwell system was used in order to evaluate the effect of the IL-10 containing cytokine mixture released from the activated CD4+ T cells on the generation of tumour or MART-1$_{27-35}$ specific T cells, respectively. (B) The T cells generated in the MLTCs were further evaluated for their IFN-γ release in ELISA in the presence of W6/32 (anti-HLA-A, -B and –C) or L243 (anti-HLA-DR) mAbs. MLTC = mixed lymphocyte tumour culture.
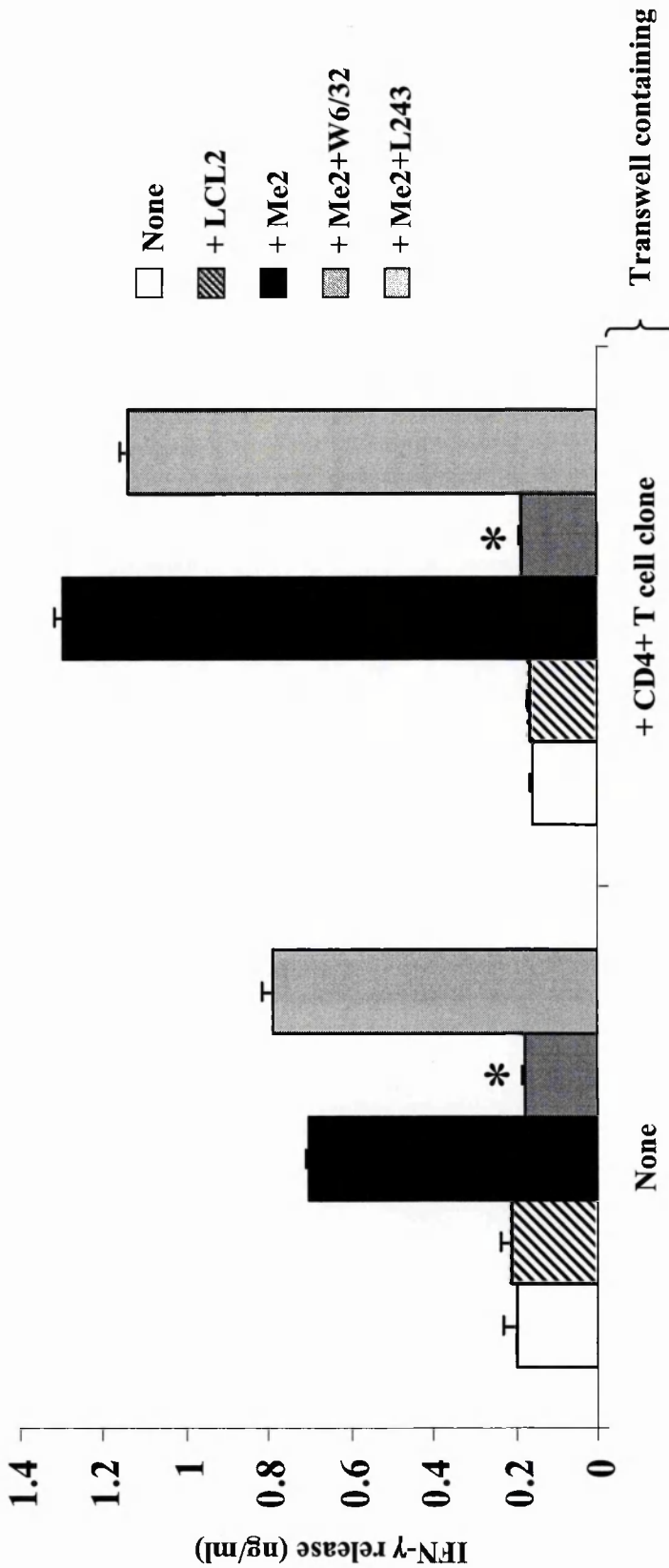
FIGURE 5.2: The supernatant of CD4+ T cell clones did not inhibit the generation of HLA class I restricted melanoma specific T cells. Peripheral blood mononuclear cells (PBMCs) (5×10^5 cells/ml) were cultured with melanoma cells (Me2) (5×10^5 cells/ml) in the lower well in the presence or absence of activated CD4+ T cells (5×10^4 cells/well) of Pt15392 in the upper transwell. After two weeks of mixed lymphocyte tumour culture (MLTC), the specificity of the generated T cells was evaluated. T cells were then incubated overnight at an E:T ratio of 1:1 with medium, with autologous LCL2 or with autologous tumour (Me2) (1×10^5 cells/well) in the absence or in the presence of anti-HLA-class I (W6/32) or anti-HLA-class II (L243) mAb. The supernatants were tested for their IFN-γ content by ELISA. Incubation in the presence of W6/32 significantly inhibits the IFN-γ release (*p<0.001) compared to control with no Ab. This indicate that, under these experimental conditions, the cytokine released by the activated CD4+ T cells did not prevent the generation of anti-melanoma T cells in the lower well. LCL2 = autologous lymphoblastoid cells, W6/32 = anti-HLA-A, -B and –C, L243 = anti-HLA-DR. One representative experiment of three is shown. Data are expressed as mean ± S.D. of triplicates.

tumour specificity. T cells were incubated with medium, T2 cells alone, T2 cells pulsed with 1 µg/ml of MART-1$_{27-35}$ peptide, the autologous melanoma, and the autologous melanoma together with mAbs W6/32 or L243. A significant blocking of the IFN-γ release (p<0.001) was observed for W6/32 indicating that a MART-1$_{27-35}$ peptide specific CD8+ T cell population was generated in the presence of the high quantity of IL-10 released from the stimulated CD4+ in the transwell (Figure 5.3). This supports the conclusion that these CD4+ T cell clones are not exerting a suppressive activity.

Thus, the IL-10 released from the anti-melanoma CD4+ T cell clones of Pt15392 showed no suppression on the generation of a T cell-mediated anti-tumour response in the lower chamber of the transwell system (Figure 5.2 and 5.3) supporting the conclusion that the IL-10 released at the tumour site may not impair the anti-tumour response. However, as IL-10 is a pleiotropic cytokine, it was interesting to investigate if other functional aspects of IL-10 could explain the high quantity produced by the anti-melanoma specific CD4+ T cells of Pt15392.

5.4 DETERMINATION OF THE IL-10 SECRETION PHENOTYPE

5.4.1 Genotyping

In order to further assess the role of IL-10 produced by the CD4+ T cells of Pt15392, an experiment was performed to analyse the genetic polymorphism of the promoter region of *IL-10* (Figure 5.4A). A commercially available kit (Cytokine Genotyping Tray) was used for PCR-single strand polymorphism (SSP). Briefly, multiple PCR reactions were set up in a multiwell-plate, each well containing a primer pair unique for each polymorphism present in the promoter regions of TNF-α (2 wells), TGF-β1 (4 wells), IL-10 (5 wells), IL-6 (2 wells) and IFN-γ (2 wells). More preciseley, genomic DNA was added to a pre-optimised mixture (D-mix), after which 10 µl aliquots were divided into each well of the
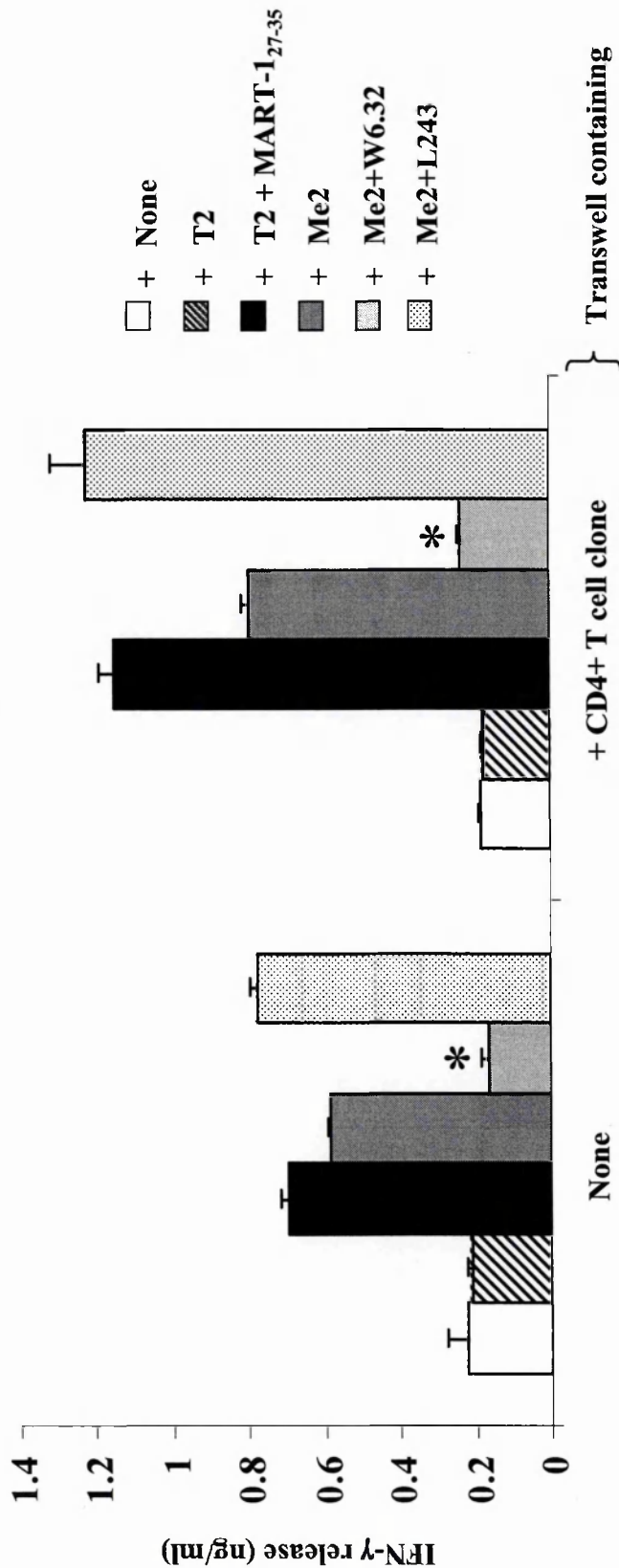
FIGURE 5.3: The supernatant of CD4+ T cell did not inhibit the generation of MART-1$_{27-35}$ specific T cells. Patient's derived PBMCs (5x10^5 cells/well) were pulsed with 1 mg/ml of the MART- 1$_{27-35}$ peptide in the lower well in the presence or absence of stimulated CD4+ T cells of Pt15392 (5x10^4 cells/well) in the upper transwell. After two weeks of culture, the specificity of the generated T cells was evaluated in a cytokine release assay by overnight incubation with the targets cells (1x10^5 cells/well) indicated above at an E:T ratio of 1:1. The IFN-γ content of the supernatants was evaluated by ELISA. Incubation in the presence of W6/32 showed a significant blocking of the IFN-γ release (*p<0.001) compared to unblocked melanoma (Me2) cells both in the presence or absence of activated TB515 cells in the transwell indicative of a MART-1$_{27-35}$ peptide specific CD8+ T cells population generated in the MLTC. T2 = TAP deficient cells, PBMC = peripheral blood mononuclear cells, W6/32 = anti-HLA-A, -B and –C, L243 = anti-HLA-DR. One representative experiment of three is shown. Data are expressed as mean ± S.D. of triplicates.

FIGURE 5.4: Cytokine Genotyping: pattering of PCR amplification bands and their association to the IL-10 genotype. (A) A schematic view of the 3 polymorphic sites in the IL-10 promoter. The polymorphic combination possible for each site is indicated above each arrow, for example the site –1082 can have either G or A base. (B) The cytokine genotyping tray is based on PCRs performed in multiwell-plates composed of small Eppendorf tubes, each containing dried pre-optimized primer pairs unique for the polymorphic sites of TNF-α, TGF-β, IL-10, IL-6 and IFN-γ. The test is based on a correlation between the sequences at polymorphic sites of these genes and the high, intermediate or low producing phenotype that they give rise to. Each plate is divided in 6 sections containing the same pattern of primers and one negative control well, making possible the assessment of the cytokine phenotypes in parallel for 6 patients. The Cytokine Genotyping scheme is to be interpreted as follow: the horizontal pattern of bands obtained for the given primer combinations of one cytokine corresponds to a specific phenotype of an individual patient, which is indicated on the left side of the scheme. The marked section (red) show the intermediate IL-10 secreting phenotype observed for Pt15392 (demonstrated in Figure 5.5).

A

The promoter region of

the IL-10 gene

START

G/A   C/T   C/A

-1082   -819   -592
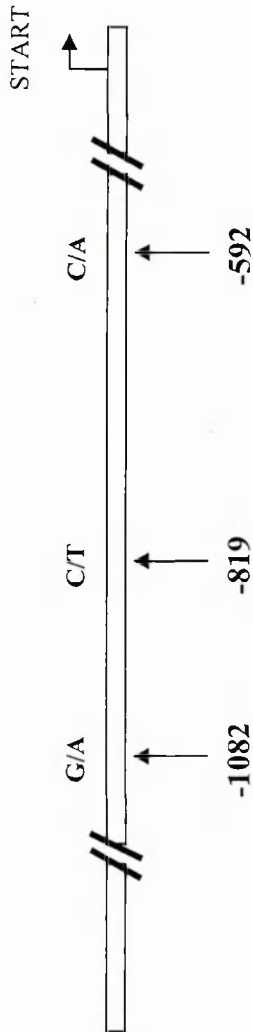
B

POSITIONS		1H/3H/ 5H/7H/ 9H/11H	1G/3G/ 5G/7G/ 9G/11G	1F/3F/ 5F/7F/ 9F/11F	1E/3E/ 5E/7E/ 9E/11E	1D/3D/ 5D/7D/ 9D/11D	1C/3C/ 5C/7C/ 9C/11C	1B/3B/ 5B/7B/ 9B/11B	1A/3A/ 5A/7A/ 9A/11A	2H/4H/ 6H/8H/ 10H/12H	2G/4G/ 6G/8G/ 10G/12G	2F/4F/ 6F/8F/ 10F/12F	2E/4E/ 6E/8E/ 10E/12E	2D/4D/ 6D/8D/ 10D/12D	2C/4C/ 6C/8C/ 10C/12C	2B/4B/ 6B/8B/ 10B/12B	2A/4A/ 6A/8A/ 10A/12A
RESULTS (mark positive locations)																	
PRODUCT SIZE (bp)		750	125	125	200	200	200	200	300	300	300	250	250	175	175	250	250
SPECIFICITY		Neg Ctrl	TNF-α promoter -308A	TNF-α promoter -308G	TGF-β1 codon 10T	TGF-β1 codon 10C	TGF-β1 codon 25C	TGF-β1 codon 25G	IL-10 promoter -1082A,-819T	IL-10 promoter -1082G,-819C	IL-10 promoter -1082A,-819C	IL-10 promoter -819T,-592A	IL-10 promoter -819C,-592C	IL-6 promoter -174C	IL-6 promoter -174G	IFN-γ intron 1 +874T	IFN-γ intron 1 +874A
TNF-α	G/G (low)																
	G/A (high)																
	A/A(high)																
TGF-β1	T/T G/G (high)																
	T/C G/G (high)																
	T/C G/C (intermediate)																
	C/C G/G (intermediate)																
	T/T G/C (intermediate)																
	C/C G/C (low)																
	C/C C/C (low)																
	T/T C/C (low)																
	T/C C/C (low)																
IL-10	GCC/GCC (high)																
	GCC/ACC (intermediate)																
	GCC/ATA (intermediate)																
	ACC/ACC (low)																
	ACC/ATA (low)																
	ATA/ATA (low)																
IL-6	G/G (high)																
	G/C (high)																
	C/C (low)																
IFN-γ	T/T (high)																
	T/A (intermediate)																
	A/A (low)																

tray. The well of the negative control was prepared with 9 µl D-mix and 1 µl of deionised water. After amplification the samples were run on a low melting agarose gel for approximatley 2 h. The individual cytokine typing bands for each polymorphism present in TNF-α, TGF-β1, IL-10, IL-6 and IFN-γ of Pt15392 appear lower than the internal control band of 750 bp (Figure 5.5). More precisely, the horizontal pattern of bands for each cytokine (i.e. the presence or absence of the individual typing bands), represents the polymorphic genotype. For example, IL-10 demonstrates the following pattern: "no band (lane 8), band (lane 9), band (lane 10), no band (11), band (lane 12)" (Figure 5.5), which corresponds to an intermediated secreting phenotype and the GCC/ACC genotype (-1082*G; -819*C and -592*C/-1082*A; -819*C and -592*C) (Perrey et al., 1998) (Figure 5.4B) present in the promoter region of IL-10. A similar interpretation of the other cytokine secreting phenotypes demonstrated low releasing TNF-α (G/G), TGF-β1 (C/C C/C) and IFN-γ (A/A) phenotypes and a high IL-6 (G/G) releasing phenotype (Figure 5.4B and 5.5).

5.4.2 Subcloning of IL-10

*5.4.2.i Subcloning of the IL-10 produced by the CD4+, class II HLA restricted anti-
tumour T cell clones*

It has been shown that one single amino acid was enough to change the imunostimulatory properties of viral IL-10 (Ding et al., 2000). Therefore, it was interesting to subclone and sequence the IL-10 produced by the CD4+ T cells of Pt15392, to directly evaluate if it could exert an immunostimulatory effect due to a similar modification in its primary structure. To address this issue, a data base search was first performed to control if additional polymorphism was present in the coding region of the human *IL-10* gene in addition to the sites of its promoter region (Hurme et al., 1998; Turner et al., 1997). All sequences that matched for *IL-10* were aligned with the full length genomic DNA and no
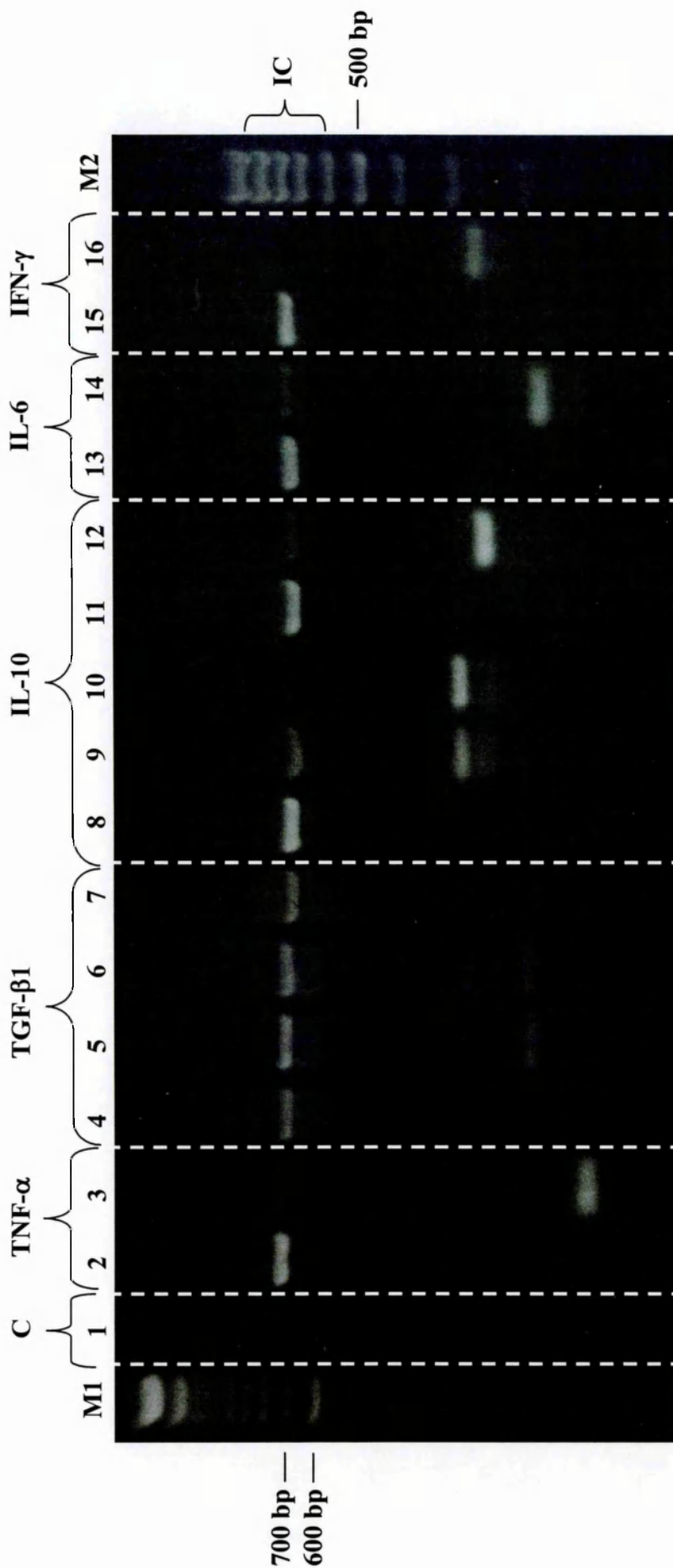
FIGURE 5.5: The long time surviving melanoma Pt15392 has an intermediate IL-10 secreting phenotype. The Cytokine Genotyping assay was performed with 30 ng/ml of genomic DNA derived from LCL cells of Pt15392 and the amplification was run according to a specific pre-set protocol. Cytokine typing bands appear lower than an internal control (IC) band of 750 bp. The result of individual cytokine genotypes were interpreted by horizontally reading the pattern of bands present for each cytokine, for example IL-10 show "no band (lane 8), band (lane 9), band (lane 10), no band (lane 11) and band (lane 12)". This pattern corresponds to an intermediate IL-10 secreting phenotype (see Figure 5.4B). The samples were visualised on a 2.5% low melting agarose gel. C = negative control; M1 = maker 1 (100 bp Ladder); M2 = Marker 2 (Low Range Marker). One representative experiment out of 3 is shown.

polymorphism was found in the coding region of the *IL-10* as differences were found to map only in the promoter region (data not shown). Furthermore, a comparison of the cDNA of *IL-10* (Accession number M57627 in the GenBank) with the genomic DNA of *IL-10* (Accession number U16720 in the GenBank), revealed that the same primers could be used to amplify the genomic DNA as well as the cDNA of *IL-10*. Subcloning and sequencing of the *IL-10* gene expressed by the CD4+ T cell clones of Pt15392, showed that it was identical to the reported *IL-10* gene (Accession number M57627 in the GenBank).

5.5 DISCUSSION

It has been shown that the original described dichotomy of Th1 and Th2 cells (Mosmann and Sad, 1996) can not explain all CD4+ T cell responses. A new category of CD4+ T cells with novel response profiles has been described: the Tr cells (reviewed in (Kelso, 1995; Mosmann and Sad, 1996)). However, this Tr subset can be even further divided into IL-10 producing Tr1 (Groux *et al.*, 1997) and TGF-β secreting Th3 cells (Inobe *et al.*, 1998). However, the high IL-10 producing CD4+ T cell clones of Pt15392 characterised here do not fit with any of the newly defined CD4+ T cell subsets (see Table 4.4). Moreover, the significance of the IL-10 in the anti-tumour response of this patient appears to be elusive, though I could not study this issue in details. Thus, in an attempt to explain the role of IL-10 released by the CD4+ T cell clones of Pt15392, two hypotheses were put forward:

(i) The high IL-10 content in the cytokine mixture released by the CD4+ T cell clones does not have an inhibitory effect;

(ii) A genetic change in the nucleotide sequence of *IL-10* may account for a non-suppressive function.

The validity of these hypotheses is discussed below.

5.5.1 Functional role of the IL-10 released by CD4+ T cells

Firstly, although releasing high amounts of IL-10 in response to tumour stimulation (see Table 4.4), the cocktail of cytokines produced by these CD4+ T cells did not show any suppressive effects on the generation of anti-tumour T cells by the autologous tumour or by peptide-pulsed autologous APC (Figure 5.2 and 5.3). This implied a non inhibitory effect of IL-10 at the concentration present in the CD4+ T cell cocktail; a result that was partially unexpected since IL-10 has been reported to work as an anti-inflammatory and immune-suppressive cytokine (Chen *et al.*, 1994; de Waal Malefyt *et al.*, 1991b; Salazar-Onfray, 1999). However, in the transwell experiments, a supernatant was evaluated that included, in addition to IL-10, also IFN-γ, GM-CSF, TNF-α and IL-2 and, therefore, the observed stimulatory effects could be simply due to the synergistic interaction of all or some of these cytokines whose stimulatory activity overcomes the IL-10 immunosuppression.

Thus, based on these considerations and according to hypothesis (i), the "dosage" or the "quality" of the IL-10 produced by the T lymphocytes of this particular patient (Pt15392), may account for the non-suppressive activity of the cytokine mixture. In addition, the notion that IL-10 indeed may have a stimulatory role has also been reported in the literature; both in humans (Go *et al.*, 1990; Rousset *et al.*, 1992; Thompson-Snipes *et al.*, 1991) and in mice (Adris *et al.*, 1999; Barth *et al.*, 1996; Suzuki *et al.*, 1995).

Moreover, the *in vitro* finding that the cytokine profile of the CD4+ T cells had non-inhibitory activity, is in agreement with data showing that from these same lymphocytes, tumour-specific CD8+ T cells could also be generated and that the same patient also developed a strong CD8+ T cell mediated anti-tumour immunity (Castelli *et al.*, 1998; Castelli *et al.*, 1999). This non-inhibitory effect on the induction of class I HLA restricted melanoma specific CD8+ T cells (Figure 5.2 and 5.3), correlates with the described lack of

inhibitory effect of IL-10 on the proliferation and growth of human CD8+ T cells when stimulated with anti-CD3 antibodies (Chen and Zlotnik, 1991). The findings reported in this and in the previous chapter are also compatible with data showing that IL-10 may have a stimulatory activity on CD8+ T cells that are already tumor- specific (Segal *et al.*, 2002) and that CD4+ T cells releasing IL-10 at tumor site may potentiate a local CD8+ T cell mediated response (Fujii *et al.*, 2001).

The results of this chapter are consistent with recent data indicating that IL-10 can be produced by either Th1 or Th2 - like clones and that the "level" of IL-10 production with respect to the level of other cytokines, like IFN-γ and IL-2, may determine whether a given cytokine cocktail results in an activation or inhibition of the generation of anti-tumour or anti-MART-1$_{27-35}$ specific T cells respectively. The level of IL-10 production may therefore be a crucial step in the regulation of the immune response.

5.5.2 Genotyping and subcloning

Until now, most publications described differences (Eskdale *et al.*, 1997a; Kube *et al.*, 1995; Morse *et al.*, 1999) and even polymorphism (Cartwright *et al.*, 1999; Hurme *et al.*, 1998; Turner *et al.*, 1997) in the upstream promoter region of the *IL-10* gene that may result in high or low IL-10 production.

In fact, the IL-10 secreting phenotypes correspond to three well defined polymorphic sites in the promoter region of *IL-10* (Hurme *et al.*, 1998; Turner *et al.*, 1997). The intermediate IL-10 secreting phenotype demonstrated by CD4+ T cells of Pt15392 (Figure 5.5) was not in agreement with the assumption that the high amount of IL-10 in the supernatant of the CD4+ T cell clones should correlate with a high IL-10 secreting phenotype. Moreover, the patient's GCC/ACC genotype (-1082*G; -819*C and -592*C/-1082*A; -819*C and -592*C) (Perrey *et al.*, 1998) corresponding to the intermediate IL-10 secreting phenotype,

is difficult to reconcile with recent data on polymorphism in IL-10. In fact, a large study of promoter polymorphism in patients with cutaneous malignant melanoma, showed that some IL-10 genotypes could be associated with the clinical prognosis and hence, be used as prognostic markers; the high releasing genotype -1082 GG was associated with horizontal and non-invasive tumours, while the −1082 AA low secreting genotype was found in patients with advanced melanoma and tumours in vertical growth phase (Howell *et al.*, 2001).

As the GCC/ACC genotype of Pt15392 represents a -1082 GA polymorphic site, it was not possible to conclude whether Pt15392 had a favourable or bad prognosis at a genetic level. Nevertheless, this patient has now been disease free for 12 years after the surgical removal of the lymph node metastasis and the high IL-10 releasing CD4+ T cell clones described herein may have been one contributing factor to the favourable clinical outcome. However, the data presented in this chapter are insufficient *per se* to allow any strong conclusion and further studies including a larger set of patients might reveal a trend or a pattern of association.

5.5.3 Conclusions

To define the role of the high IL-10 releasing cytokine profile of the CD4+ T cells from Pt15392 in regulating the tumour specific immune response, additional experiments should be performed. In order to further explore the role of IL-10 it would be crucial to analyse the effect of recombinant IL-10 or anti-IL-10 neutralising antibodies, in the transwell system described here.

Moreover, the suppressive mechanism of Tr cells has been described to be independent of cytokine production and to require cell-cell contact (Chai *et al.*, 1999; Taams *et al.*, 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000), but I did not perform this type

of experiment. However, on the basis of the data presented in Chapter 4 and 5, the CD4+ T cells obtained from Pt15392 are not likely to belong to the IL-10 releasing Tr cells involved in maintaining tolerance and suppressing autoimmunity (Groux *et al.*, 1997).

In this chapter and Chapter 4, data have been presented that show that the cytokine profile released by the CD4+ T cell subsets of Pt15392 is similar but not identical to the profile of Tr cells (Table 5.2). These findings thus suggest that Pt15392 CD4+ T cell subsets could constitute a novel group of T lymphocytes with still undefined functional properties.

In conclusion, the characterisation of CD4+ T cell clones together with the molecular analysis of IL-10 implies a role for IL-10 as an immunostimulatory cytokine in the anti-melanoma response observed in Pt15392.

The analysis of the anti-tumour CD4+ T cell response in other long time surviving melanoma patients with emphasis on the cytokine release, might provide additional information regarding the relevance of this type of CD4+ T cells in tumor protection. However, the particular features observed for the CD4+ T cells of Pt15392, could remain a unique case.

Although the data presented in this chapter were intriguing, they were not investigated further as the main objective of this project was to identify the target antigen for the CD4+ T cell mediated immune response. With this perspective, an invariant chain (Ii) – cDNA fusion library was constructed and screened for antigen identification (Chapter 6 and 7).

Table 5.2: A comparison of the profiles of cytokines secreted by the high releasing IL-10 CD4+ T cell clones of Pt15392 (T$_{15392}$) and the known cytokine profiles of CD4+ T cell subsets. The classical CD4+ T helper (Th) cells are functionally divided into (i) Th1 cells participating in cell-mediated inflammatory reactions and releasing IL-2 and IFN-γ and, (ii) Th2 cells secreting IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and which are active in humoral responses. In addition to the cytokines indicated below, both subsets also release IL-3, TNF-α and GM-CSF. Immunosuppressive, reulatory (Tr) CD4+ T cells are divided into a Tr1 and one Th3 subset. The Tr1 cells secretes high levels of IL-10, low amounts of IFN-γ, TGF-β and TNF-α, but no IL-4 or IL-5, while Th3 cells are characterised by their high release of TGF-β. Th3 cells also produce IL-4 and IL-10, but only minimal levels of IFN-γ. The CD4+ clones from Pt15392 (T$_{15392}$) release a high IL-10 containing Th1 profile, which is different to all the other desribed CD4+ subsets. ND = not done. This table has been adapted from (Wakkach et al., 2000).

SUBSET	IL-2	IFN-γ	TGF-β	TNF-α		IL-4	IL-5	IL-6	IL-10
		Th1 cytokines					**Th2 cytokines**		
[1]Th1	+	+	+	+		-	-	-	-
[1]Th2	-	-	-	+.		+	+	+	+
[2]Tr1	-	+	+	+		-	+	-	+++
[3]Th3	-	+/-	+++			+	-	-	+
T$_{15392}$	+	+	-	+		-	ND	ND	+

1) Originally described for mouse (Mosmann et al., 1986) and for human (Del Prete et al., 1993).
2) Originally described for mouse and human in (Groux et al., 1997).
3) Originally described for mouse (Chen et al., 1994b) and for human (Fukaura et al., 1996).

Chapter 6

CONSTRUCTION AND SCREENING OF AN Ii-cDNA FUSION LIBRARY: IDENTIFICATION OF A POSITIVE CONSTRUCT

6.1 INTRODUCTION

The identification of tumour associated antigens (TAAs) has had a crucial impact on the development of tumour immunotherapy: in particular the elucidation of the molecular nature of TAAs recognised by T cells has opened up a new perspective for the vaccination of cancer patients (reviewed in (Rosenberg, 1997)). The discovery of TAAs was mainly achieved by the development of cDNA library screening by T lymphocytes (Boon, 1993), a technique that allows fast cloning and provides new tools for designing vaccines for cancer immunotherapy. In addition to providing moleculary defined TAA peptides potentially useful for clinical intervention, increased insight into the nature of class I HLA restricted TAAs has been achieved (reviewed in (Coulie *et al.*, 2001; Van den Eynde and Boon, 1997; Wang and Rosenberg, 1999)). Nevertheless, it soon became clear that although essential for mediating an anti-tumour response, the quantity and possibly quality of CD8+ T cells generated *in vivo* by different immunological manipulations were *per se* insufficient to activate a strong anti-tumour reaction that could lead to a significant tumour regression. One of the reasons for such a limited clinical response could be found in the lack of MHC class II epitopes in the vaccine used and, therefore, in a lack of activation of CD4+ T cells.

In fact, several studies have demonstrated that CD4+ T cells play an essential role at different levels of the anti-tumour responses (reviewed in (Pardoll and Topalian, 1998; Toes *et al.*, 1999; Topalian *et al.*, 1996)). As helper T cells, CD4+ T cells, (i) augment and sustain the cytolytic immune response by cross-priming of CD8+ T cells (Ridge *et al.*, 1998), (ii) are involved in the generation of CD8+ T cells (Surman *et al.*, 2000), and (iii) are required for maintaining CD8+ T cell numbers and possibly also to promote CD8+ T cell infiltration of the tumor (Marzo *et al.*, 2000).

Vaccination studies in mice (Mandelboim *et al.*, 1995) and recent trials in humans (Huang *et al.*, 2002) have demonstrated the importance of both CD8+ and CD4+ T cells in tumour destruction. The prominent role of CD4+ T cells has been further corroborated by their capacity to eradicate MHC class II deficient tumour cells in mice (Mumberg *et al.*, 1999; Ossendorp *et al.*, 1998) and recent findings indicate that a CD4+ T cell mediated anti-tumour mechanism that involves IFN-γ release and inhibits angiogenesis (Beatty and Paterson, 2001; Qin and Blankenstein, 2000). Thus, successful vaccination with tumour antigens relies on the activation of CD8+ as well as CD4+ T cells (reviewed in (Greten and Jaffee, 1999)). However, the mechanisms that underline these synergistic actions remain to be fully elucidated.

Thus, in order to provide better tools for immune intervention in cancer, it is fundamental to characterise molecularly TAAs restricted not only by class I HLA, but also those seen in the context of class II HLA. However, the development of a standard technique for cloning genes encoding HLA class II-restricted TAAs has been hampered by the complex nature of the MHC-class II processing pathway (reviewed in (Geuze, 1998)). The method for the identification of CD8+ T cell epitopes uses recipient cells transfected with plasmids encoding tumour cDNA. The cytoplasmatic localisation of these plasmids leads to the entrance of the encoded proteins into the class I HLA processing pathway. However, modifications in the cDNA library construction and in the recipient cells can provide an engineered system that directs the entrance of endogenously synthesised proteins into the class II HLA processing pathway. This can be achived by fusing the tumour derived cDNA in frame with with an ER (endoplasmatic reticulum) retention signal, provided by the first 80 amino acids of the Ii (Odorizzi *et al.*, 1994; Pieters *et al.*, 1993; Teyton *et al.*, 1990).

The resulting chimeric protein, once produced by the biosynthetic machinery of the transfected cells, will be preferentially targeted to the endoplasmatic reticulum (ER) thereby facilitating entrance into the class II HLA processing pathway (Chervonsky *et al.*, 1994; Odorizzi *et al.*, 1994; Tan *et al.*, 1997), where the peptide loading will occur in different intracellular compartments (reviewed in (Fineschi and Miller, 1997; Geuze, 1998)). Furthermore, in order to provide a specific system for monitoring of a CD4+ T cell mediated antigenic response, recipient cells must be transfected with the polymorphic class II HLA β-chain which can assemble with the endogenously expressed α-chain and form a recombinant class II HLA allele (reviewed in (Cresswell, 1994; Pieters, 2000)).

In this chapter I have used an innovative molecular approch to identify candidate TAAs recognised by CD4+ T cells obtained from Pt15392. This antigen cloning strategy is based on the availability of specific immunological reagents; i.e. (i) established tumour reactive CD4+ T cell clones (Chapter 4), (ii) subcloned HLA alleles expressed by the patient under study, and (iii) a tumour derived cDNA library (presented in this chapter).

Parts of the work with the library construction have been performed in collaboration with Dr. Luisa Novellino.

6.2 AIMS OF THE CHAPTER

- Optimising a strategy for construction of a cDNA library capable of directing inserts into the endogenous antigen processing pathway.

- Library screening

6.3 RESULTS

6.3.1 Rationale for cDNA library construction and screening

The main steps required for cDNA library construction and screening are shown in Figure 6.1. Messenger RNA (mRNA) was isolated from patient derived tumour cells and then converted into cDNA. The synthesised cDNA was then ligated to adapters and fractionated by liquid chromatography in order to select only large cDNA-adapter inserts (> 500bp). These selected tumour-derived cDNAs were cloned into an expression plasmid. The plasmid used for library construction was pEAK8, an eukaryotic expression plasmid in which the transcription of the cloned insert is driven by the EF-1 alpha promoter. This plasmid has been engineered to contain the first 80 amino acids of the Ii in frame with the multiple cloning site, at which the cDNA was inserted (Figure 6.2). This procedure resulted in a fusion protein containing the NH_2 terminal amino acids derived from the Ii protein followed by the amino acid sequence encoded by the tumour-derived cDNA. The Ii confers the proper cellular localisation of the fusion protein to the class II processing pathway. The detailed steps used to screen this cDNA library are reported below.

In order to screen the Ii-cDNA fusion library it was necessary to transfect the library into appropriate recipient cells. A complex set of molecular steps must co-operate inside the recipient cells to allow expression of CD4+ T cell epitopes on the cell surface, as outlined in Figure 6.3. In particular it is very important to use highly transfectable recipient cells (herein 293 cells), which express a complete HLA-class II processing machinery. To this aim, 293 cells engineered to express the class II trans-activator (CIITA) protein, 293/CIITA cells, were used. It has been shown that CIITA is a key molecule for the physiological transcription of genes encoding the HLA-DR, -DQ and -DP alleles, as well as the other proteins belonging to the HLA-class II machinery, like the Ii and HLA-DM
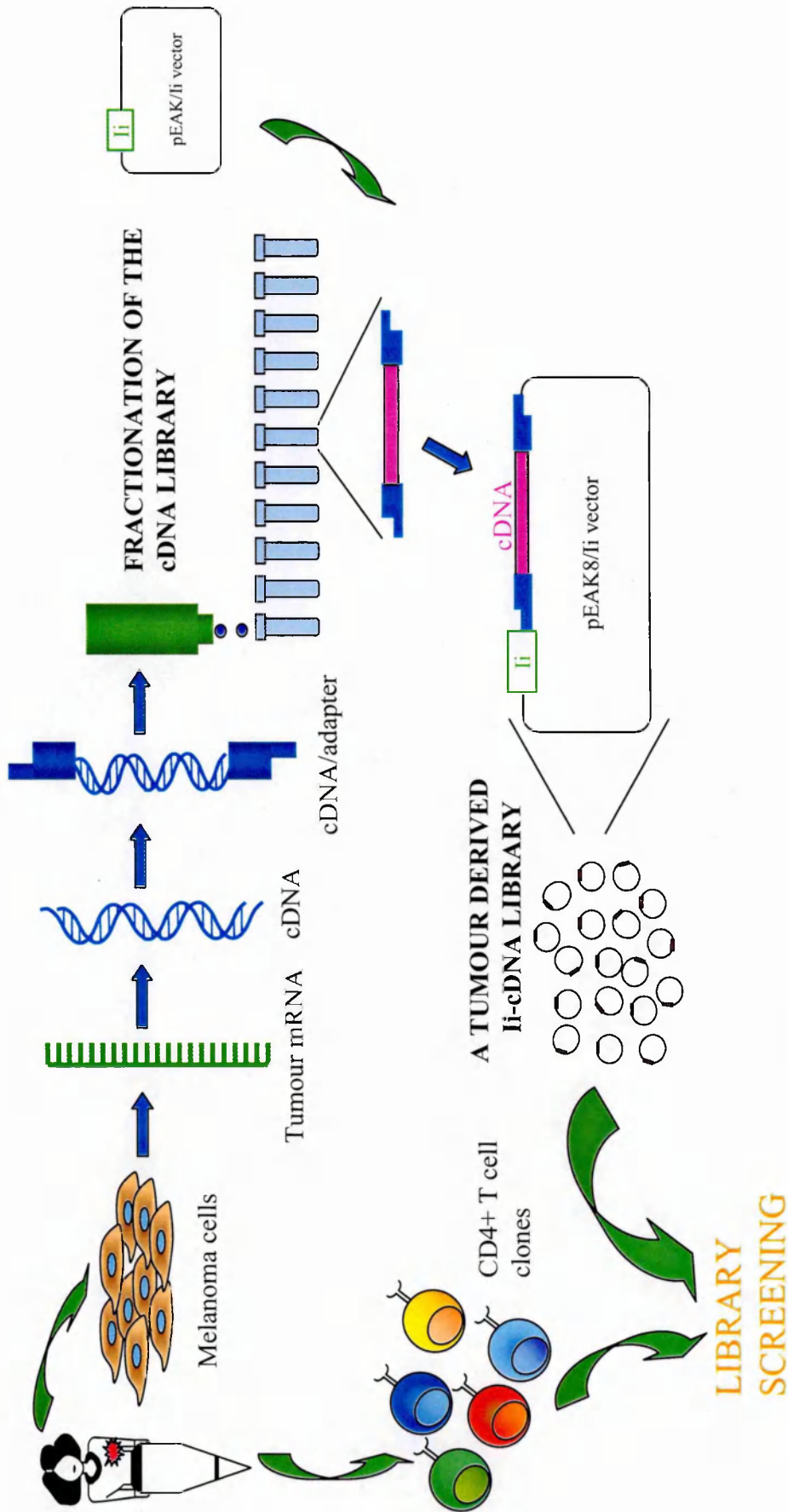
FIGURE 6.1: A schematic view of the construction and screening of an Ii-cDNA library. Messenger RNA (mRNA) was extracted from tumour cells of Pt15392, converted into cDNA and linked to adapters. In a next step, the cDNA was size fractionated by column chromatography to obtain uncontaminated cDNA fragments of suitable length, after which the appropriate cDNA/adapter fragment was ligated with the invariant chain (Ii) of an expression vector, here pEAK8. This Ii- cDNA fusion library was then submitted for several rounds of screening with autologous CD4+ T cell clones.

FIGURE 6.2: The construction of an invariant chain (Ii) - cDNA fusion library by ligation of the cDNA-adapter insert to the pEAK8/Ii vector. Messenger RNA (mRNA) was prepared from melanoma cells extracted from a metastatic lymph node of Pt15392. The reverse transcription was performed by the aid of a primer-adapter providing asymmetry in the cDNA sequence as it inserted new restriction sites as for example *Xba* I (indicated above by a red line). The first cDNA strand was then used as a template for the synthesis of the second strand. The ligation efficiency of the double stranded blunt-ended cDNA, which constitutes a poor substrate for the T4 ligase, was then maximized by the addition of a *Bst* XI adapter providing a *Bst* XI overhang suitable for vector ligation. The cDNA/adapter sequence was then cut by *Xba* I to obtain asymmetric stick ends allowing directional cloning into the pEAK8 vector. Full sequences for the *Xba* I primer-adapter and the *Bst* XI adapter are shown, the dotted line show the precise cutting pattern for *Xba* I. The bold grey letters highlight the nucleotides of the *Xba* XI cutting site, while the bold green letters represent the cutting site of *Bst* XI. The library was constructed following the SuperScript Plasmid System procedure.

mRNA 5' —— AAAAA - 3'

⇩ **Addition of the Not I primer-adapter**

mRNA 5' —— AAAAA - 3'   *Xba* **I site**

3'   T T T T T GACTAGT**TCTAGA**TCGCGAGCGGCCGCCC - 5'

←————————— *Xba* I primer-adapter ————————→

⇩ **1st strand synthesis**

1st strand 3' ▬▬ T T T T T GACTAGT**TCTAGA**TCGCGAGCGGCCGCCC - 5'

←→ **cDNA**   ←————— *Xba* I primer-adapter —————→

⇩ **2nd strand synthesis**

1st strand 3' ▬▬ GACTAGT**TCTAGA**TCGCGAGCGGCCGCCC - 5'

2nd strand 5' ▬▬ CTGATCA**AGATC**TAGCGCTCGCCGGCGGG - 3'

←→ **cDNA**   ←————— *Xba* I primer-adapter —————→

⇩ **Ligation of the *Bst* XI adapter**

CTGGAAAG ▬▬ GACTAGT**TCTAGA**TCGCGAGCGGCCGCCC**CTTTCCAGCACA**

ACAC GACCTTTC ▬▬ CTGATCA**AGATC**TAGCGCTCGCCGGCGGGGAAAGGTC

←——————→ ←→ ←——————————————→ ←——————→
***Bst* XI adapter**   **cDNA**   *Xba* I primer-adapter   ***Bst* XI adapter**

⇩ **Digestion with *Xba* I**

CTGGAAAG ▬▬ GACTAGTT

ACAC GACCTTTC ▬▬ CTGATCA**AGATC**

←——————→ ←→ ←—————————→
***Bst* XI adapter**   **cDNA**   *Xba* I primer-adapter

Ligation to the pEAK8/Ii vector

Kpn I   *Nco* I   *Bst* XI   *Eco* RI   *Asc* I   *Ecr* RV   *Hind* III   *Bst* XI   *Xba* I   *Not* I

EFα promoter   **Ii**₁₋₈₀

pEAK8/Ii

Amp   ORI

The pEAK8/Ii vector was cut with *Bst* XI and *Xba* I.

FIGURE 6.3: Detailed view of the strategy for the transfection the invariant chain (Ii) -cDNA library from melanoma cells of Pt15392. Recipient 293 cells, engineered to constitutively express the class II trans-activator, was double transfected with the polymorphic β-chain HLA-DRβ1*10011 (DR10) and pools of the Ii-cDNA fusion library. CIITA indirectly induce the expression of the HLA class II antigen processing machinery. This assists the assembly of the class II HLA allele, i.e. the DR-10 β-chain associates with the internally provided α-chain. The Ii-cDNA fusion protein binds to the class II HLA complex, after which the signal sequence in the cytoplasmic tail of the Ii direct this complex to the cell surface. During this route, the Ii-cDNA fusion protein become degraded and mediate the possibility for the peptide to bind to the groove of the class II HLA molecule and become presented for CD4+ T cells on the cell surface. The library screening was performed in an autologous system by the addition of CD4+ T cell clones characterised from a long time surviving patient (Pt15392). The supernatants from these cultures can be evaluated for their IFN-γ release by ELISA.
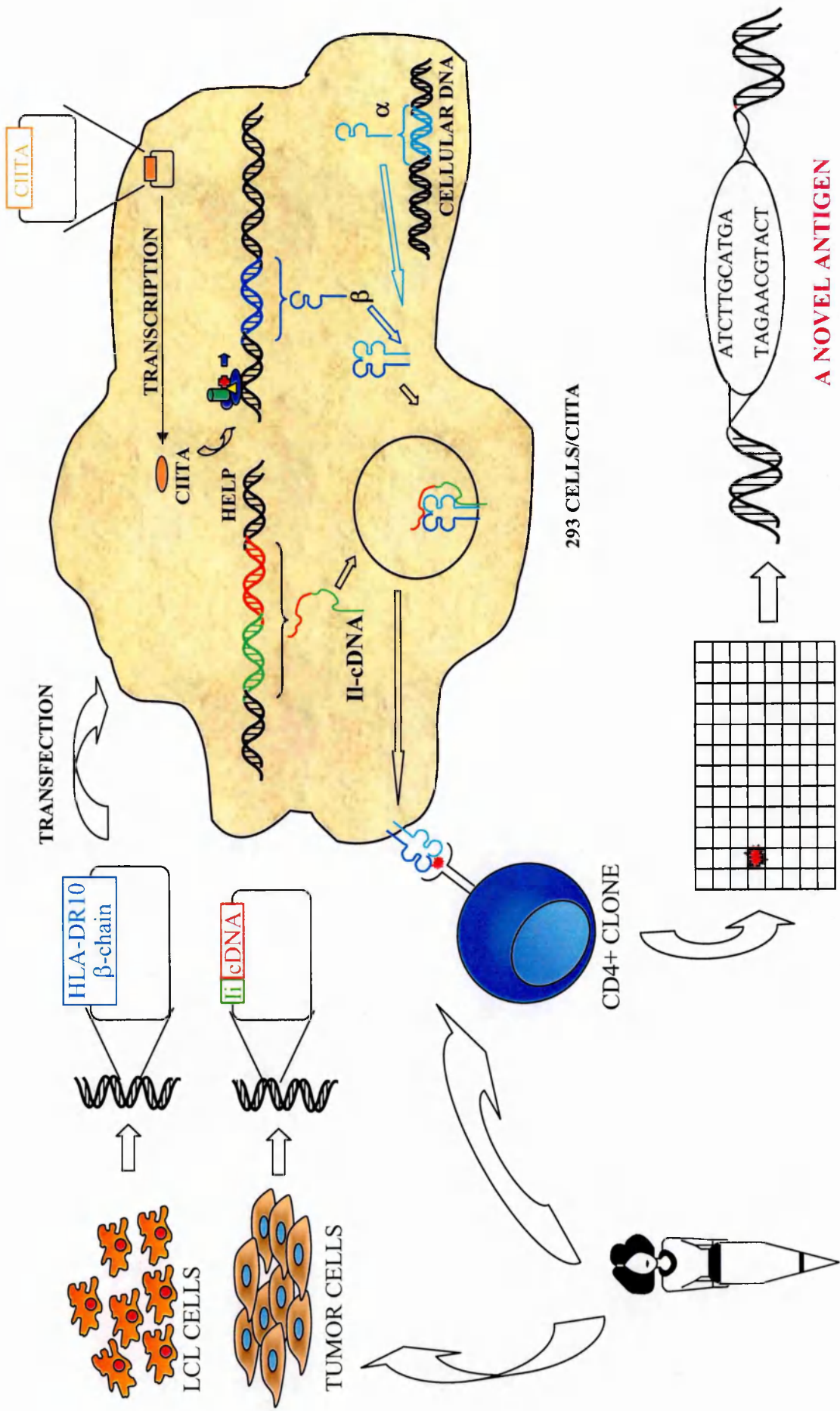
molecules (Chang and Flavell, 1995; Kern *et al.*, 1995; Liu *et al.*, 1999; Wright *et al.*, 1998). Moreover, 293/CIITA cells were genetically modified to express the HLA-DR alleles of melanoma Pt15392 (DRβ1*01021 and DRβ1*10011). The α-chain of HLA-DR was intrinsically provided by the 293 cells, while the polymorphic β-chain (Andersson, 1998) was cloned from LCL cells of Pt15392 and transfected into 293 cells.

Prior to transfecting recipient cells with the Ii-cDNA fusion library, the library was transformed into *E.coli* cells and divided into pools of approximately 100 bacteria carrying independent recombinant inserts. This number of constructs has been quantified as suitable for an efficient and quantitative throughput screening of a large number of samples (reviewed in (De Plaen *et al.*, 1997)). Plasmid DNA was extracted from these bacterial pools and separately transfected into modified 293/CIITA recipient cells expressing the proper HLA allele derived from Pt15392. This allele conferred specificity into the system and worked as a restriction element for the T cell clone. The transfected cells were then incubated overnight with the T cell clone whose antigen has to be identified. Finally, T cell activation was monitored by evaluating the amount of IFN-γ released in the medium using a commercially available ELISA. IFN-γ release thus indicates T cell triggering and the presence of a tumour-derived cDNA encoding the nominal antigen originally recognised by the T cell clone used for the screening.

6.3.1.i   Construction and qualitative evaluation of the Ii-cDNA fusion library

The high quantitative and qualitative yield obtained for the isolated polyA mRNA is a prerequisite to obtain a high quality library corresponding to 10^6 clones according to the SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning. This protocol indicates that 1 – 5 µg of mRNA are sufficient for the synthesis of a good cDNA library.

Thus the FastTrack protocol was applied for mRNA isolation and a yield of 6.6 µg of polyA mRNA was obtained from 40×10^6 melanoma cells.

6.3.1.ii Library construction: oriented expression

We chose to construct a directional Ii-cDNA fusion library, as it minimises the number of clones needed for the screening. The cDNA was genetically engineered to express two-staggered terminus with different restriction sites. The first strand synthesis was copied using as template the tumour-derived mRNA and as primer an oligo (dT) primer containing the *Xba* I restriction site at the 5' terminus (Figure 6.2). The first strand DNAs were converted into blunt-ended second strand cDNA and subsequently ligated to the *Bst* XI adapters.

The recombinant cDNA inserts will express 5' *Bst* XI and 3' *Xba* I terminus after digestion with the *Xba* I the enzyme (Figure 6.2). Thus, this approach makes possible the generation of cDNAs with asymmetric ends allowing a directional cloning.

6.3.1.iii Library construction: fractionation

The selection of large cDNAs (> 500 bp) by size fractionation polished the cDNA by any residual adapters and by all the fragments generated by the double digestion. This procedure avoided the production of recombinant plasmid containing irrelevant inserts. Moreover, cDNA smaller then 500 bp do not represent full-length copies of mRNA transcript. Thus, by selecting large cDNA inserts we improved the quality of our library.

With the purpose of obtaining the highest possible yield, the amount of cDNA obtained after the different passages of first and second strand synthesis was not measured, but the total quantity was used for the adapter ligation. The cDNA yield was measured after size fractionation. As explained in the Material and Methods, cDNA fractions #3 to #12 could

be considered to contain large inserts with minimal contamination of non-ligated adapters and smaller cDNAs. To estimate the quantity of cDNA in these selected fractions an ethidium-bromide staining was performed. Samples #3 to #6 did not contain any cDNA, while the amount of the cDNA of the fractions #7 to #12 is indicated in Table 1. According to the SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning, a cDNA yield of 5-10% of the initial mRNA can be obtained. We obtained a total mass of 469 ng of fractionated cDNA (Table 6.1), that represents approximately 7% of the initial 6.6 µg of the melanoma-derived mRNA used for the library construction. Moreover, at individual level, fraction #10 was the most concentrated sample. Among the remaining five fractions, one differed markedly from the others showing a concentration as low as 0.5 ng/µl. Altogether, 4 high standard preparations with an estimated concentration of 3 ng/µl could be defined from the size fractionation.

Table 6.1. Amounts of cDNA obtained in fractions #7 to #12 after size fractionation. These fractions are considered as containing large cDNA inserts.

FRACTION	FINAL VOLUME[1]	CONCENTRATION	MASS
# 7	35 µl	0.5 ng/µl	18 ng
# 8	12 µl	3 ng/µl	36 ng
# 9	35 µl	3 ng/µl	105 ng
# 10	37 µl	4.5 ng/µl	167 ng
# 11	12 µl	3 ng/µl	36 ng
# 12	36 µl	3 ng/µl	108 ng

1) Volume remaining in each fraction after the use of 5 µl for a fluorescent evaluation of cDNA content.

6.3.1.iv Library construction: quantitative estimation

The ligation of cDNA to the vector backbone as well as the bacteria transformation was carefully optimised to reach maximum efficiency. In order to obtain an approximate evaluation of which approach would confer the highest transformation efficiency to our Ii-cDNA fusion library, we compared two independent systems of transformation, chemical transformation versus electroporation into *E.coli* ElectroMAX™ DH10B™ competent cells. The transformations reported above were performed with empty pcDNA3.1(-)/Hygro vectors produced 1.5×10^5 positive colonies/µg (Q_C) by chemical transformation and 8.5×10^7 positive colonies/µg (Q_E) by the ElectroMAX approach (Table 6.2A). The latter approach appeared to be 600 times more efficient and the numbers of colonies obtained fell 80 times above the range indicated for a high quality library. The superiority of the ElectroMAX protocol was due to the fact that the DNA was introduced into target cells by electroporation, which is more efficient than chemical conditions.

As additional steps for the optimisation of library construction, experiments were set up aimed at evaluating the quality of the backbone vectors, selected as possible recipients for the expression library and opened up in the cloning site *Bst* XI and *Xba* I. For these quality controls, cDNA inserts were substituted with a green fluorescent protein (GFP) - derived fragment having compatible *Bst* XI and *Xba* I sticky ends. The GFP fragments were ligated into the *Bst* XI and *Xba* I sites of pEAK8/Ii, pEAK8.5 and pcDNA3.1(+) vectors. The *E.coli* ElectroMAX™ DH10B™ competent cells were too costly to be used to set up the conditions of the system and, therefore, we performed a chemical transformation representative of an equal transformation with the library. The mean value of transformation efficiency achieved by the chemical procedure was 3.3×10^3 positive colonies/µg (Q_{GFP}).

Table 6.2: Calculation of the number of transformants that can be expected in the Ii-cDNA fusion library applying the ElectroMAX approach for transformation. A) The transformation efficacy, Q, obtained by different approaches. The values for the ElectroMAX electroporation and the chemical transformation, Q_E and Q_C respectively, were obtained for empty vectors, while Q_{GFP} is the mean value of chemical transformations performed with different vectors carrying GFP as an insert; a setting resembling the conditions that would be used for transformation of the library. The Q_{GFP} efficiency was further used to calculate the approximate number of transformants (N_t) that could be expected when performing the ElectroMAX electroporation with the library. B) The total number (tot-N_t) of expected transformants in the library using 80ng of vector in the ligation reaction.

A

The ratio of efficiency

ElectroMAX (Q_E): 8.5x10^7 colonies/µg

Chemically (Q_C): 1.5x10^5 colonies/µg

$$Q_E / Q_C = 6x10^2 = \kappa$$

Chemically (Q_{GFP}): 3.3x10^3 colonies/µg

The approximate number of transformants (N_t)

$N_t = \kappa \times Q_{GFP} = 6x10^2 \times 3.3x10^3$ colonies/µg $= 2.0x10^6$ colonies/µg

B

The total number (tot-N_t) of transformants in the library

Mass to use of vector (m_{vector}) = 80 ng

Tot-$N_t = N_t \times m_{vector} = 2.0x10^6$ colonies/µg x 0.080 µg $= 1.6x10^5$ colonies

---

This efficiency was multiplied by the factor of efficiency obtained for *E.coli* ElectroMAX™ DH10B™ competent cells (Q_E) (Table 6.2A). The equation puts the number of theoretical transformants possibly obtained with the ElectroMAX approach to 2.0×10^6 per µg. Our results indicate the high quality of all of our backbone vectors and the high standard of our experimental procedures.

Finally, according to the guidelines indicated in the SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning the ratio between the vector and cDNA quantity is a critical parameter affecting the total number of colonies contained in the future library. Thus, considering these limits, in the library ligation I decided to use a molecular ratio of 2/1 for the cDNA insert and pEAK8/Ii vector i.e. 25 ng cDNA (approximately 1000 bp) and 80 ng pEAK8/Ii vector (6400 bp).

Setting these conditions, the number of expected recombinant clones in our Ii-cDNA fusion library should be close to 1.6×10^5 (Table 6.2B) independent clones. This number is consistent with the generation of a high quality cDNA library, comprehensive of relatively low abundant transcripts.

6.3.1.v   Library construction: cDNA and vector ligation

The amounts of cDNA and pEAK8/Ii vector to be used in each ligation were carefully evaluated (se the calculations above) to theoretically obtain 1.6×10^5 transformants in the library (Table 6.2B), a value indicative of a comprehensive high quality library. To perform the library ligation, 4 of the obtained fractions containing large cDNA inserts (Table 6.1) were chosen as reagents. More precisely, fractions #9, #10, #11 and #12 all were in the same range of concentration and fraction #9 and #10 were chosen as representative samples. In addition, fraction #7 was pooled with fraction #8 to include all the cDNA information contained in the relevant fractions for the library ligation. All the 3

cDNA fractions (#7 + 8; #9 and #10) were subjected to individual ligation reactions with the pEAK/Ii vector and the ligated cDNAs (L#7+8, L#9 and L#10) were immediately electroporated into bacteria (described below).

6.3.1.vi Library construction: transformation

The 3 cDNA libraries L#7+8, L#9 and L#10, were thus submitted to electroporation into *E.coli* ElectroMAX™ DH10B™ Competent Cells. Each electroporation reaction was performed in duplicate resulting in a total volume of 2 ml per each ligation. 50 μl of each electroporation was used for quality control and serial dilutions and plating were set up in order to determine the titer of the library. The independent electroporated bacteria were then kept overnight at 4°C and amplified according to the Millipore protocol (se below) after the assessment of its quality was completed.

6.3.1.vii Qualitative library evaluation

Qualitative evaluation of the recombinant plasmids obtained with the cDNA fractions (#7+8, #9 and #10) was performed using a PCR-based approach. DNA from each colony was amplified with primers specific for the pEAK8/Ii sequencing spanning the cloning site. The presence and the length of the cDNA insert was deduced from the length of the band obtained in this amplification. Bands of 700 bp matched to the empty cloning site containing only the Ii, while bands showing a higher molecular weight identified positive fragments. The appropriate length of the cDNA inserts was obtained by subtracting the molecular weigh of the amplified sequences with 700 bp. The results shown in Figure 6.4 clearly indicated many characteristics for each individual ligation. To focus on some important features, the L#7 + 8 library contained cDNA inserts of variable length, indicative of the variability of mRNA transcripts contained in the original tumour cells. On the other hand, this library contained very few transformants (Table 6.3) and a large

FIGURE 6.4: A quantitative and qualitative evaluation of different fractions of the Ii-cDNA library performed PCR with colonies. The invariant chain (Ii) – cDNA library was constructed by transformation into *E.coli* cells and plating onto LB/Amp plates. After incubation overnight, single colonies were directly analysed by RT-PCR. Each gel shows an upper and one lower raw of samples, where each lane corresponds to the sample of one single colony. The molecular weight of each cDNA insert was calculated by subtracting the length of the obtained bands with the 700 bp, which corresponds to the empty cloning site containing only the Ii. Library (L) L#7+8 and L#10: lane 1 and 28 = 1 kb ladder; lane 2 - 27 = cDNAs from single colonies. L#9: lane 1 and 25 = 1 kb ladder; lane 2 - 24 = cDNAs from single colonies. bp = base pair.

Library L#7+8
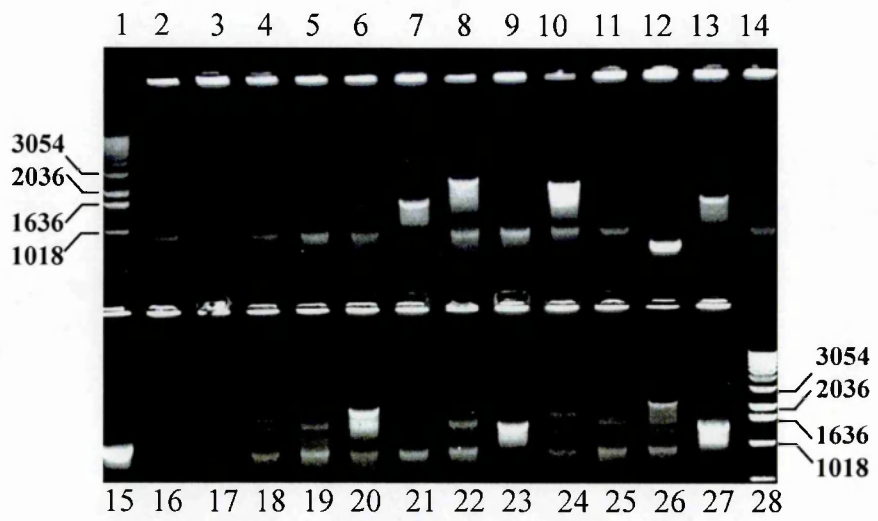
Library L#9
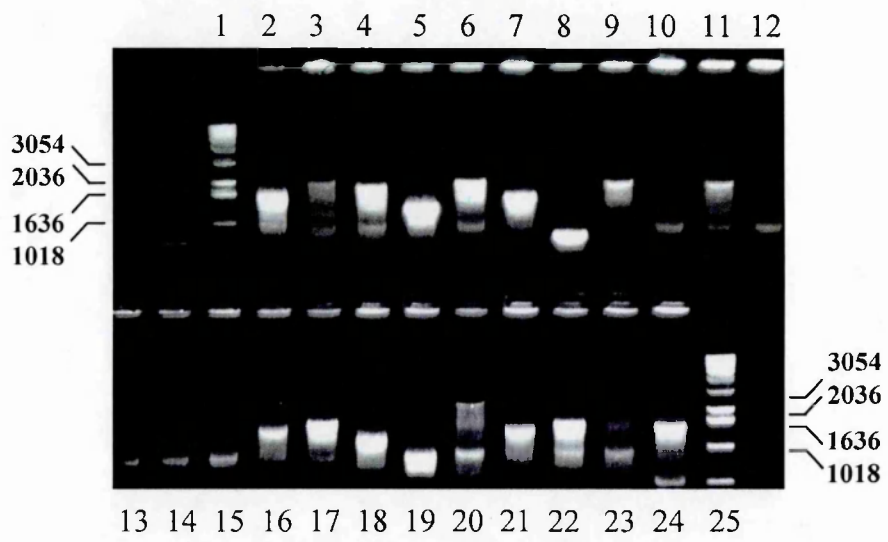
Library L#10
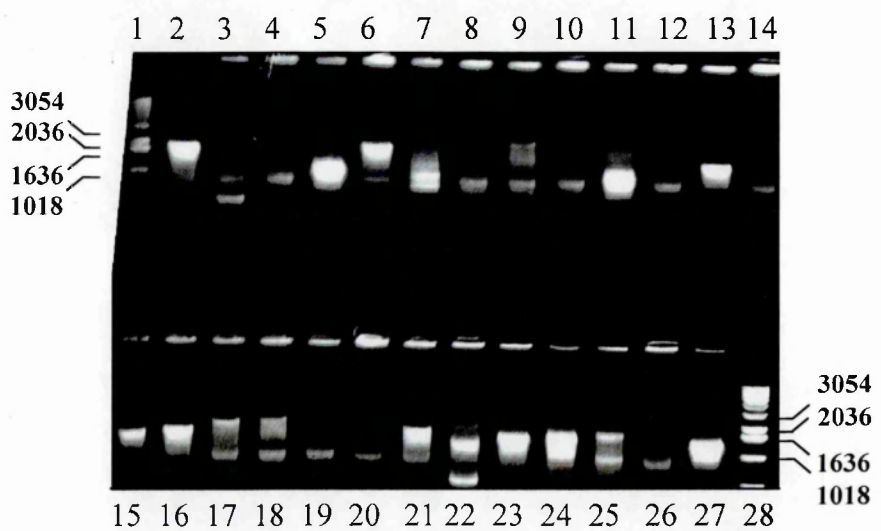
Table 6.3: Qualitative and quantitative features of the Ii-cDNA fusion libraries. *E.coli* DH5α cells were electroporated with the Ii – cDNA fusion library and subsequently plated onto LB/Amp plates that were left for overnight incubation at 37°C. The % of recombinants corresponds to the total number of single colonies obtained for each library after plating onto LB/Amp plates. The length show the range of bands present in each library. The transformation efficiency shows the number of colonies obtained per μl and the final column show how many total colonies that were obtained for each library. No = number.

LIBRARY	% RECOMBINANTS	LENGTH	TRANSFORMATION EFFICIENCY	TOTAL No
L#7+8	30%	600-1800 bp	26 colonies/μl	52000
L#9	60%	600-1300 bp	5.5 colonies/μl	11000
L#10	50%	500-1200 bp	10 colonies/μl	20000

number of colonies just contained an empty plasmid. On the contrary, the L#9 and L#10

libraries contained a much higher number of positive recombinants. In addition, they also

showed a similar yield, both carrying cDNA inserts ranging in size from 700 bp to 1300

(Table 6.3). However, 50% of the L#10 library was represented by short inserts of just 500

- 600 bp, while only two recombinants with insert of 1000 bp or more were detectable. As

an example, lane 15 and lane 16 (Figure 6.4) do contain short cDNA, while the band in

lane 22 should represent only the empty vector. A high percentage of long inserts reaching

the 1300 bp in size was included L#9 library. Thus, the L#9 Il-cDNA fusion library was

shown to constitute a more comprehensive library, as it contained cDNA inserts with a

wider range of molecular weights and the highest number of recombinants (60%) carrying

cDNA inserts of proper length.

The total number of colonies obtained for each electroporation was determinated taken into

account the remaining volume and the bacteria titer (Table 6.3). Library L#9 and L#10

both demonstrated a low transformation efficiency with only 5.5 colonies/µl and 10

colonies/µl respectively, while L#7 + 8 was at least 3 times more efficient, with 26

colonies/µl (Table 6.3). The total number of recombinants for each electroporation is

reported in Table 6.3. This quantitative data revealed that the total number of recombinant

plasmids obtained in each ligation was 10-fold lower than the theoretical expected yield of

1.6×10^5 transformants (Table 6.2).

6.3.1.viii Library amplification

Library amplification was only performed for the ligations of fraction L#9 and L#10

ensuring the highest percentage of plasmids carrying cDNA insert with the correct length.

Bacteria derived from electroporation L#9 and L#10 with a carefully evaluated titer (see

quantitative evaluation) were derived and grown in liquid cultures containing an average

of 100 bacteria, were inoculated in 96 deep well blocks in a final volume of 1.5 ml of media containing the selective antibiotic ampicillin. This method allowed a rapid high throughput method for plasmid minipreparations, outlined in the Millipore MultiScreen®Assay System protocol.

6.3.1.ix Preparation of the recipient cells 293 suitable for the library screening

The 293/CIITA recipient cells were employed for the genetic expression of a specific HLA class II processing designed to reconstitute the HLA-DR haplotype of Pt15392, and to assure the expression of restriction elements for the CD4+ T cell clones (Figure 6.3). This was achieved by the use of 293/CIITA cells, which showed a stable 98% expression of the HLA class II alleles (Figure 6.5), due to infection with a retroviral vector expressing CIITA. However, the polymorphic DRβ1*01021 and DRβ1*10011 β-chains (see section 2.3.5) had to be provided to the system by transient transfection during the screening procedures. This engineered cells allowed the highly specific expression of fully assembled class II HLA alleles, as the non-polymorphic HLA-DR α-chain, endogenously supplied by the cellular DNA of the 293/CIITA cells, could join with the transfected β-chains of the HLA-DR alleles.

6.3.1.x Screening of the Ii-cDNA fusion library and identification of a positive pool conferring activation of a CD4+ T cell clone.

Chapter 4 described the functionally characterisation of the CD4+ T cell repertoire of Pt15392 and among the defined clones, TB515 was selected for screening of the Ii-cDNA fusion library. This clone demonstrated a high specificity and, when stimulated with the autologous tumour, it released a high amount of IFN-γ (Figure 6.6) and showed anti-tumour lysis even when used at a very low effector:target (E:T) ratio (Figure 6.7). Unfortunately, since no anti-HLA-DR10 or -DR1 specific antibodies are commercially available, it was not possible to anticipate which DR allele acted as restriction element in
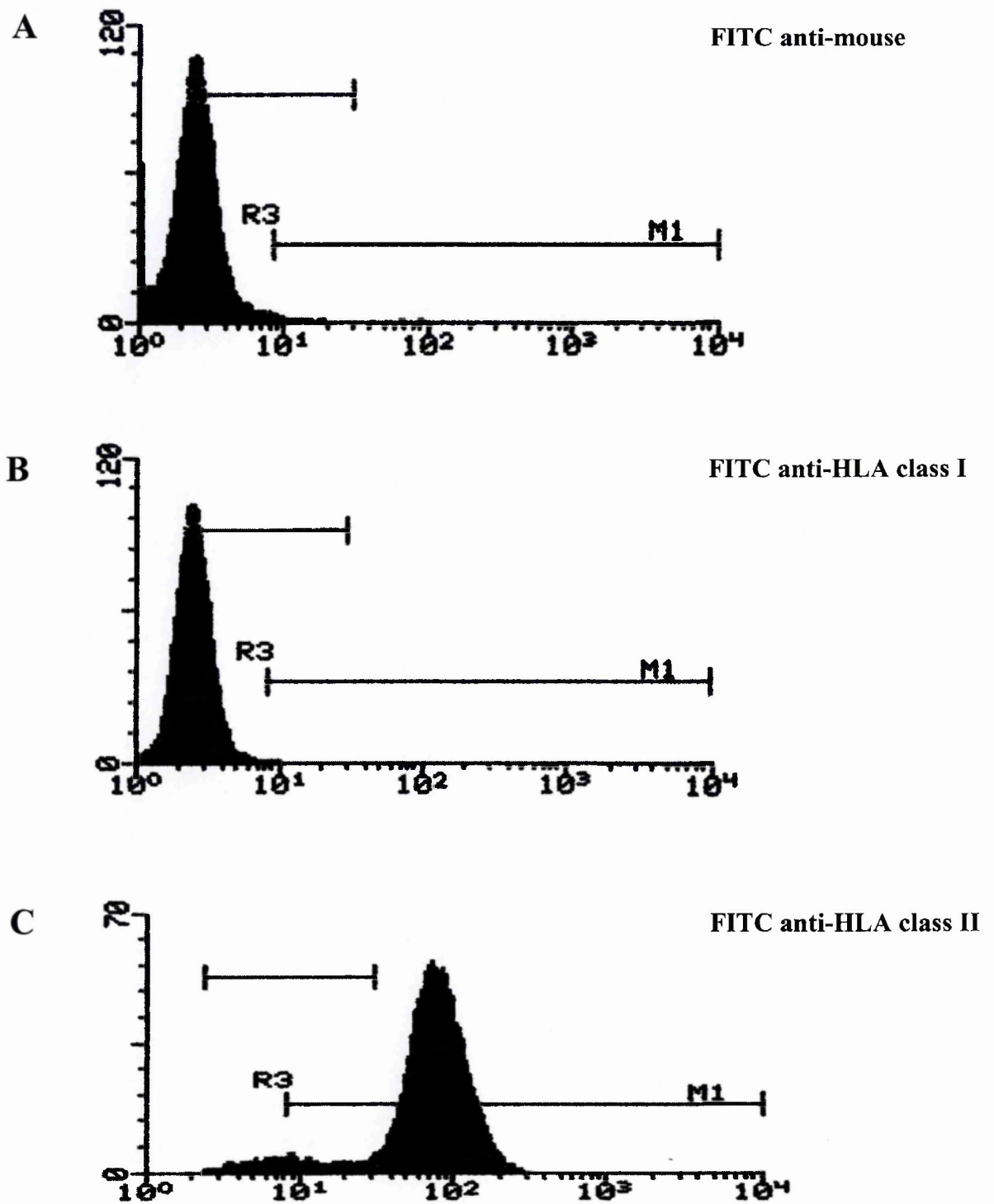
FIGURE 6.5: Class II HLA expression on 293-EBNA cells stably transfected with CIITA. Fluorometric analyses of the surface expression of class I and class II HLA alleles on 293-EBNA cells constantly expressing CIITA (293/CIITA) with (A) FITC anti-mouse Ab; (B) FITC anti-HLA-A, -B and –C (W6/32) mAb; (C) FITC anti-HLA-DR (L243) mAb. The 293/CIITA cells are positively stained to almost 98%.

IFN-γ release (pg/ml)
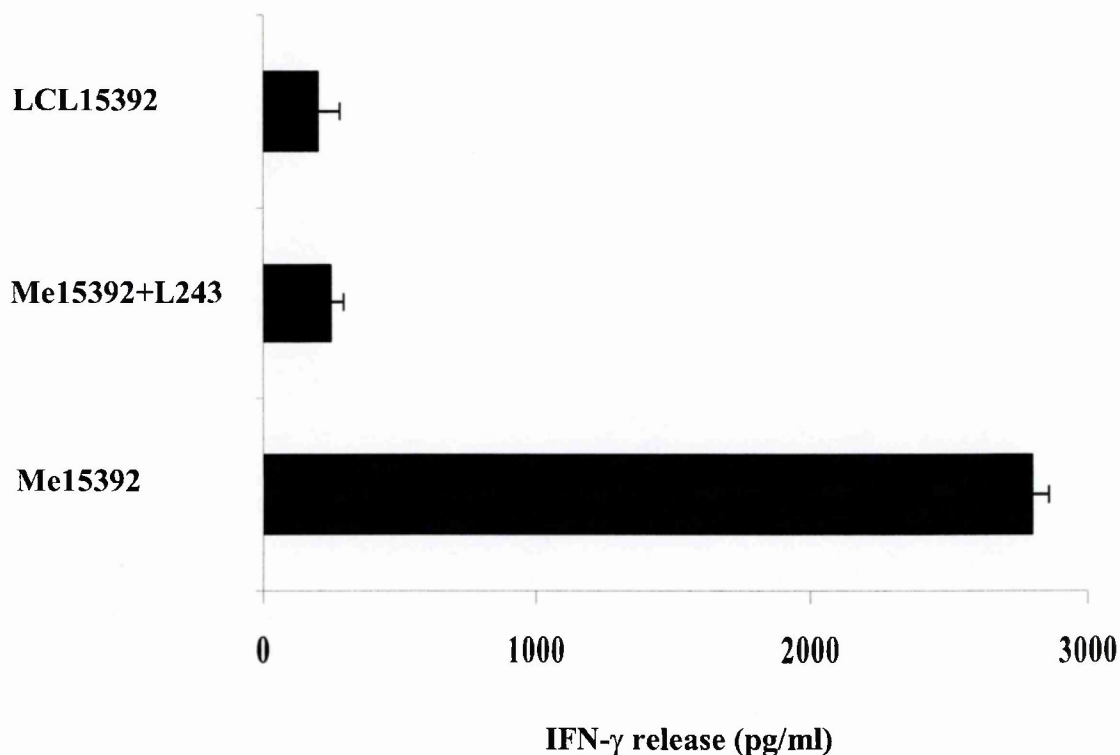
FIGURE 6.6: Reactivity of clone TB515 against the autologous tumour. The autologous melanoma (Me15392) or LCL (LCL15392) cells were incubated with 5x10³ cells of clone TB515 over nigth at an E:T ratio of 1:1. Me15392 induced a significant IFN-γ release from clone TB515 (p<0.001). The supernatants were collected and evaluated for their IFN-γ content by ELISA. The anti-HLA-DR antibody L243 added in the test significantly ihnibited the cytokine release (p<0.001). Results are shown as mean ± S.D. of two experiments.
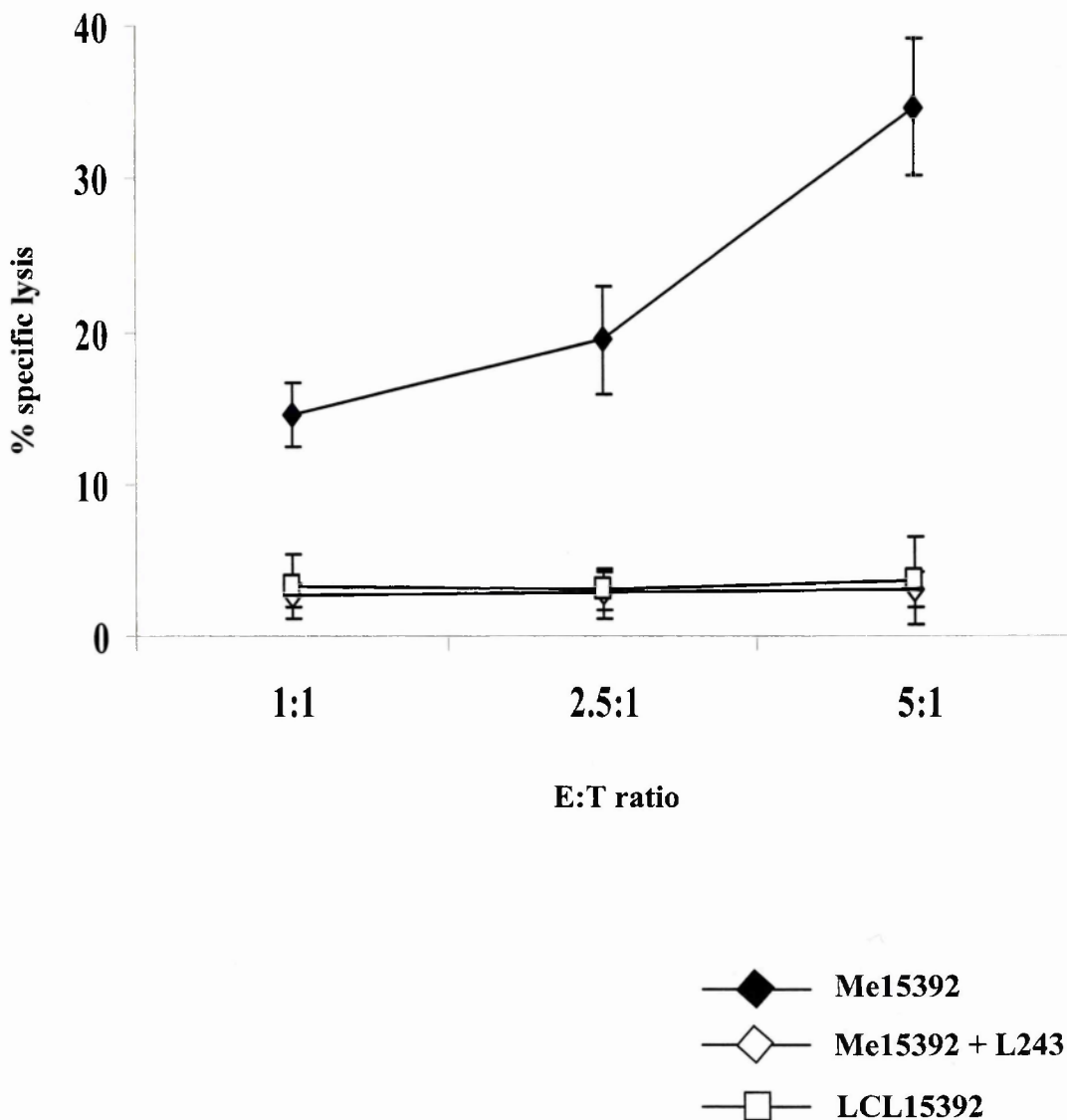
FIGURE 6.7: Cytotoxic activity of clone TB515 against the autologous tumour. Clone TB515 (5×10^3 cells/well) was incubated for 5 h with labelled autologous melanoma (Me15392) or LCL cells (LCL15392) (control) at indicated E:T ratios. The cytotoxic activity was evaluated in a standard 4 h ^{51}Cr-release assay. Lytic activity was significantly blocked (p<0.001) by the addition in the test of the anti-HLA-DR antibody L243. Results are mean ± S.D. of three separate experiments.

the tumour recognition by TB515. For this reason, a double library screening had to be performed using either the DRβ1*01021 and DRβ1*10011 molecules independently. However, the melanoma cells of Pt15392, clearly expressed HLA-DR and could be used as positive controls in the screening procedure (Figure 6.8).

As a last parameter, to ensure that the expression level of the cDNA was detectable in our system, the GFP was used as an internal control for transfection: a good fluorescence was observed down to 1 ng/μl of cDNA. This indicated that the system can allow detection of one single cDNA construct, expressed together with 100 others in a pool, using only 200ng of DNA for transfection. Thus, the tumour derived Ii-cDNA fusion library was submitted for screening by the available CD4+ T cell clone TB515 by co-transfection of either the HLA-DRβ1*01021 or -DRβ1*10011 allele together with individual cDNA pools ($\approx$ 100 cDNAs/pool) into 293/CIITA cells. Indeed, one positive clone was detected after the screening of $5x10^4$ pools restricted in HLA-DRβ1*10011 (Figure 6.9), while no positive clones could be identified after additional evaluation of as many as $1x10^5$ Ii-cDNA constructs restricted in HLA-DRβ1*01021. The positive clone, named pool #D2, was re-analysed several times with independent transfections and confirmed to mediate CD4+ T cell recognition.

6.4 DISCUSSION

In this chapter, the genetic methodology for cloning of class II HLA restricted TAA has been presented. The reported qualitative and quantitative features of the Ii-cDNA library (Table 6.3 and Figure 6.4) and the engineered 293 cells represented an optimal system for antigen identification. However, the limitation with the Ii-cDNA fusion library approach, with respect of using a normal cDNA library, is that a triple number of clones need to be screened to reach an optimum number of correctly expressed cDNAs. In fact, the Ii can be
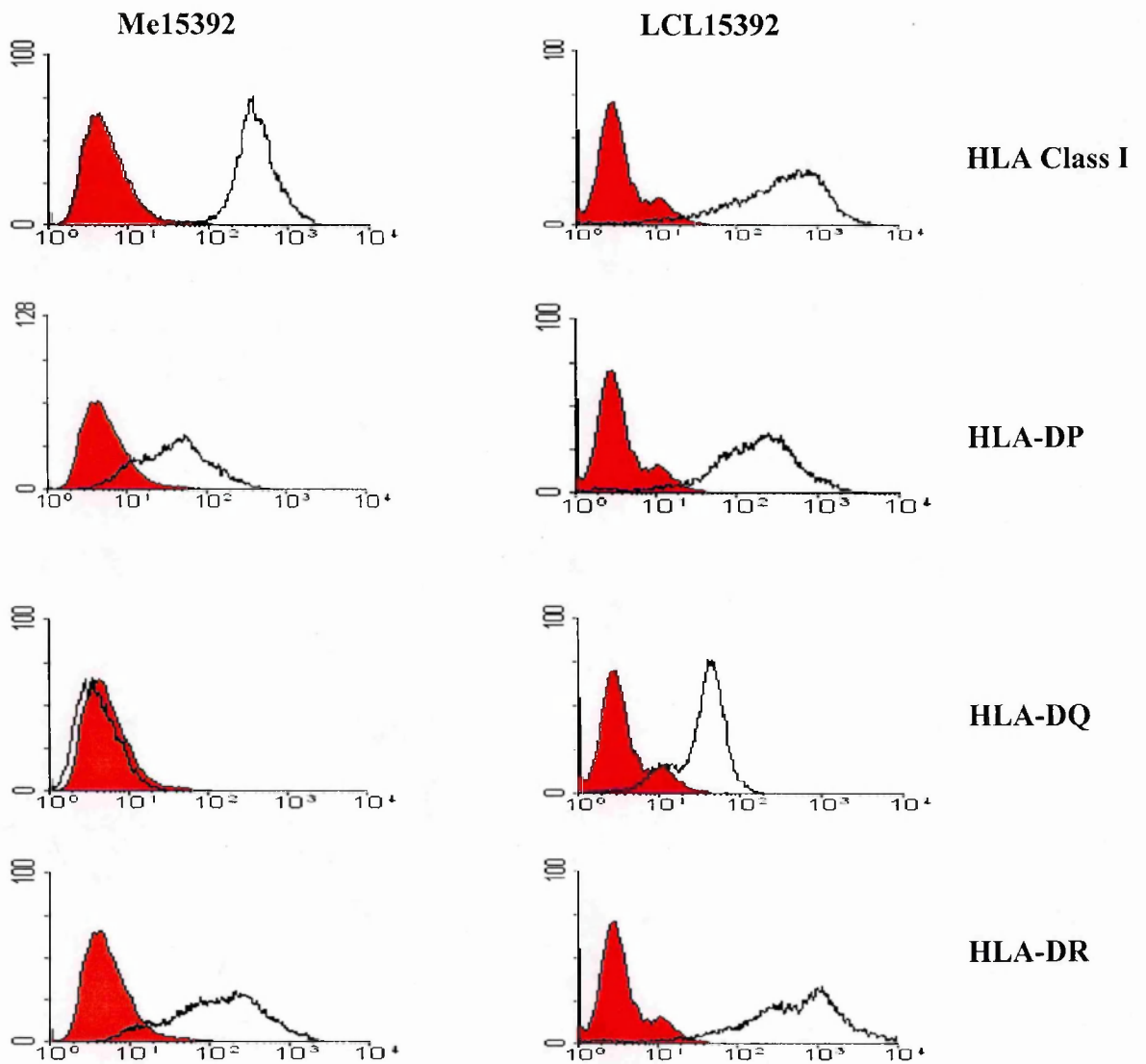
Figure 6.8: Characterisation of the class II HLA expression on Me15392 cells. Fluorometric analyses of the surface expression of class I and class II HLA alleles on autologous melanoma cells (Me15392) (left panels) and LCL cells (LCL15392) (right panels). The cells were stained with the anti-HLA-A, -B and –C mAb W6/32 and the anti-DP, -DQ and -DR mAb. Red profiles represent baseline unspecific fluorescence (negative control). The LCL15392 cells express all alleles of Pt15392, while Me15392 was positively stained for HLA class I and HLA-DP and –DR, but did not express HLA-DQ.
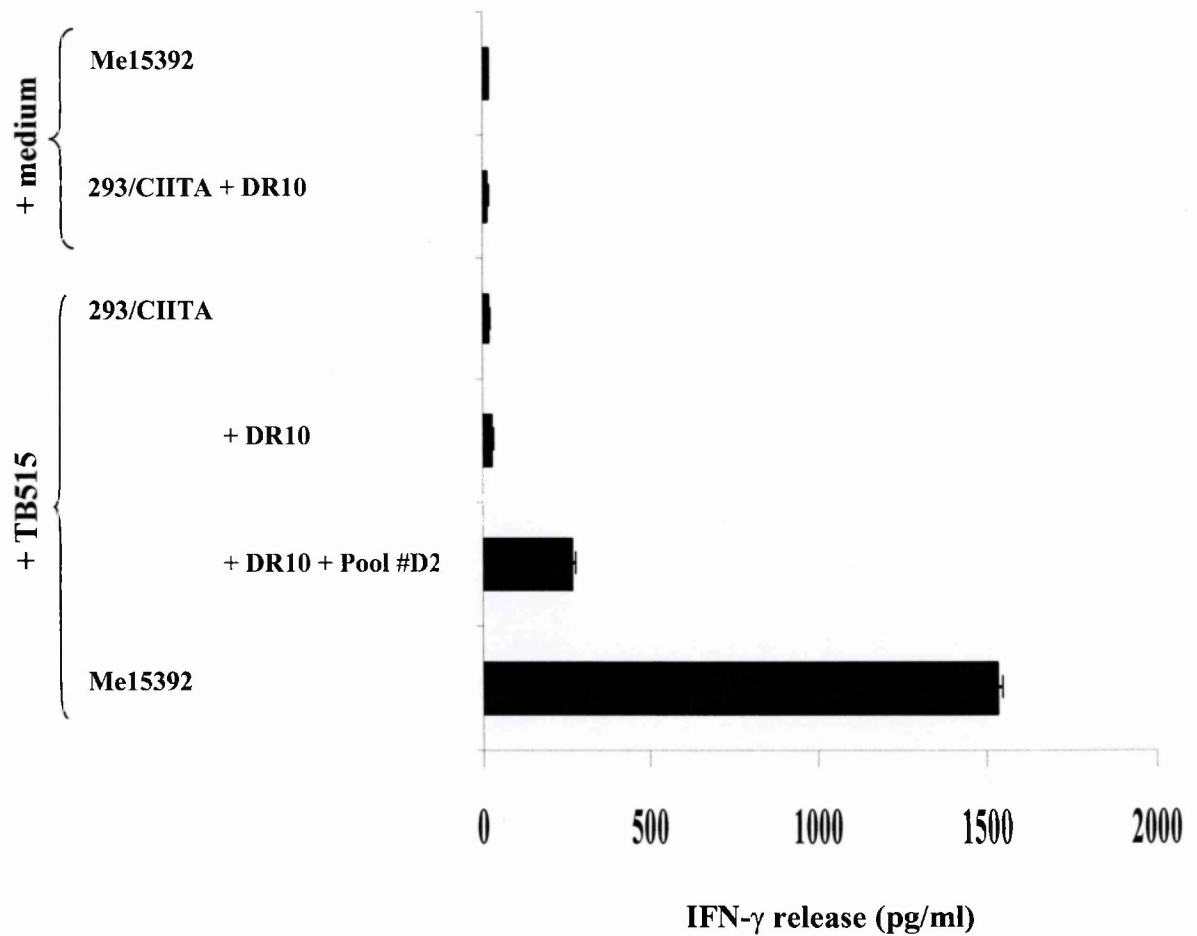
Figure 6.9: Recognition of a a positive HLA-DRβ1*10011 restricted pool of the Ii-cDNA fusion library. Recipient 293 cells (1×10^4 cells/well), constitutively expressing the class II trans-activator (CIITA), were double transfected with 150 ng of either the HLA-DRβ1*01021 or -DRβ1*10011 (DR10) allele together with individuals pools of an invariant chain (Ii) – cDNA fusion library (approximately 100 Ii-cDNAs/pool). The cells were incubated for 24 h before the addition of 2×10^4 cells/well of the CD4+ T cell clone TB515. After overnight incubation, the supernatants were evaluated for their IFN-γ content by ELISA. Autologous melanoma cells (Me15392) were used as positive control. The screenings of the library were performed twice for each allele with single wells representing 50 different pools. Results are collected from 3 experiments and represent the mean ± S.D. No positive pools were found using HLA-DRβ1*01021 (not showed).

ligated to any of the 3 open reading frames of the cDNA insert. However, one advantage provided by the use of an Ii-cDNA fusion library is that it assures the delivery of the cDNA construct into the HLA class II pathway of antigen processing.

The method use here required functional characterised CD4+ T cells as initial reagents. Other strategies, like reverse immunology (reviewed in (Schultze and Vonderheide, 2001)) and computerised peptide algorithms (Maier *et al.*, 1994; Rammensee *et al.*, 1999) allow the prediction of potential peptides of class II HLA-restricted antigens, but they require to know the antigenic sequence. These methods also benefit from the fact that the antigen under study can be pre-selected, which is an advantage when TAAs have to be identified from cancers other than melanoma, as culture of other neoplastic cells has proved difficult. Moreover, it is possible to identify naturally processed epitopes by performing biochemical dilution of epitopes presented on the HLA molecules on the cell surface, but this is a cumbersome procedure and does not allow a genetic targeting of genes expressed by the tumour (Mandelboim *et al.*, 1994).

The results presented in this chapter demonstrate that clone TB515 was involved in an anti-tumour response. As antibodies specific for these alleles not are commercially available, this result indicated the presence of a tumour antigen restricted in either HLA-DRβ1*01021 or HLA- DRβ1*10011 allele expressed by Pt15392. While antigens such as gp100 (Li *et al.*, 1998), MART-1/Melan-A (Zarour *et al.*, 2000), CDC27 (Wang *et al.*, 1999a), TPI/m (Pieper *et al.*, 1999) and LDLR/FUT (Wang *et al.*, 1999b) encode epitopes restricted by HLA- DRβ1*0101, no antigen has previously been described as restricted by HLA-DRβ1*10011. This implies two possibilities; first, T cell clone TB515 may recognise either a new or already described epitope restricted by HLA-DRβ1*0101 or, second, that

clone TB515 has mounted an immune response against a novel antigen restricted by HLA-DRβ1*10011.

Thus, in order to assess the nature of the antigen recognised and define the exact allele restriction, clone TB515 was employed in the screening of the Ii–cDNA library in the presence of either of these alleles. One positive pool of the library was identified as restricted by the class II HLA allele DRβ1*10011 (Figure 6.9), whereas no positive pool could be found by co-transfecting the HLA-DRβ1*01021 allele (not shown). This result clearly indicated the presence of a novel TAA, restricted by HLA- DRβ1*10011 and uniquely expressed by the melanoma cells of Pt15392. It also opens many challenging questions about the identity of this unique antigen and the features of its immunogenic epitope, an information that may contribute to a better understanding of the anti-CD4+ T cell mediated tumour response in Pt15392.

Chapter 7

IDENTIFICATION OF THE PROTEIN AND DEFINITION OF THE EPITOPE OF THE UNIQUE ANTIGEN PTPR-κ/m

7.1 INTRODUCTION

Identification of epitopes from TAAs enabled clinical approaches for cancer treatment and the availability of such immunogenic peptides is also instrumental to better define the role of T cells in the anti-tumor response. In fact, such epitopes have also a prominent role in the evaluation of T cell associated systemic immune responses (reviewed in (Melief and Kast, 1995; Melief *et al.*, 1996; Ressing *et al.*, 1996)). The experiments described in this chapter are focused on the identification of the antigenic protein expressed by the melanoma cells of Pt15392 representing the specific target for clone TB515 (as reported in Chapter 6). Moreover, the data reported in this chapter provide a fine molecular characterisation of the immunogenic peptide contained in such protein. This peptide constitutes the nominal epitope for the TB515 CD4+ T cell clone.

7.2 AIMS OF THE CHAPTER

- Identification of the gene conferring recognition by the CD4+ T cell clone TB515.

- Definition of the immmunogenic epitope of this antigen.

7.3 RESULTS

7.3.1 Construct/sequence identification

7.3.1.i Molecular approaches for sub-cloning the positive pool of the Ii-cDNA library

In the genetic approach for library screening, the Ii-cDNA library has been divided into pools each containing approximately 100 constructs carrying recombinant inserts. The identification of one positive pool, #D2, in this library (as reported in Chapter 6) was the first step to the molecular definition of the gene encoding this antigen. The subloning procedures used to isolate the single cDNA encoding the antigenic protein is shown in Figure7.1.

FIGURE 7.1: The molecular strategy to the single construct: the subcloning strategy to identify a single positive construct from pool #D2 of the Ii-cDNA fusion library of Pt15392. The first pool identified as positive, pool #D2, in the library screening was further subcloned in three subsequent cycles. Briefly, an aliquot of pool #D2 was elctroporated into *E.coli* cells, which were grown for 24 h in deep well culture blocks. The plasmid DNA was then purified by the Millipore multi-screen approach. Recipient 293 cells, constitutively engineered to express the class II transactivator (CIITA), 293/CIITA cells, were double-transfected with the restriction alleles HLA-DRβ1*01021 or –DRβ1*10011 and pools of plasmid DNA; 293 cells were then tested for their ability to stimulate IFN-γ release from the CD4+ T cell clone TB515. The positive subpool inducing the highest IFN-γ release, was selected for an additional round of subcloning. After 3 rounds of sububcloning, a single construct had been cloned and sequenced.
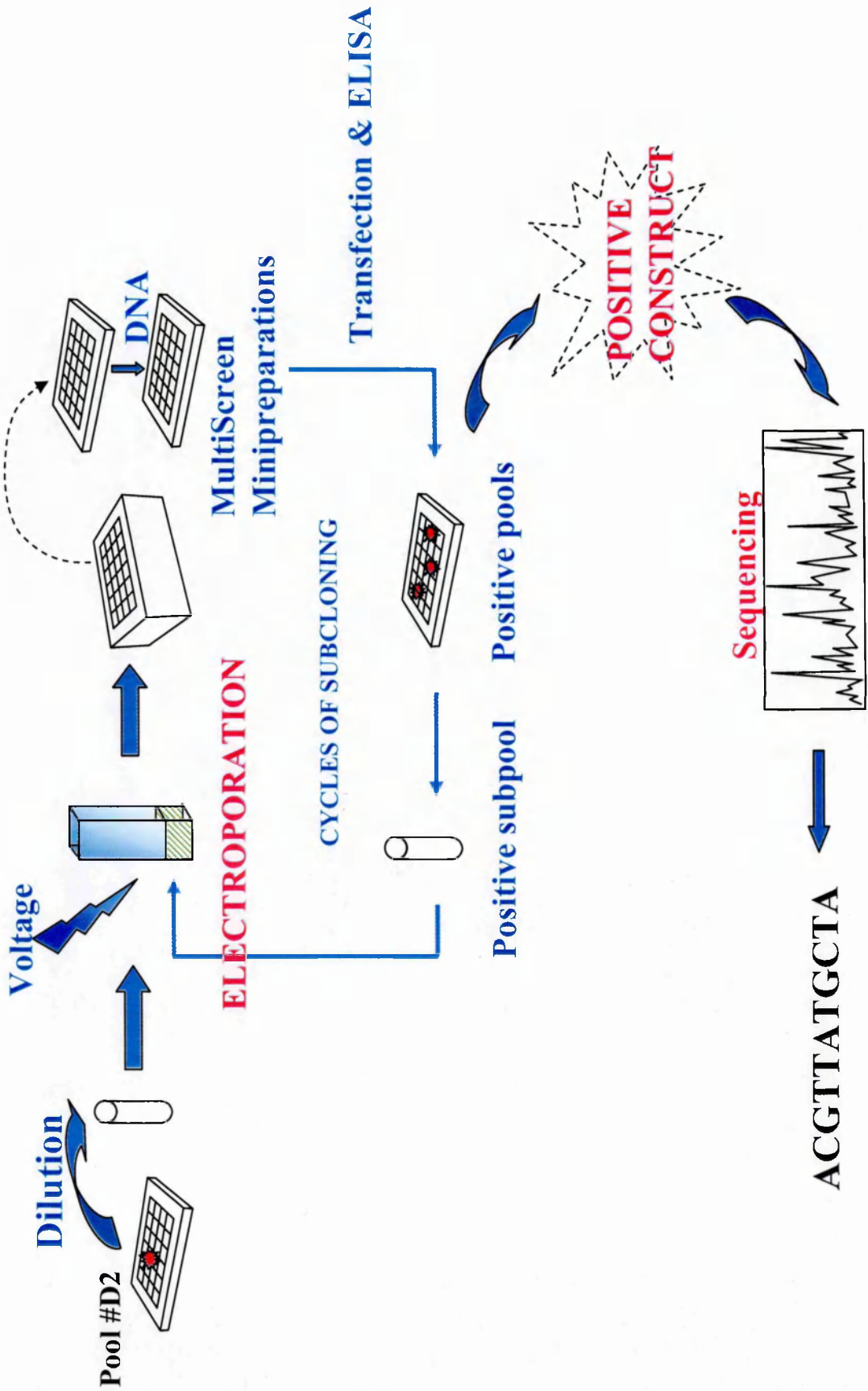
Firstly, an aliquot of the positive pool #D2 was submitted for electroporation into *E.coli* ElectroMAX™ DH10B™ competent cells. After titration, the electroporated bacteria were seeded in a deep well culture block at 150 bacteria per well. Plasmid DNA was prepared from these new subpools by the Millipore MultiScreen®Assay System protocol, and after transfection into 293/CIITA cells, 10 new pools were identified as being able to stimulate TB515 (Figure 7.2). The pool showing the highest IFN-γ release, #F6, was chosen for further evaluation. Secondly, the DNA pool #F6, selected from the 10 pools that resulted positive in the previous screening was subjected to a second cycle of sub-cloning and electroporated under the same conditions previously used for pool #D2. Subpools of 23 bacteria each were then grown in a deep well culture block of 96 wells. Plasmid DNA of these cultures was prepared following the Millipore MultiScreen®Assay System protocol and upon transfection, 23 positive pools were identified out of 189 tested. One of them, sub-pool #C8, was selected for further purification. In the third and last round of subcloning DNA from subpool #C8 was eletroporated in bacteria, and plated on soft agar containing ampicillin. Single growing colonies were then inoculated in liquid media. Plasmid DNA was extracted from 35 bacteria cultures, each derived from a single independent colony, by traditional mini-preparations following the Quiagen protocol. Finally, in this last step of the subcloning, plasmid DNA encoding these single inserts was transfected into 293/CIITA cells together with the HLA-DRβ1*10011 restriction allele. Among them, the DNA of Clone #11 was best recognised by clone TB515 (not shown) and thus identified as the putative insert encoding the protein containing the immunogenic epitope.
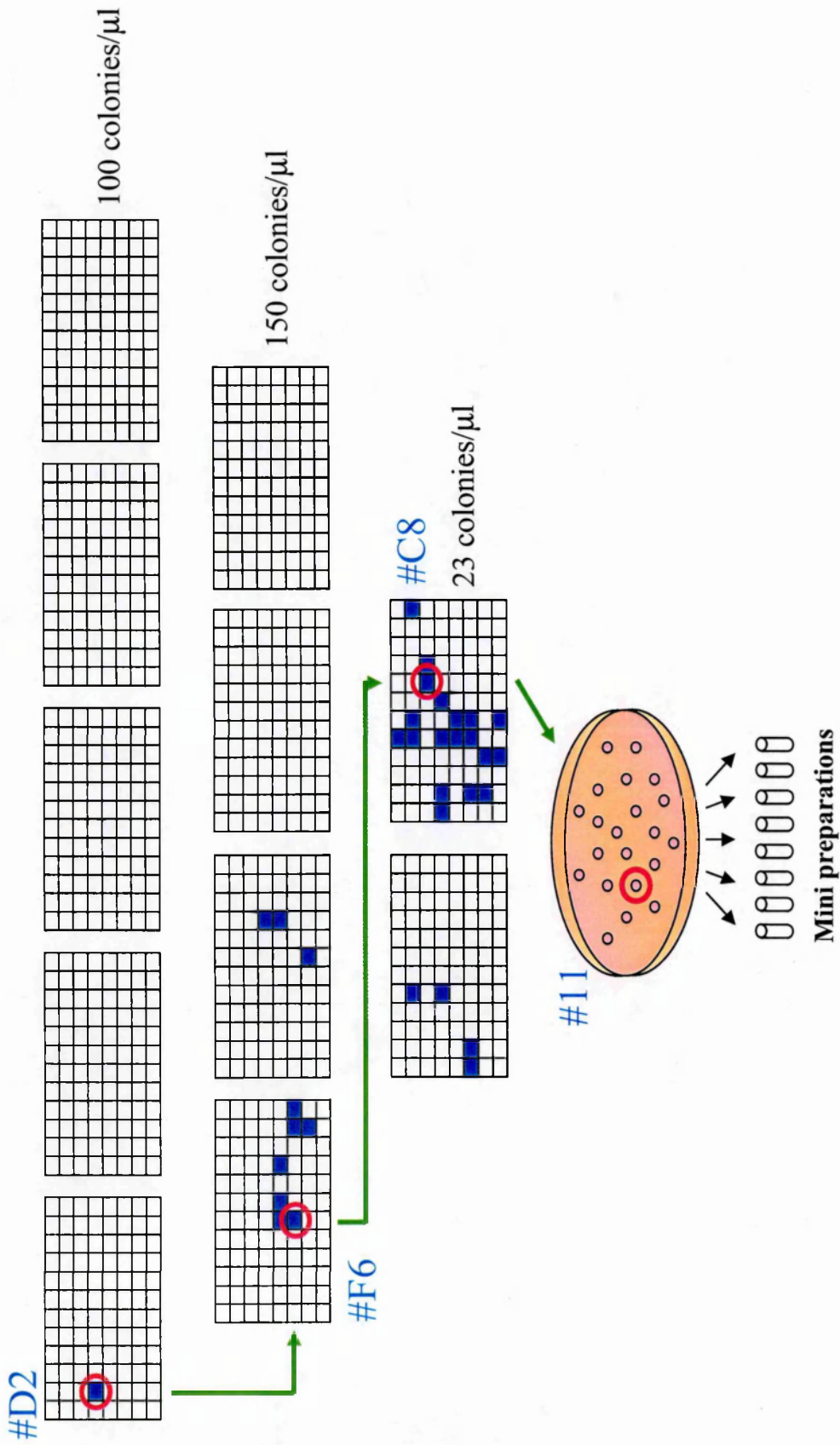
FIGURE 7.2: The subcloning procedure. Subcloning of the positive pool #D2. The number and positions of all positive subpools obtained in each cycle of subcloning are indicated. Plasmid DNA from pool #D2, #F6 and #C8 were all prepared by the Millipore multi-screen approach, while single DNA from colonies were prepared by standard mini-preparation. The titer (colonies/μl) of each subpool is indicated to the right.

7.3.2 Database search and sequence identification

7.3.2.i Characterisation of the insert from Clone #11

As a first step to characterise our positive Clone #11, DNA form Clone #11 was amplified with primers specifically spanning the cloning site of the pEAK8/Ii expression vector. A band of 1500 bp was amplified by this set of primers. The empty cloning site of the pEAK8/Ii vector spans 800 bp, i.e. 500 bp derived from the cloning site and 300 bp derived from the Ii (Figure 7.3A). Thus, by subtracting the molecular weight of the inserts carried by Clone #11 (1500 bp) and the pEAK8/Ii vector (800 bp), the positive clone #11 was found to encode an insert of 674 bp (Figure7. 3B).

7.3.3 Database analysis of insert #11

The 700-bp sequence of Clone #11, i.e. the individual construct of the Ii-cDNA library that mediated a positive recognition by the CD4+ T cell clone TB515, was compared with sequences in Gene Bank by using the Entrez site available on the web (http://www.microbiology.adelaide.edu.au/learn/index.htm). The computerised analysis showed that the sequence of Clone #11 was 99% identical to a previously reported sequence of the human *protein tyrosine phosphatase receptor kappa, the PTPR-κ gene* (Accession number NM-002844.2). The fine comparison between the two sequences revealed that in the cDNA derived from melanoma, a point mutation had occurred at position 2249 where a nucleotide replaced the corresponding wild type (wt) base g. The triplet sequence of the mutation codon encodes an R (Arg) while the wt sequence contains a G (Gly) (Figure 7.4). The mutated version found in the melanoma cells of Pt15392 will hereafter be referred to as PTPR-κ/m.

7.3.3.i An internal ATG codon directs the PTPR-κ/m expression

By the sequence analysis it was evident that the cDNA insert was not in frame with the
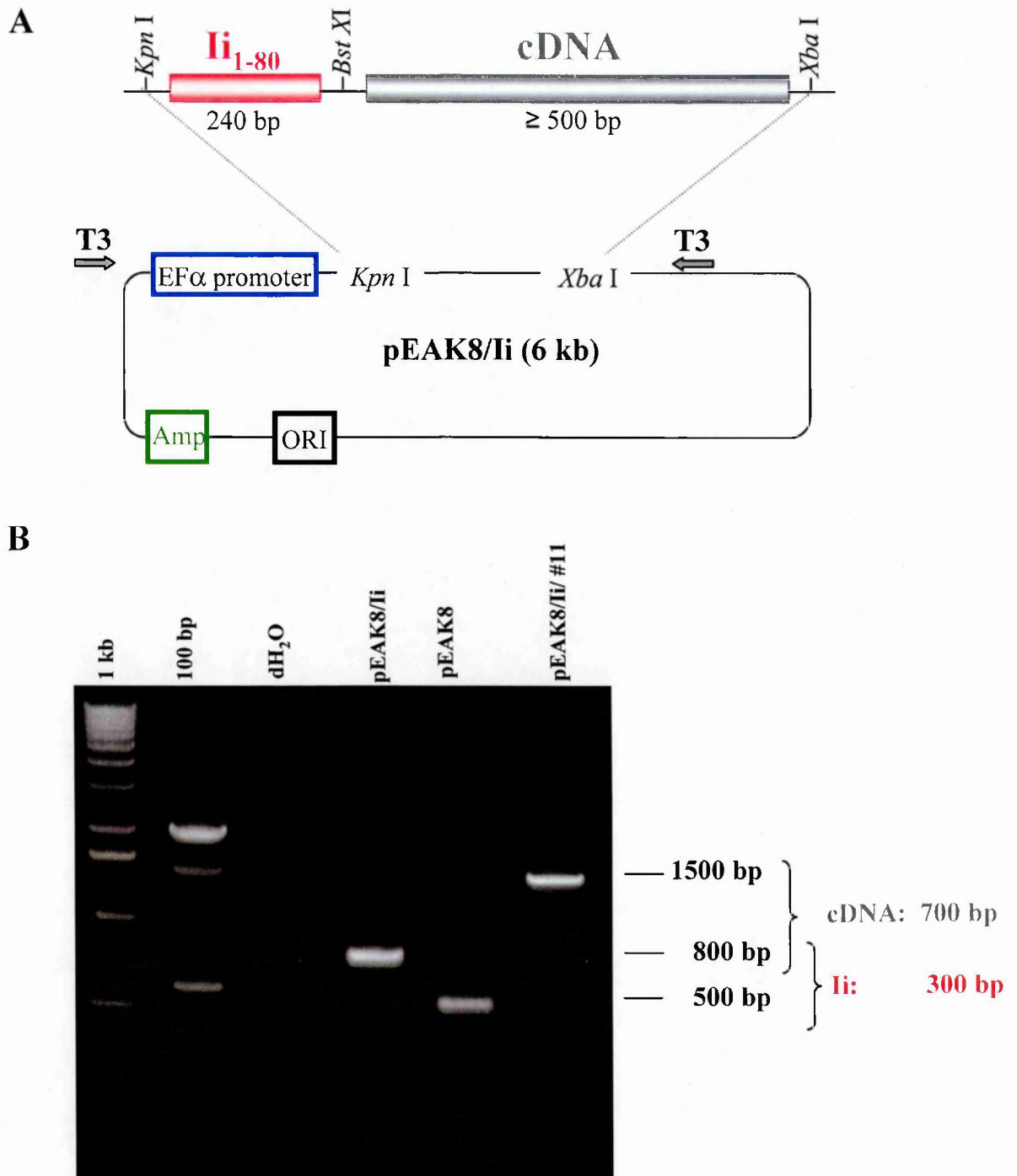
FIGURE 7.3: The positive cDNA #11 encodes a 700 bp insert. (A) A schematic view of the invariant chain (Ii) - cDNA fusion insert in the polylinker of the pEAK8 vector. The first 80 amino acids of the Ii_{1-80} cover 240 bp (red bar), which have been ligated with tumour derived cDNA (grey bar) selected to contain bands of minimal 500 bp after size fractionation. (B) PCR performed using T3 forward primer and T7 reverse primer (grey arrows in A) with the empty pEAK8 vector alone and vector constructs containing either only the Ii or the full Ii-cDNA fusion insert. The 1 kb and 100 bp ladders were used to size molecular weight of the DNA bands.
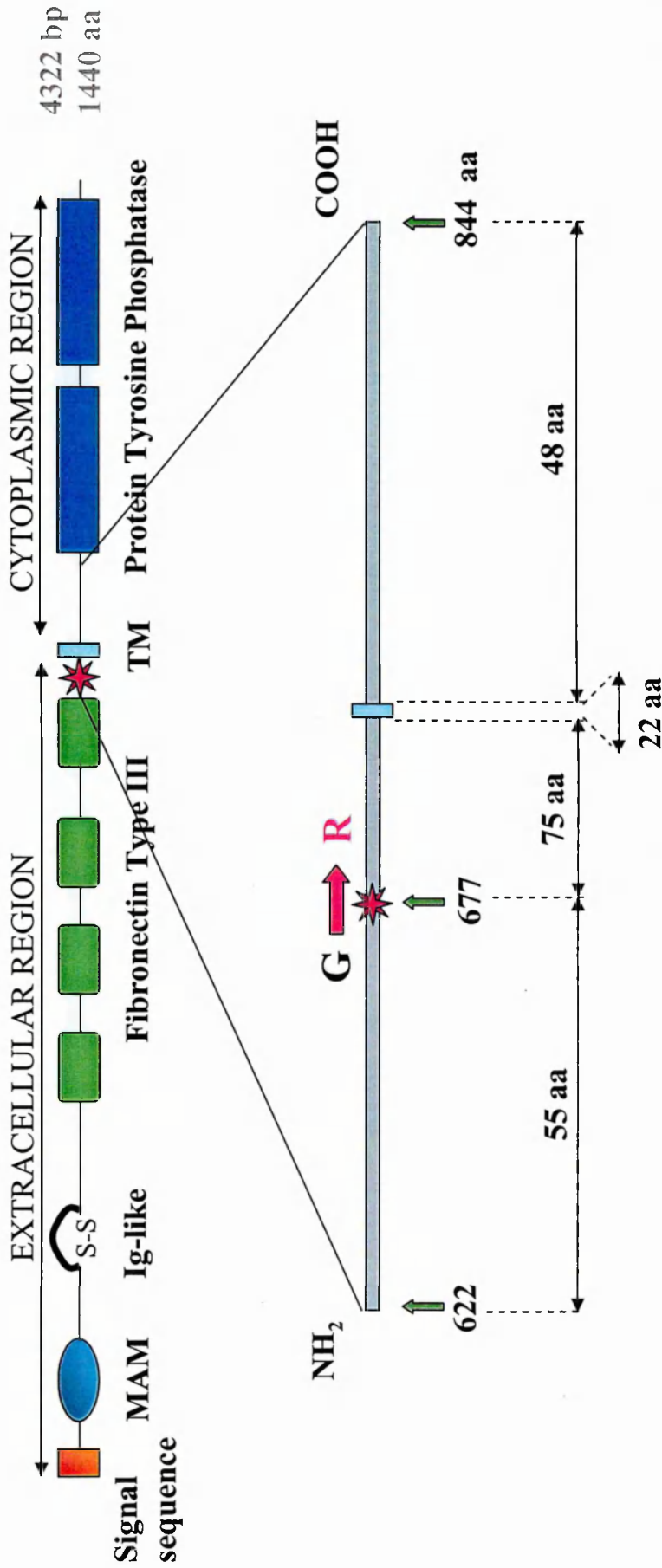
FIGURE 7.4: Clone #11 encodes a 674 bp insert that shows 99% homology with the tyrosine phosphatase receptor kappa (PTPR-κ/m (Accession number NM-002844.2) is characterised by the presence of different domains: the extracellular region contains one signal sequence, a MAM (Meprin/A5/μ) domain, an immunoglobulin (Ig) like domain and four fibronectin type III domains, while two protein tyrosine phosphatase domains are present in the cytoplasmic region. The clone isolated from Me15392 library (grey bar) extends between amino acid 622 and 844 and contains a G (glycine) to R (arginine) point mutation at position 677 of the protein, as indicated with the star. The mutation is located 101 amino acids after the fourth fibronectin III domain, i.e. in the extracellular part of the protein, more precisely 75 amino acids above the transmebrane (TM) region. The full length gene is encoded by 4322 base pairs (bp) and translated to a 1440 amino acid (aa) long protein.

initial ATG inside the Ii chain. Therefore the part corresponding to the PTPR-κ/m cDNA was not translated into the PTPR-κ/m protein. The existence of an inner ATG directing the translation of the cDNA was postulated. By analyzing the sequence of cDNA#11, a Kozak-like sequence ((g/a)nn<u>atgg</u>) was found preceding the presence of two internal ATG codons (base pair position 2165 and 2204) in the GenBank NM-002844.2 sequence), both in frame with the authentic first starting methionine of the *PTPR-κ* gene (Figure 7.5A). In order to evaluate (i) if cDNA #11 could be translated independently from the Ii and (ii) which impact the Ii had on its processing and presentation, subcloning was performed: cDNA #11 was shifted from the pEAK8/Ii vector and ligated to the pcDNA3.1 vector. This new construct, lacking the Ii sequence, was then transfected into 293/CIITA cells together with the HLA-DRβ1*10011 allele. Indeed, cDNA #11 was efficiently recognised by the specific T cells (Figure 7.5B). This result demonstrated that the PTPR-κ/m protein encoded by cDNA #11 gained access to HLA-class II processing without the requirement of the 80 amino acid of the Ii chain. In addition, the recognition of cDNA #11 alone demonstrated that the internal ATG positioned at 2165 in the GenBank NM-002844.2 sequence is active in directing the protein translation.

7.3.3.ii   A polyclonal CD4+ T cell response against the PTPR-κ/m antigen

The insert Clone 11 was also transfected into 293/CIITA cells in the presence of HLA-DRβ1*01021 or -DRβ1*10011 and tested for recognition by the other, previously characterised CD4+ T cell clones TB515, TB39 and TB48. All the three clones, although expressing different TCR specificity, were induced to release significant amounts of IFN-γ (Figure 7.5B and 7.6). This indicated that their nominal epitopes were all derived from PTPR-κ/m and presented by the HLA-DRβ1*10011 allele. On the basis of these results it may be concluded that the PTPR-κ/m antigen induced a strong and polyclonal immune response at the tumour site.

Figure 7.5: The invariant chain is not needed for HLA class II processing and T cell recognition. (A) The first 80 amino acids of the invariant chain (Ii$_{1-80}$) have been ligated to cDNA#11, as schematically shown. Sequence analysis of this insert revealed that the cDNA insert was not in frame with the ATG of the Ii. Two internal ATG start codons (violet triangles) contained in cDNA#11 and corresponding to the ATG at position 649 and 662 of the PTPR-κ cDNA (Accession number NM-002844.2) are indicated. The ATG codon of the invariant chain (Ii) is indicated by a white triangle.

(B) The derived cDNA#11 was ligated into the pcDNA3.1 vector and 150 ng of this new construct (cDNA#11/pcDNA3.1), lacking the Ii sequence, was transfected into 293/CIITA cells (5x10^4 cells/well) together with 150 ng of the HLA-DRβ1*10011 (DR10) allele. As comparison, transfection was also performed using the original construct (cDNA#11/pEAK8/Ii) derived from the tumour derived Ii-cDNA library. After over night incubation with 1.5x10^4 cells/well of clone TB515, the supernatants were evaluated for their IFN-γ content by ELISA. The autologous tumour (Mel5392) (1x10^4 cells/well) was used as a positive control. No statistically significant difference in T cell recognition was observed between cDNA#11 alone (cDNA#11/pcDNA3.1) or ligated to the Ii (cDNA#11/pEAK8/Ii) indicating that processing and presentation of PTPR-κ/m is independent from the Ii. CIITA = class II trans-activator. Results are collected from 3 experiments and represent the mean ± S.D. of two replicates.
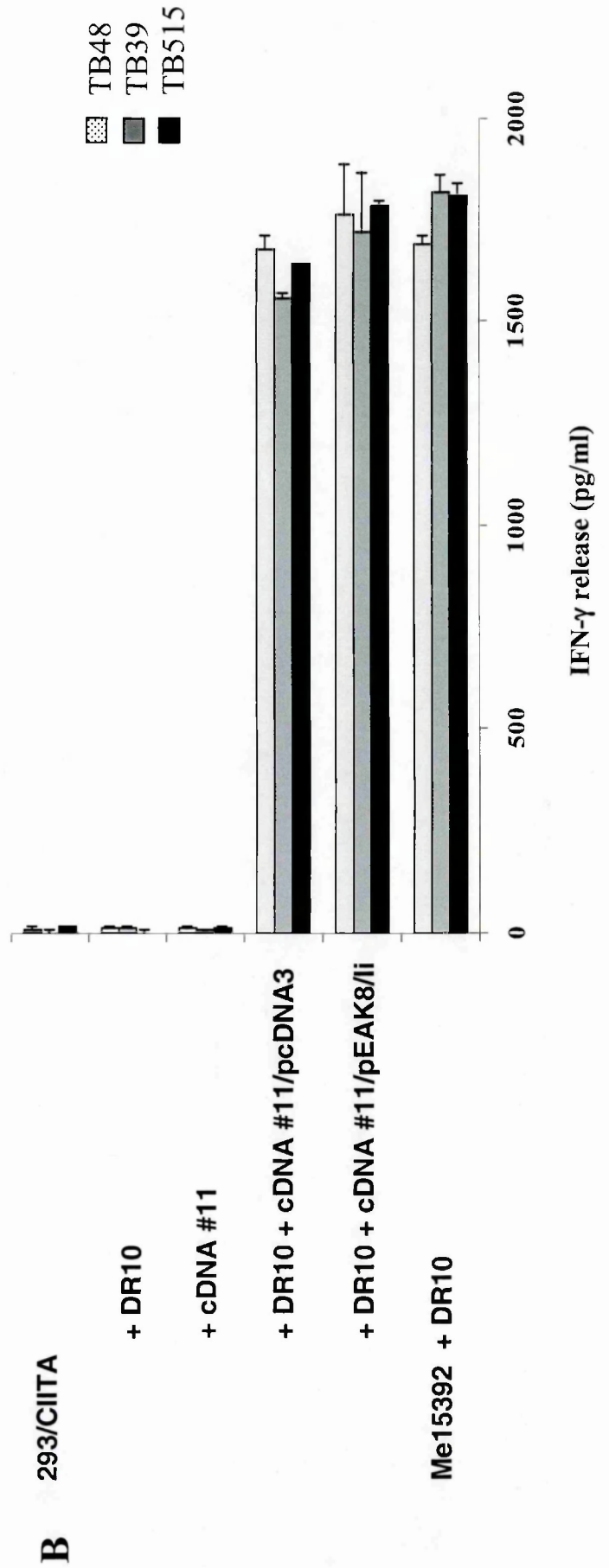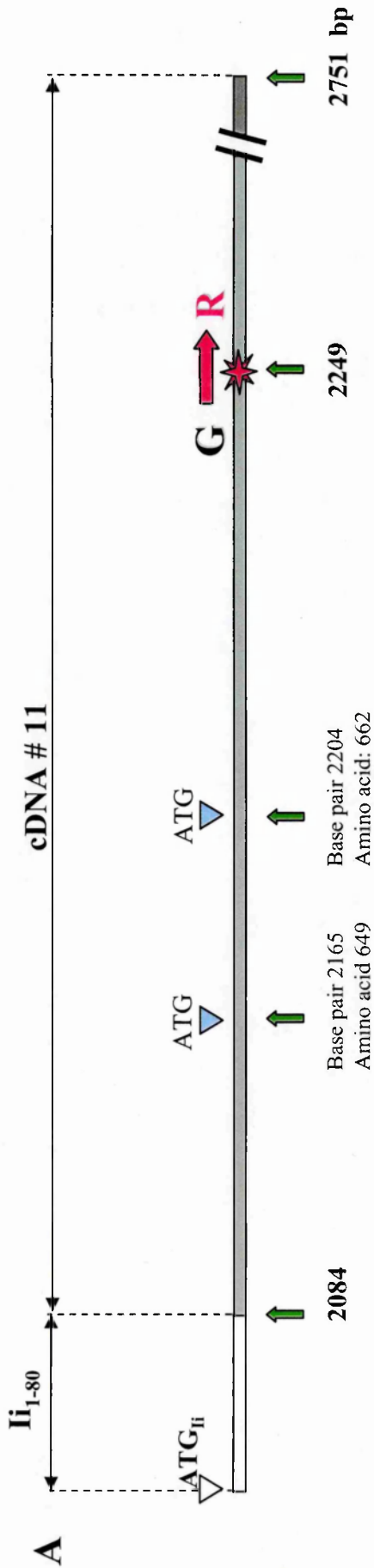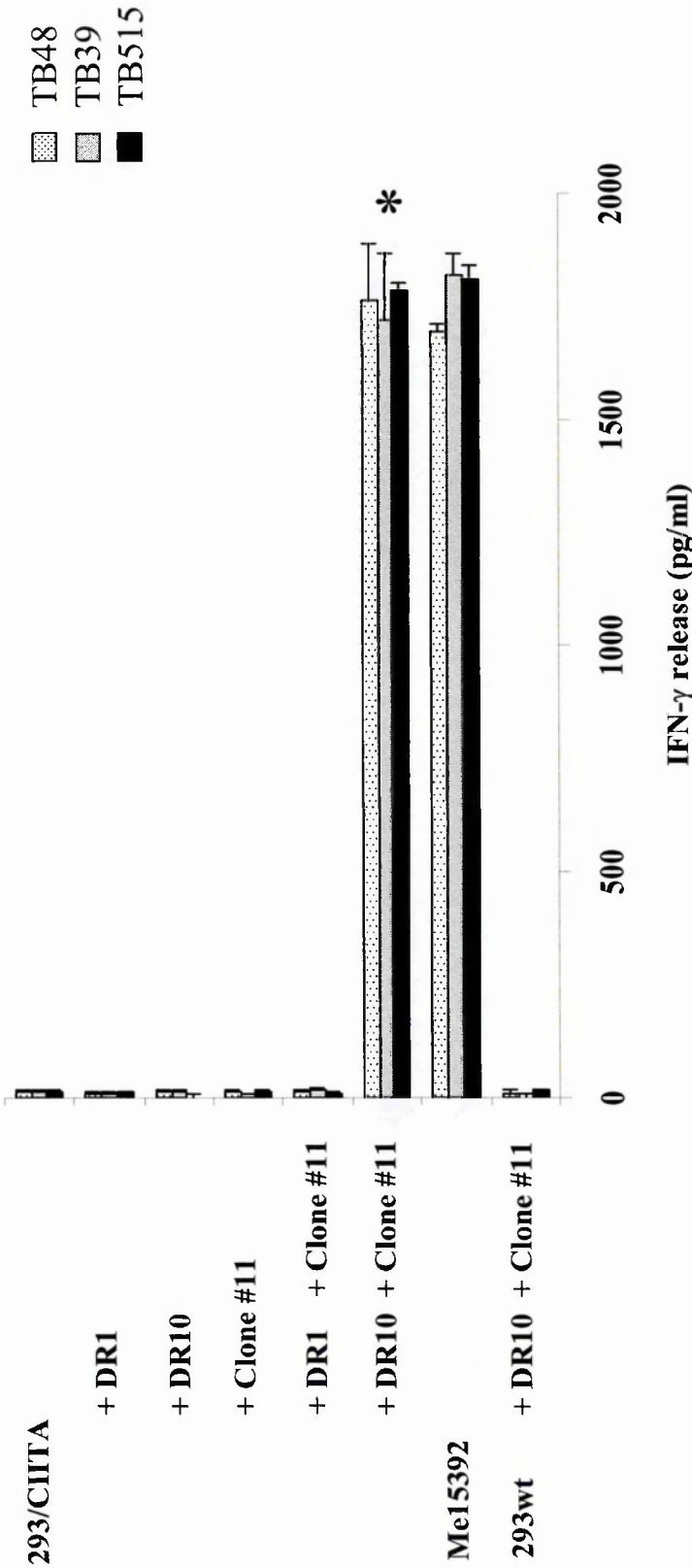
Figure 7.6: A polyclonal CD4+ T cell response directed against the PTPR-κ/m gene. 293/CIITA cells (5x10⁴ cells/well) were transfected with 150 ng of either HLA-DRβ1*01021 (DR1) or HLA-DRβ1*10011 (DR10) and Clone #11(PTPR-κ/m) of the Ii-cDNA fusion library respectively, and then incubated overnight with 1.5x10⁴ cells/well of TB515, TB39 or TB48. Autologous melanoma cells (Me15392) (1x10⁴ cells/well) were used as a positive control. The IFN-γ content in the supernatant was evaluated by ELISA. 293/CIITA cells transfected with the DR10 allele and Clone #11 induced a significant higher IFN-γ release (*p<0.001) compared with control cells transfected with DR10 only, with the DR1 allele and Clone #11, or with the DR1 allele alone. No significant difference in the polyclonal response was observed between the autologous tumour and 293/CIITA cells transfected with DR10 and Clone #11. The results are mean ± S.D. from 3 experiments performed in duplicates. CIITA = class II trans-activator; wt = wild type.

7.3.3.iii Identification of the immunogenic region of PTPR-κ/m

As the point mutation was found to be crucial for the recognition of the antigen by T cells, 3 minigenes (M) spanning the region containing the mutated nucleotide were synthesised by amplification with primer F2, starting approximately 150 bp upstream of the mutated site in association with the reverse primers EPR1, EPR2 and EPR3 located downstream of the forward primer F2 (Figure 7.7A). The amplified products were ligated into the pcDNA3.1D/V5-His-TOPO vector and co-transfected into 293/CIITA cells together with the HLA-DRβ1*10011 encoding plasmid. The minigene M1, corresponding to a short region of 202 bp surrounding the mutation, was recognised by clone TB515 (Figure 7.7B). Furthermore, the minigene M2 (179 bp) was recognised, though less efficiently, while the shortest minigene, M3 (159 bp), was unable to stimulate any response by Clone TB515. This implies that the small stretch of 7 amino acids (NLPEPAPF), by which minigene M2 differs from M3 is important for the interactions with the T cell receptor and sets the restriction for the COOH end (Figure 7.8). Therefore, a stretch of 28 amino acids extending from this region and in the direction of the NH_2 end was choosen as a replica sequence for the design of putative peptides constituting the epitope of Clone TB515. This result was also fully in accordance with our hypothesis that the mutation has a role in the recognition.

7.3.3.iv Identification of the TB515 epitope derived from PTPR-κ/m

HLA-DRβ1*10011 is an allele with an unknown distribution (reported at http://www.ashi-hla.org) and although the development of new assay systems has facilitated the definition of binding motifs for HLA class II molecules (Southwood et al., 1998), no binding anchors have yet been defined for HLA-DRβ1*10011 to assist in the prediction of the putative epitope. Thus, in order to further limit the putative epitope region and to understand which amino acids are important for recognition, a set of 16-mer overlapping peptides spanning

FIGURE 7.7: Definition of the immunogenic region of the *PTPR-κ/m* gene with the aid of amplified minigens. (A) The positions of the primers used to construct minigens M1 (202 bp), M2 (179 bp) and M3 (159 bp) are schematically indicated. They are located in a region extending from base pair (bp) 2000 to 2300 of the *PTPR-κ/m* gene. The star indicates the site of the G to R point mutation, which is located at position 2249 in the nucleotide sequence. Two violet triangles indicate the approximate position of two internal start codon (ATG) in the *PTPR-κ/m* sequence. The precise base pair locations of the primers are as follow: F2 (2091-2110 bp): 5'-gtgctcctatcagtgcttat-3'; EPR1 (2273 – 2093): 5'-ccgattgtcaccacagtgaa-3'; EPR2 (2256 – 2070): 5'- gggcaggctcaggta-3' and EPR3 (2236 – 2250): 5'-gggcaggctcaggtaggtttccg-3'. M1 was amplified with F2/EPR1, M2 with F2/EPR2 and M3 with F2/EPR3. M = minigene; F = forward; EPR = epitope primer.

(B) Minigens M1, M2 or M3 (150 ng) were individually transfected into 293/CIITA cells (1x10^4 cells/well) in the presence 150 ng of the HLA-DRβ1*10011 (DR10) allele and then incubated with 1.5x10^4 cells/well of either CD4+ T cell clone TB515, TB39 or TB48. M1 and M2 transfected cells significantly stimulated IFN-γ release from all 3 CD4+ T cell clones TB515, TB39 and TB48 (*p<0.05) compared to un-transfected cells. Transfection of M3 was not significantly different from the control indicative of M3 lacking a sequence important for immunogenic recognition and sensitization of these CD4+ T cell clones for induction of IFN-γ release. The results represent the mean ± S.D. of 3 experiments performed in duplicate for each sample.

1st ATG   2nd ATG

▽   ▽

M2 MECYQPVTYQNAMSGGAPYYFAAELPPRNLPEPAPF

     649   662   677

M3 MECYQPVTYQNAMSGGAPYYFAAELPPR

          YQNAMSGGAPYYFAAELPPRNLPEPAPF

                            ↑
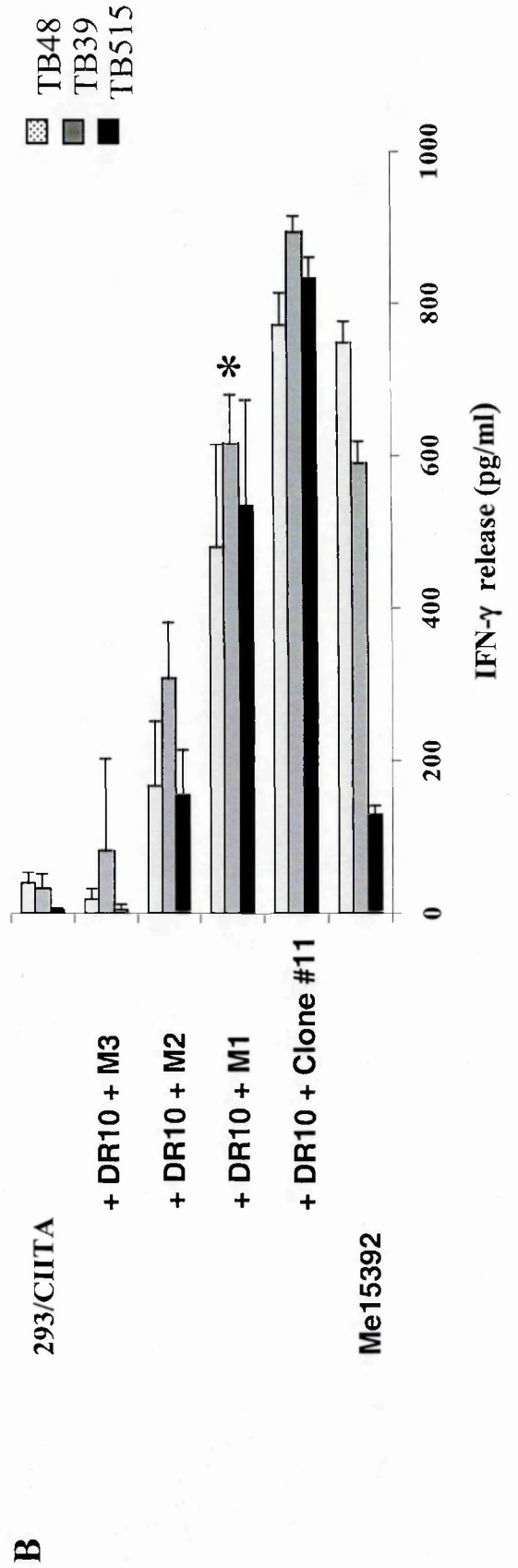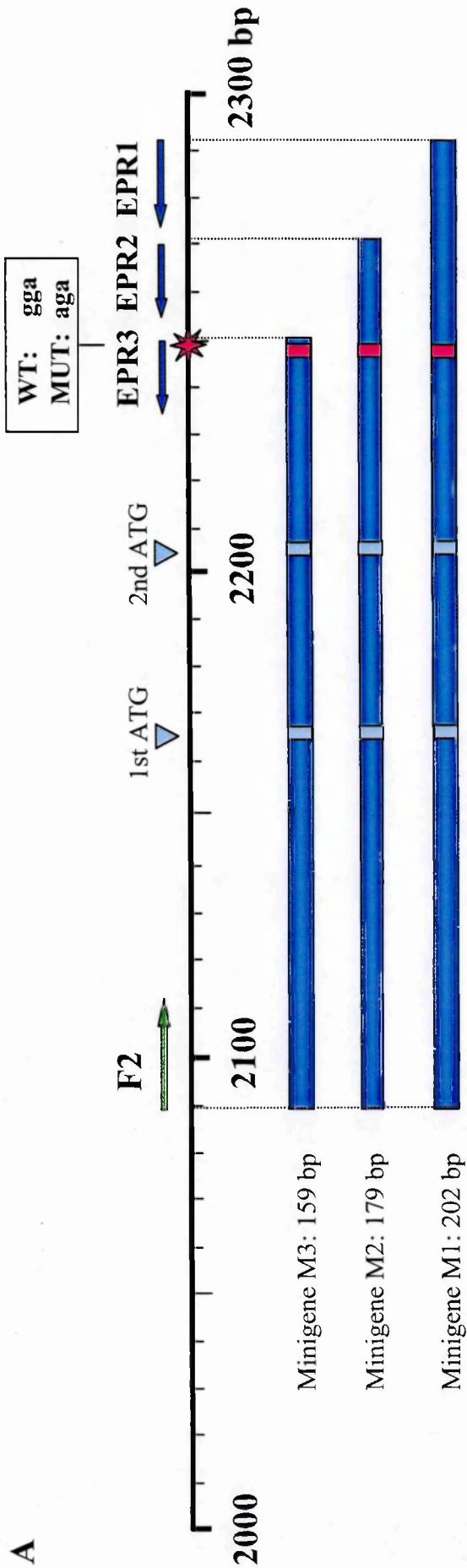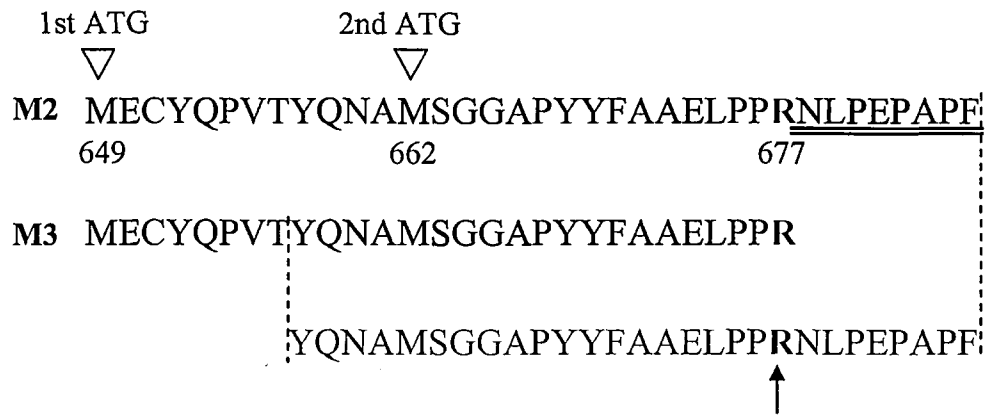
Figure 7.8: Selection of an immunogenic region of PTPR-κ/m minigenes suitable as a replica sequence for the design of peptides. The amino acid sequences of minigene M2 and M3 is shown. The mutated base R (in read), is located at position 677 of the PTPR-κ/m protein and the two internal ATG codons at position 649 and 662 are indicated. As shown in Figure 7.7 T cell recognition was markedly reduced for M2, but fully lost for M3. This showed that the truncated region NLPEPAPF (marked by a double line) at the COOH end of M2 (shown in Figure 7.7) was important for recognition and indicated that the immunogenic epitope should extend further towards the NH_2 end of the minigenes. Based on these data, a region of 28 amino acid was chosen as a replica sequence for the design of peptides. The arrow indicated the position of the mutated amino acid.

the mutated R of the *PTPR-κ/m* gene were designed from the selected region shown in Figure 7.9. These peptides were pulsed at different concentrations onto autologous LCL and evaluated for the ability to stimulate the clone TB515.

The best stimulation was achieved by Peptide 2, i.e. PYYFAAELPP**R**NLPEP (mutation in bold), PTPR-κ/m$_{667-682}$, that showed a dose response curve (Figure 7.9) and, in addition, induced IFN-γ release at a concentration of only 10 nM. As all the 4 tested peptides were well recognised, no conclusion could be made regarding the precise peptide COOH and NH$_2$ ends. However, peptides overlap with an 11-mer core sequence, located between the second Y$_{669}$ (position 669) at the NH$_2$ end and the L$_{679}$ at the COOH end, which contains the G→R point mutation at amino acid 677. Thus, these results indicated that the epitope of the PTPR-κ/m gene should be located within this core sequence. Moreover, the significance of the point mutation (residue R) in immune induction was clearly demonstrated in a pulsing experiment where Peptide 5, corresponding to the wild type sequence, was unable to stimulate IFN-γ release form clone TB515 (Figure 7.10A), while Peptide 2, carrying the mutation, induced a strong T cell recognition.

Peptide 2 was used as a model sequence also in the assessment of the putative binding anchors for the NH$_2$ and COOH ends. First, the crucial role of the Y$_{669}$ as a residue for the NH$_2$ end of the peptide was demonstrated by the abolished capability of modified peptides to induce IFN-γ release (Figure 7.10B). More precisely, Peptide 6, ending at the F residue and Peptide 7, having the Y$_{669}$ substituted with a neutral G, were not recognised by TB515 when pulsed onto LCL cells. Second, a marked impairment in the stimulatory capacity was observed when the L was removed from the COOH end, as observed after pulsing with Peptide 9 (Figure 7.10C). Removal of the N$_{678}$ residue also abrogated the recognition. Taken together, these results indicate that the minimal PTPR-κ/m epitope is represented by

Peptide 1: YFAAELPPRNLPEPAP

Peptide 2: PYYFAAELPPRNLPEP

Peptide 3: GAPYYFAAELPPRNLP

Peptide 4: GGAPYYFAAELPPRNL

Core sequence
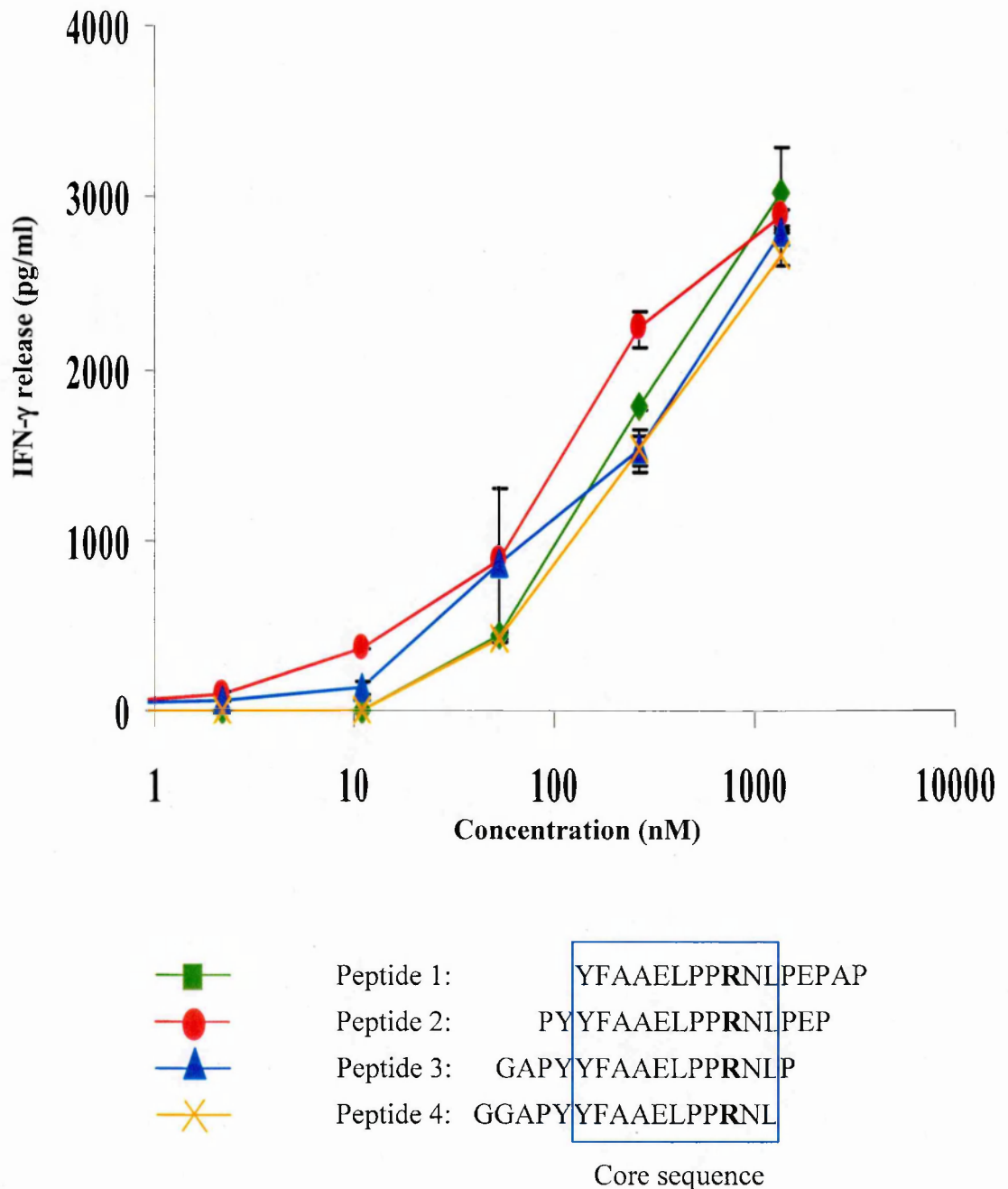
FIGURE 7.9: The definition of the immunogenic core region of the PTPR-κ/m gene. Autologous LCL15392 cells (5000 cells/well) were pulsed with increasing concentrations of overlapping 16-mer peptides for 2h and then incubated overnight with 1×10^4 cells/well of the CD4+ clone TB515. The supernatants were evaluated for their IFN-γ content by ELISA. Boxed amino acids indicate the herein defined core sequence. Data are representative of the mean ± S.D. of 3 individual experiments performed in duplicates.
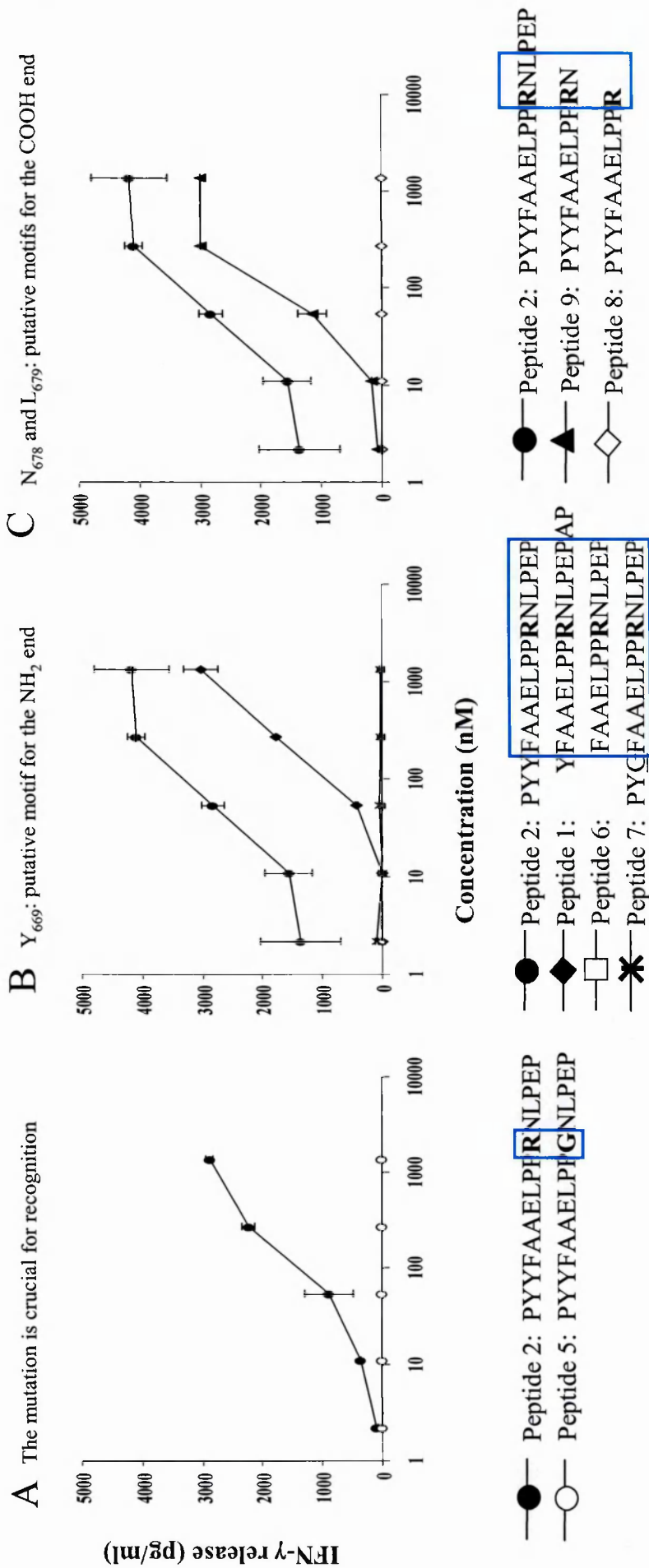
FIGURE 7.10: Definition of the immunogenic PTPR-κ/m peptide. Autologous LCL15392 cells (5000 cells/well) were pulsed with increasing concentrations of the indicated synthetic peptides for 2 h, and then used to stimulate IFN-γ release from Clone TB 515 (1x10⁴ cells/well). The mutated base R (position 677) and the wild type base G are shown in bold. (A) TB515 cells were stimulated with the mutated (Peptide 2) or wild type (Peptide 5). (B) Clone TB515 stimulated with the NH₂ end truncated Peptide 1 and 6 compared with the control Peptide 2 and a peptide (Peptide 7) having the putative binding anchor Y substituted with a G (underlined). (C) Clone TB515 stimulated with peptides truncated at the COOH end compared with the control peptide (Peptide 2). The boxes indicate (A) the amino acids influencing T cell recognition, (B) the herein defined core sequence and (C) amino acids positioned at the COOH end that are critical for recognition. Results are mean ± S.D. of three separate experiments.

the TB515 clone corresponds to 11-mer core sequence $YFAAELPPRNL_{669-679}$, while Peptide 2 constitutes the best recognised epitope. Finally, given the previously observed polyclonal T cell response against Clone #11 (Figure 7.6) and minigenes M1 and M2 (Figure 7.7), experiments were set up in order to estimate the individual responses of the T cell clones TB515, TB39 and TB48 against the mutated (Peptide 2) and wild type (Peptide 5) peptides. All three clones showed a specific recognition of Peptide 2, with clones TB39 and TB48 showing similar response curves (Figure 7.11).

7.4 DISCUSSION

Pt15392 showed a favourable clinical course and was therefore selected and assessed for the presence of an anti-tumour immune response at a cellular (as described in Chapter 3 and 4) and molecular level (described in this chapter).

7.4.1 Characteristics of the PTPR-κ/m gene

The subcloning of the positive pool defined in Chapter 6 led to the identification of a immunogenic cDNA insert of 667 bp (Clone #11). The insert of Clone #11 was 99% identical to the human *PTPR-κ* gene and corresponded to a region located between amino acid 622 to 844 of the membrane spanning region with a G (Gly) to R (Arg) point mutation at position 677, i.e. in the extra-cellular part of the protein (Figure 7.4). These data demonstrate the identification of PTPR-κ/m as a novel TAA, though sequence and protein structure of the wild type protein were already known (Yang *et al.*, 1997).

The *PTPR-κ* gene encodes an open reading frame of 4322 bp, which becomes translated into a mature protein of 1440 amino acids. This protein is expressed in several normal tissues such as brain, thymus, testis, colon, liver, pancreas, stomach, kidney, and placenta and also in mammary carcinoma (Yang *et al.*, 1997). The *PTPR-κ* gene belongs to the
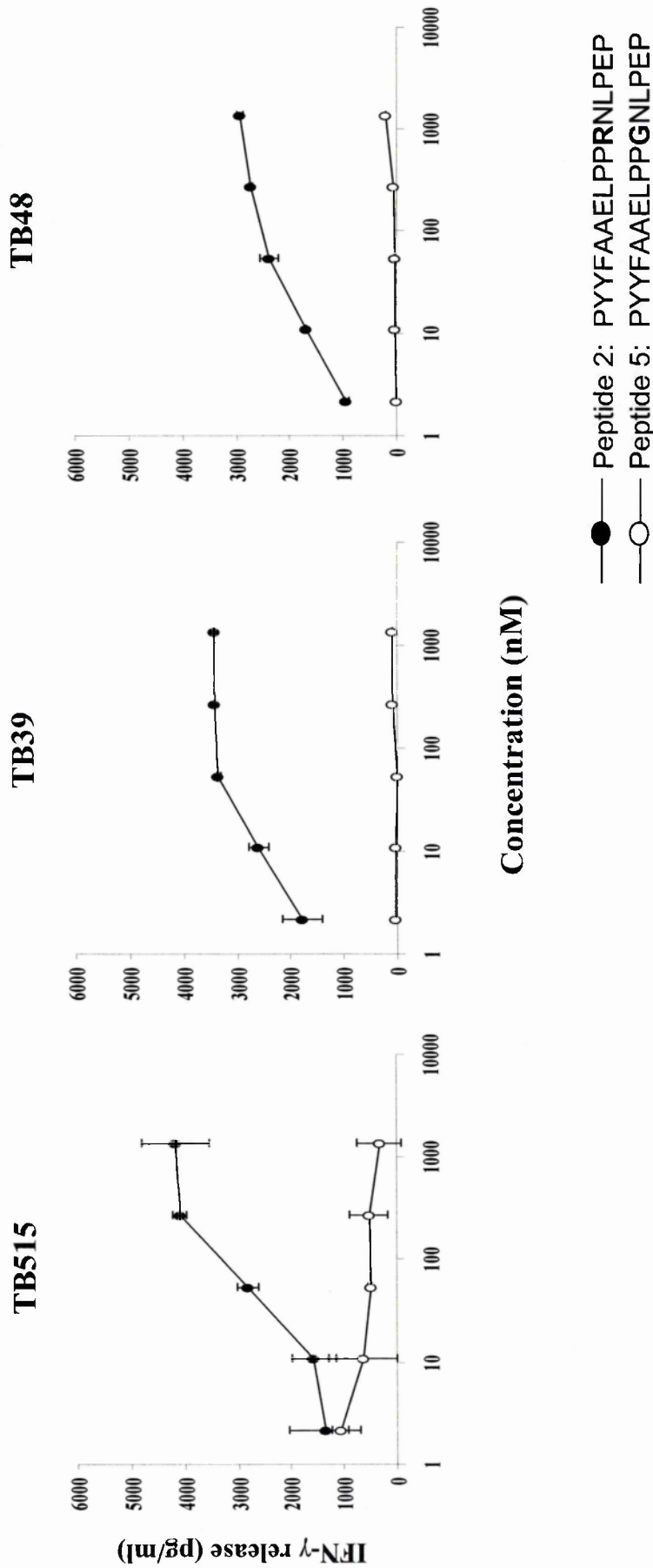
FIGURE 7.11: Recognition of the mutated PTPR-κ/m peptide by 2 additional CD4+ T cell clones. Autologous LCL15392 cells (5000 cells/well) were pulsed with increasing concentrations of the mutated (Peptide 2) or wild type (Peptide 5) of the PTPR-κ for 2 h, and then used to stimulate IFN-γ release from the CD4+ T cell clones TB 515, TB39 and TB48 (1x10⁴ cells/well). Only the mutated peptide was recognised. Results are representative of the mean ± S.D. of 3 separate experiments.

large enzyme family of protein tyrosine phosphatases (PTPases). This protein family can be further subdivided into receptor like or non-receptor like PTPases. The *PTPR-κ* gene relates to the former group together with four other sub-members: PTPR-ρ,-μ, -λ and -π (Cheng *et al.*, 1997; Crossland *et al.*, 1996; McAndrew *et al.*, 1998).

The PTPR-κ protein is composed of an extracellular portion (753 amino acids), a transmembrane domain (22 amino acids) and a cytoplastic region (665 amino acids) (Yang *et al.*, 1997), as illustrated in Figure 7.4. The cytoplasmatic portion of *PTPR-κ* gene, with two protein tyrosine phosphatase domains arranged in tandem, is classic for members of the PTPases and defines *PTPR-κ/m* gene as a member of this group (Sap *et al.*, 1994; Zhang *et al.*, 1998). Moreover, the *PTPR-κ* gene share structural similarities with cell adhesion molecules, owing to the presence of an extracellular region encoding an Ig-like domain, a meprin-A5 antigen-PTP μ (MAM) domain and four fibronectin type III repeats (Figure 7.4).

7.4.2 No need for the invariant chain

The fusion of the Ii to selected gene sequences is a molecular way to assure a specific delivery of library inserts to endocytic compartments. However, it does not address the question of whether the guidance by the Ii is required also for T cell recognition in the physiological context. My experiments show that transfection of the cDNA #11 without Ii, resulted in a CD4+ T cell recognition comparable to that induced when delivered as a fusion protein. Thus, in this applied system the identified protein, although endogenously produced, is *per se* able to gain access to HLA-class II processing compartments (Figure 7.5).

However, in contrast to the results presented herein, it has recently been documented that the Ii is a molecular prerequisite for recognition of HLA class II restricted antigens, as reported for the fusion protein BCR-ABL b2a2 (ten Bosch *et al.*, 1999). This recombinant protein was immunogenic only in combination with the Ii. However, the mechanism of action of Ii in determining the immunogenic potential of endogenous protein mainly relies on its ability to guide protein to distinct cellular compartments where they would otherwise not be present. Data supporting this conclusion have been provided in the case of the CDC-27 antigen (Wang *et al.*, 1999a).

The reasons for these opposing requirements for the Ii are not known. However, the observation that the CD4+ T cell recognition of the PTPR-κ/m epitope is independent from the Ii guidance opens several questions about antigen processing and presentation. One enigma is where the loading of the PTPR-κ/m epitope takes place in the recipient cells; the engineered 293 cells should be considered a closed cellular system with an optimal expression of both the internal and supplied genes involved in the class II HLA machinery, due to the up regulatory capacity of CIITA (Chang and Flavell, 1995). In the absence of the supplied Ii, the 293 cells will continue to express their internal Ii, while the synthesised PTPR-κ/m antigen will not be artificially chaperoned into the endocytic peptide loading compartments. Instead the PTPR-κ/m protein is forced to enter the endocytic route from a cytoplasmic location which involves entrance into the endoplasmatic reticulum, transfer to the Golgi network and endosome transport to the cell surface.

One alternative for this would be that the PTPR-κ/m protein follows the same pathways as cytoplasmatic proteins which take advantage from sorting sequences for entrance into endocytic trafficking. For example, the Tyr-Gln-Thr-Ile signal in the cytoplasmic tail of the lysosomal protein LAMP-1, enabled expression of a chimeric gene guiding the E7 model

TAA of the human papilloma virus to the endosomal/lysosomal compartments (Wu *et al.*, 1995). Likewise, a di-leucin motif contained in the melanosomal transport signal (MTS) has been reported to assist TAAs like tyrosinase, gp100 and Trp-2 to this route (Wang *et al.*, 1999c). However, as the PTPR-κ/m protein lacks such known signal sequences this scenario should not be possible in this instance.

A second alternative would be that the cytosolic PTPR-κ/m protein uses the Ii independent pathway for HLA class II antigen processing; i.e. recycling of membrane receptors (reviewed in (Geuze, 1998; Hiltbold *et al.*, 1998)) and HLA-DR molecules (Pinet *et al.*, 1995; Pinet and Long, 1998). This endogenic route is probably applied by another defined TAA: the EhA3 membrane receptor (Chiari *et al.*, 2000). It is thus possible that the PTPR-κ/m receptor become recycled to endosomal/lysosomal system directly from the membrane and then re-routed as an antigenic peptide cargo loaded onto HLA molecules guided by a leucin motif in their β-chain (Pinet *et al.*, 1995).

In addition, indications of a third alternative pathway comes from studies with immature DCs which demonstrate that extracellular peptide loading may occur (Santambrogio *et al.*, 1999a; Santambrogio *et al.*, 1999b) and that the Ii may be dispensable for MHC expression (Villadangos *et al.*, 2001). Collectively, these findings may argue for proteolysis of the PTPR-κ/m receptor in an extracellular milieu with loading to empty HLA class II molecules for recycling (Santambrogio *et al.*, 1999b).

It is known that endogenous proteins can enter to the HLA class II pathway (Sanderson *et al.*, 1995), but a dichotomy for intracellular trafficking has been described for HLA-DR restricted CD4+ T cell epitopes. Indeed, MAGE-3, an antigen of cytosolic origin, encodes epitopes which either can reach the cell surface (Manici *et al.*, 1999; Schultz *et al.*, 2000)

or not (Chaux *et al.*, 1999b). Taken together, these findings reveal the complexity of the intracellular trafficking pathways for class II HLA restricted antigens (reviewed in (Robinson and Delvig, 2002)). Thus, the loading compartment for CD4+ T cell antigens which, like PTPR-κ/m, are independent from chaperon help of the Ii, remains to be defined. However, such "Ii independent epitopes" may be important for inducing CD4+ T cell responses against tumours in physiological situations, e.g. when as tumour cells cannot function as professional APCs and therefore, may have inadequate HLA class II machinery.

7.4.3   The definition of the epitope

The HLA-DR locus has been frequently described as a restriction element for CD4+ T cell defined TAAs, but PTPR-κ/m is the first antigen with an epitope restricted to the HLA-DRβ1*10011, as this allele has not previously been reported as an restriction element for tumour antigens. The results of the library screening (Chapter 6) anticipated the presence of such a novel antigenic epitope restricted by the HLA-DRβ1*10011 allele. The results presented herein describe the PYYFAAELPPRNLPEP, PTPR-κ/m$_{667-682}$, as the immunogenic epitope of the *PTPR-κ/m* gene. Substitution of the mutated base (R) completely abrogated the T cell recognition (Figure 7.10A) and underscored the essential role for the G→A point mutation in providing immune recognition.

Moreover, HLA-DRβ1*10011 is an allele with an unknown distribution (reported at http//:www.ashi.hla.org under Resources/allele frequencies) and although the development of new assay systems has facilitated the definition of binding motifs for HLA class II molecules (Southwood *et al.*, 1998), no definitive binding anchors have yet been defined for HLA-DRβ1*10011. However, another study proposed the presence of a Y residue as a putative binding anchor in position 2 or 3 of the HLA-DRβ1*10011 allele (Hickling *et al.*,

1990). These data are in accordance with the observation that further truncation of our PTPR-κ/m peptide YFAAELPPRNL defined core sequence, results in loss of recognition. This implied that the Y_{669} at the NH_2 end and the L_{679} at the COOH end may represent amino acids residues with putative binding anchors for the interaction between this peptide and the TCR.

The position of the mutated amino acid is located in the extracellular region between the IV fibronectin III domain and the transmembrane region (Figure 7.4); future work should be aimed at exploring whether this mutation may affect the function of the PTPR–κ protein.

7.4.4 Conclusions

Upon screening of an Ii-cDNA fusion library, constructed from RNA purified from a metastatic melanoma, we succeeded in identifying PTPR-κ/m as a novel TAA. The results presented in this chapter show the crucial role of the point mutation for the recognition of autologous anti-tumour specific CD4+ T cells.

Taken together these data allow us to add the *PTPR-κ/m* gene to the increasingly growing list of identified TAAs recognised by T cells. The point mutation makes PTPR-κ a unique antigen, probably with a high immunogenicity. In fact a strong polyclonal CD4+ anti-tumour response could be detected *in vitro* against the PTPR-κ/$m_{667-682}$ epitope. This has probably contributed to the favourable clinical course of the melanoma patient whose tumour bears this particular mutation. Whether the mutation, in addition to determining the immunogenicity of this protein, is also affecting its physiological functions remain to be determined.

Chapter 8

DISCUSSION AND FUTURE PERSPECTIVES

8.1 DISCUSSION

8.1.1 Background

Cutaneous malignant melanoma is a neoplasm of multi-genetic origin, whose progression to an aggressive phenotype involves a step-wise accumulation of mutations in genes critical for cell proliferation, differentiation and death. Although a genetic model for melanoma transformation does not exist, several genes have been characterised that confer susceptibility to melanoma. For example cyclin-dependent kinase 4 (*CDK4*) is a gene involved in regulation of the cell cycle progression, while the melancortin-1 receptor (*MC1R*) conferes susceptibility, and *p53* and *N-ras* are connected to defects in cell signalling (reviewed in (Castellano and Parmiani, 1999)). Other genes that activate transforming activity of melanom are the apoptotic protease activating factor - 1 (*APAF-1*) (Soengas *et al.*, 2001), *BRAF* (Davies *et al.*, 2002) and *WNT5A* (Weeraratna *et al.*, 2002). Of note the observation that *BRAF* appears to be mutated in approximately 60% of melanomas.

It is also known that melanoma expresses genes that encode antigens that can be recognised by T cells and activate the immune system against the tumour (reviewed in (Renkvist *et al.*, 2001; Van den Eynde and Van der Bruggen, 2001)). The molecular identification of such T cell-defined TAAs has opened new avenues for an immunological based treatment of cancer patients. However, vaccination strategies that use antigenic peptides have met with limited success, with only 10-30% of patients demonstrating tumour regression (reviewed in (Parmiani *et al.*, 2002a)). This may be due to the many mechanisms of tumour escape (reviewed in (Perez-Diez and Marincola, 2002)), suboptimal activation of the immune system (Gervois *et al.*, 1996), low peptide affinity, T cell unresponsiveness or to TCR signal impairment (reviewed in (Whiteside, 1999)).

Nevertheless, the few patients achieving clinical response demonstrate that (i) it is possible to break self tolerance against TAAs deriving from normal proteins, (ii) tumour cells can be immunogenic *in vivo* and (iii) under certain conditions such immune response translates into clinically effective tumour rejection. However, the knowledge of the precise mechanisms mediating tumour eradication is still inadequate. Therefore, immunological monitoring in those patients responding to treatments or spontaneously experiencing tumour regression may be crucial for dissecting the *in vivo* immunological mechanisms potentially leading to clinical responses (review (Parmiani *et al.*, 2002a)).

8.1.2 Choice of patient

The overall study of this thesis has been focused on the functional characterisation of the antigen-specific response that may have accompanied the positive clinical evolution observed in a patient (Pt15392) who is still disease-free 12 years after surgical resection of a lymph node metastatic melanoma.

This patient was selected for several reasons. First, she showed a long disease-free period without therapeutic interventions except the early lymph node resection. Thus, patients with a favourable clinical evolution might represent examples of activation and maintenance of strong anti-tumour responses. Secondly, Pt15392 displayed a T cell mediated immunity directed against class I HLA-restricted epitopes derived from the TRP-2 and gp100 proteins (Castelli *et al.*, 1999). Therefore, it was interesting to explore whether (i) this CD8+ T cell-mediated response was also associated to the presence a CD4+ T cell response and (ii) such a CD4+ T cell response could be directly involved in the establishment of an anti-tumour systemic immunity, eventually (Castelli *et al.*, 1999) contributing the positive clinical evolution of the disease. Thirdly, initial characterisation of various CD4+ T cell clones suggested that, beside gp100 and TRP-2, additional tumour-

associated proteins could have been immunogenic in this patient. The molecular definition of such immunogenic determinants could represent an initial step in clarifying the immunological mechanisms eventually involved in the favourable clinical evolution experienced by Pt15392.

8.1.3 Identification of a CD4+ T cell epitope

Several data indicate a pivotal role of CD4+ T cells in anti-tumour responses (Toes *et al.*, 1999) and reviewed in (Pardoll and Topalian, 1998; Wang, 2001). However, the diverse molecular requirements for the generation of peptides presented by class II HLA have hampered the identification of CD4+ T cell epitopes. Recently, an implementation of the molecular approach used for the identification of class I HLA-restricted TAAs has been modified and adapted to identify HLA-class II presented epitopes (Wang *et al.*, 1999a). The use of a similar method included the construction of a chimeric Ii - cDNA library in which tumour-derived cDNAs were fused to the first 80 amino acids of the Ii forcing the presentation of the encoded protein into the HLA-class II processing pathways. I used this method for antigen screening that resulted in the molecular identification of the TAA expressed by Me15392 and recognised by autologous HLA-DR-restricted T cell clones. A mutated form of the PTPR-κ was identified as a melanoma-specific antigen. In fact, the cDNA cloned from Me15392 cells was homologous to the PTPR-κ sequence (Accession number NM-002844.2) with the exception of a g→a point mutation at nucleotide position 2249. This mutation led to an amino acid change in the PTPR-*κ* protein and the mutated nucleotide changed a neutral glycin (G) to a positively charged arginine (R). The *PTPR-κ* mutated gene was shown to be responsible for the generation of an immunogenic epitope classifying this protein as a novel TAA.

The mutation in *PTPR-κ/m* gene generated an antigenic epitope presented by the HLA-DRβ1*10011 allele to the CD4+ T cells clones isolated from tumour infiltrated lymph nodes. The best recognised peptide corresponded to the PTPR-κ/m$_{667-682}$ epitope, PYYFAAELPPRNLPEP (mutation in bold), as T cell recognition was lost or strongly impaired by further delimitation of the NH$_2$ or COOH ends. The mutated R is crucial in determining the immunogenicity of the peptide since the corresponding wild type peptide did not stimulate any of the CD4+ T cells evaluated in this thesis.

8.1.4 The role of the mutation in the transformation process

The main DNA damage linked to carcinogenesis of the skin occurs in cyclobutan pyrimidines (Bykov *et al.*, 1999) and is caused by ultraviolet radiation (UVR), known to be a high risk factor for melanoma. As the point mutation in the *PTPR-κ/m* gene occurred in the guanine base, a pyrimidine, it may be possible that this mutation had a role in the transformation process, though recent data demonstrated that the repair kinetics of UVR induced DNA lesions in patients with cutaneous melanoma was not reduced compared to healthy controls (Xu *et al.*, 2000).

8.1.5 CD4+ T cell defined antigens belong to the category of unique tumour antigens

Among the four defined groups of TAAs (reviewed in (Renkvist *et al.*, 2001; Van den Eynde and Van der Bruggen, 2001)) (shared , differentiation, widely expressed and unique antigens), the presence of a mutation crucial for T cell recognition qualifies PTPR-κ/m as a melanoma-specific unique TAA. Although several unique antigens have been described for cytotoxic T cells, hitherto only three of them, CDC27 (Wang *et al.*, 1999a) and TPI/m (Pieper *et al.*, 1999) from melanoma, and HPV-E7 (Hohn *et al.*, 1999) from cervic carcinoma, have been derived from mutated proteins recognised by CD4+ T cells.

Unfortunately, given the present technology, unique TAAs are not suitable for large scale vaccination programs, because their expression is restricted by and large to individual patients. However, in mouse models, unique antigens have been shown to be more immunogenic than shared antigens (Dudley and Roopenian, 1996) and shown to function as tumour-rejection antigens (Prehn and Main, 1957).

Nevertheless, PTPR-κ/m extends the group of class II HLA-restricted unique TAAs and though not promising for vaccine development and clinical approaches in other subjects, its nature as a protein tyrosine phosphatase may contribute to increase our knowledge of tumour biology. Indeed, in addition to their structural diversity, the complexity of PTPases, as cellular enzymes, as membrane receptors and regulators of kinases, indicate the PTPR-κ/m as an interesting antigen for biological studies.

8.1.6 PTPR-κ/m: association with tumour suppression

The PTPases include several structural groups. The PTPR-κ protein belongs to a receptor-like group, which has a domain topology composed of one meprin/A5/μ antigen (MAM) domain, one Ig-like domain and four fibronectin III repeats in the extracellular region and a cytoplasmic region encoding two tandemly repeated tyrosine phosphatase domains (reviewed in (Brady-Kalnay and Tonks, 1995; Hickling *et al.*, 1990; Li and Dixon, 2000)). Moreover, while the functions of most TAAs are unknown, PTPR-κ/m belongs to a large family of PTPase associated with signal transduction, cell adhesion and neoplastic transformation (reviewed in (Fischer *et al.*, 1991)). Although the knowledge about PTPases is limited (reviewed in (Petrone and Sap, 2000)), several findings suggest a role for PTPR-κ/m as a potential tumour suppressor gene.

Firstly, a 20% down regulation of the PTPR-κ and PTPR-π phosphatase submembers has been observed in primary and metastatic melanoma cell lines (McArdle *et al.*, 2001), an observation compatible with a role for PTPR-κ in tumour suppression. Secondly, the PTPR-κ gene has been physically mapped to a putative tumour suppressor region, 6q22.2-22.3 (Zhang *et al.*, 1998). Thirdly, as a membrane receptor protein with tyrosinase phosphatase activity, PTPR-κ has been implied in signalling (reviewed in (Zondag and Moolenaar, 1997)). Fourthly, the PTPR-κ protein has a complex structure with several extracellular domains (Figure 7.4 and 8.1) of which particularly the meprin/A5/μ (MAM) domain has been associated with cell-cell interactions (Sap *et al.*, 1994; Zondag *et al.*, 1995). Moreover, it has been shown that PTPR-κ is dependent on cell density and that it becomes upregulated by signals induced by cell-cell interaction (Fuchs *et al.*, 1996). Since loss of cellular connections via cell adhesion is a typical feature contributing to neoplastic phenotype (reviewed in (Johnson, 1999; Ruoslahti, 1996)), these data strongly associate PTPR-κ/m with tumourigenesis.

Another special feature of PTPR-κ is represented by post-transcriptional modification (Jiang *et al.*, 1993). More precisely, a furin-like endoprotease cleaves a signal sequence located between amino acid 640-643 in the fourth fibronectin type III domain, after which the halves of the domain become non-covalently connected (Figure 8.1). The protein then appears as an interrupted structure on the cell surface. This extracellular region of PTPR-κ has a pronounced role in homophilic cell-cell interaction, i.e. interaction between the same type of surface molecules on two opposing cell membranes (Sap *et al.*, 1994; Zondag *et al.*, 1995).

Taken together, these data point towards a role of the PTPR-κ/m protein as a possible regulator of signal transduction and cell adhesion thus suggesting that alterations
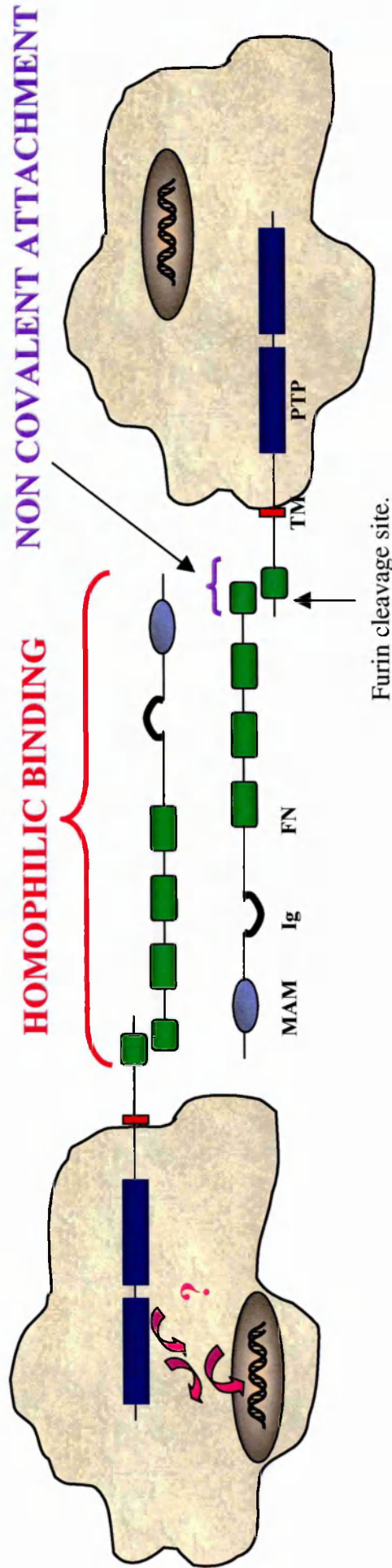
FIGURE 8.1: A schematic view of homophilic binding through the extracellular regions of the *PTPR-κ gene*. After enzymatic cleavage by furin, an endopeptidase ,the two protein halves remain associated by a non-covalent attachment [Jiang, 1993 #335]. The the meprin/A5/µ domain (MAM) domain has been suggested to mediate the homophilic cell-cell interaction for PTPR-κ (Zondag *et al.*, 1995), while both the MAM domain and the Ig domain have been indicated in this interaction for PTPR-µ (Zondag *et al.*, 1995; Brady-Kalnay *et al.*, 1994). One can speculate that there may be a coupling between homophilic interaction and signal transduction, but this is still an open question, as symbolic indicated by the question mark " ? ". MAM = Meprin/A5/µ domain; Ig = immunoglobulin domain; FN = fibronectin III domains; TM = transmembrane region and PTP = protein tyrosine phosphatase domains.

(mutations) of this gene may have a role in neoplastic transformation. In fact, the mutation could hinder the negative regulatory role of this tyrosine phosphatase and, therefore, contribute to malignant transformation by placing a cellular oncogene, target for the negative regulation by PTPR-κ/m, under a constant activation state.

Thus, the results presented in this thesis may contribute also to a new view of the PTPR-κ protein (Figure 8.2). In fact, previously, PTPR-κ/m was known as a membrane receptor (1), whose dimerization via homophilic interactions induced downstream signalling events (reviewed in (Zondag and Moolenaar, 1997)) possibly linked to malignant transformation (Sap et al., 1994) (2). My data described herein depict PTPR-κ/m as a novel tumour specific antigen (3). However, few examples exist of TAAs being directly involved in neoplastic transformation and progression, and the T cell epitopes derived from murine oncogenic proteins such as p53 (Noguchi et al., 1994) and ras (Abrams et al., 1996; Peace et al., 1994; Skipper and Stauss, 1993) have been defined by reverse immunology approaches. Mutated fibronectin is the only TAA, identified by using a genetic approach and recognized by TILs, for which the point mutation generating the immunogenic peptide was also proved to be associated with the malignant phenotype (Wang et al., 2002). In fact, the mutation described by Wang and co-workers (Wang et al., 2002) was shown to directly interfere with the formation of extracellular matrix and disrupt cellular connections. There is no such evidence for the mutation in PTPR-κ, but it is nevertheless intriguing that such mutation occurred in a protein with phosphatase activity and with defined roles in important cellular processes like signalling and cell cycle regulation.

PTPR-κ/m is the first phosphatase described as a TAA, although previous publications have reported mutated epitopes deriving from proteins with kinase activity (Chiari et al., 2000; Harashima et al., 2001). All together these findings stress the importance of enzymes
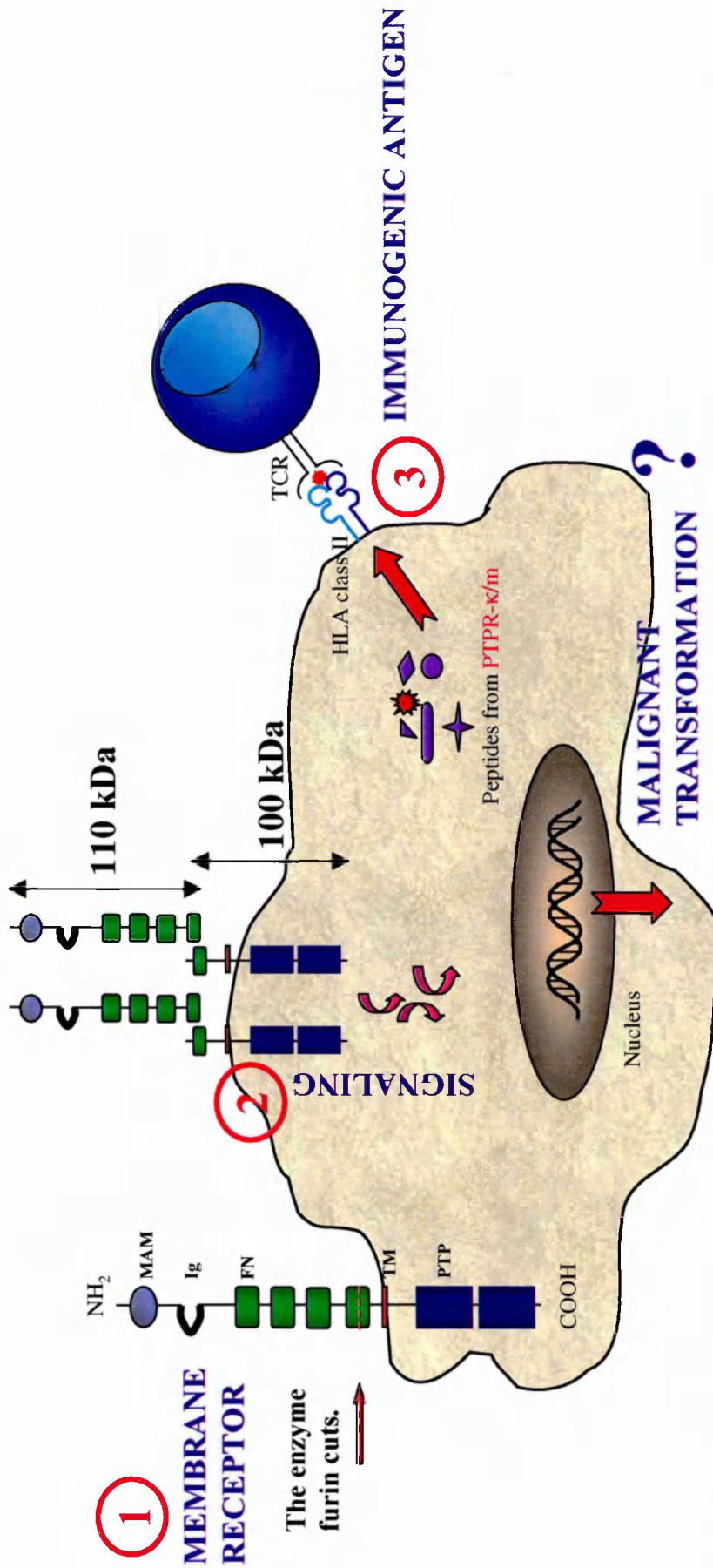
FIGURE 8.2: A schematic view summarising the proposed functions of *PTPR-κ/m* gene as 1) a membrane receptor; 2) a signalling protein and 3) a tumour antigen.

After synthesis, PTPR-κ become expressed as a 210 kDa precursor protein on the cell surface. Its fourth fibronectin domain harbour a signal sequence, present in amino acid residue 640-643, which is involved in a post-translation processing step of this protein. An endopeptidase called furin cleave the intact protein at this site, which result in the expression of two associated subunits: one extracellular 110 kDa product containing the NH_2 end and a 100 kDa spanning the cell membrane expressing the cytoplasmic parts and the COOH end. TCR = T cell receptor; MAM = Meprin/A5/μ domain; Ig = immunoglobulin domain; FN = fibronectin III domains; TM = transmembrane region and PTP = protein tyrosine phosphatase domains.

having a role in dephosphorylation and phosporylation of proteins and involved in the regulation and balance of internal cellular events, as potential targets for an immune-mediated recognition and as source of unique as well as shared TAAs. This set of proteins may therefore constitute an unexplored reservoir for novel undefined tumour-specific antigens.

8.1.7 Immunogenicity of unique tumour-antigen

PTPR-κ/m has been identified as a unique TAA in a melanoma patient who is still disease free 12 years after surgery that removed a lymph node metastasis and was not followed by therapeutic interventions. Given that also other unique TAAs, like CDK-4 (Wolfel *et al.*, 1995), MUM-1 (Coulie *et al.*, 1995), MUM-2 (Chiari *et al.*, 1999) and MUM-3 (Baurain *et al.*, 2000), have been identified in long term surviving melanoma patients, it is possible that immune responses against mutated TAAs are strong enough to eliminate or to keep at bay tumour cells (reviewed in (Parmiani *et al.*, 2002b)) (Table 8.1). Therefore, such TAAs may constitute the best TAAs for therapeutic interventions despite their restriction to individual patients (reviewed in (Gilboa, 1999a)). Indeed, although never observed for CT antigens, spontaneous regression for melanoma has been reported as determined by T cell reaction against a TAA encoded by a mutated myosin gene (Zorn and Hercend, 1999b). Thus, PTPR-κ/m may represent a TAA with a strong immunogenic potential associated with its point mutation.

Table 8.1. TAAs with a putative role in neoplastic transformation. Possible strategies for tumour cells to take command of the cellular regulation and facilitate neoplastic transformation. EMC = extracellular matrix.

ANTIGEN	IMPLIED FUNCTION	REFERENCE
SART-2	Signal transduction of cellular proliferation	Nakao *et al.*, 2000
CDK4/m	Cell cycle regulation	Wölfel *et al.*, 1995
Hsp70-2M	Cell cycle regulation	Gaudin *et al.*, 1999
[1]β-catenin	Cell adhesion	Robbins *et al.*, 1996
CASP-8	Apoptosis signalling pathways	Mandruzzato *et al.*, 1997
[1]Fibronectin	ECM formation	Wang *et al.*, 2002
Lck	Anchorage independent growth	Harashima *et al.*, 2001
[a]PTPR-κ/m	Tumour suppression, cell-cell adhesion	Novellino *et al.* 2002 (submitted)

1) Also implied in cell-cell contact.

8.1.8 CD4+ T cell response in Pt15392

Indeed PTPR-κ/m was able to induce in Pt15392 a strong immune response in the surgically removed, infiltrated lymph node. In fact, several CD4+ T cell clones obtained from such lymph nodes were found to be reactive against the autologous melanoma and recognised the PTPR-κ/m peptide.

The T cell mediated anti-tumour response occurring in the tumour-infiltrated lymph nodes of this patient was analysed at clonal level and CD4+ T cell clones were established from metastatic lymph nodes and then characterized *in vitro* for their functional activities.

The cloning procedure of the established TALs resulted in the isolation of several CD4+ T cell clones that have been assigned to 5 different groups on the basis of composition of their TCR. Library screening led to the identification of the PTPR-κ/$m_{667-682}$ as the epitope for the CD4+ T cells of three of these groups. Interestingly, a predominant expression of TCRVA3, TCRVA13 and TCRVB21 was observed in the clones recognizing the PTPR-κ/$m_{667-682}$ epitope. Unfortunately, T cells belonging to the two other groups were not available for the final epitope screening, but the composition of their TCR, also including TCRVB21 or TCRAV13, strongly suggests that they too were directed against this same epitope.

All the CD4+ T cell clones demonstrated the characteristics of full T cell activation; i.e. IL-2 secretion, antigen specific proliferation (data not shown) and lysis of the antigen positive tumour cells. Sensitization of these clones with the PYYFAAELPPRNLPEP peptide at a concentration as low as 10 nM demonstrated a T cell activation fulfilling all the above parameters. The half maximum release of IFN-γ was achieved at approximately 100 nM, suggesting that these CD4+ clones expressed TCRs with a high affinity/avidity

against this epitope. As shown for class I HLA-restricted epitopes (Loftus *et al.*, 1998), mutated peptides may be able to induce T cells that, bearing high affinity TCRs, are more efficient in the recognition of their nominal MHC/peptide complexes expressed at the cell surface of tumour cells.

Cytokines released from activated CD4+ T cells help to shape the direction of an immune response. Therefore, attention was focused on the estimation of the exact cytokine profile of the CD4+ T cell clones shown to participate in the response to the autologous tumour. Remarkably, an atypical cytokine pattern was observed, since the anti-melanoma CD4+ T cell response directed against the unique PTPR-κ/m antigen had been accompanied by a Th1 dominating, but high IL-10 releasing cytokine profile. This finding is compatible with recent reports which suggest a less dogmatic view of the Th1/Th2 function and define an important role for CD4+ T cells with new cytokine profiles in tumour responses (Brady *et al.*, 2000; Segal *et al.*, 2002; Shimizu *et al.*, 1999).

However, the possible contribution of this previously un-described cytokine profile to the favourable clinical outcome in Pt5392 remains elusive. Though IL-10 is known to be immunosuppressive (as reviewed in (Wakkach *et al.*, 2000)), these IL10-secreting CD4+ T cells did not display inhibitory effects on the *in vitro* generation of an anti-tumour T cell mediated response in Pt15392. Moreover, the findings reported in this thesis are also consistent with previous data showing that IL-10 may have a stimulatory activity on CD8+ T cells that are already tumour-specific (Fujii *et al.*, 2001). CD4+ T cells releasing IL-10 at tumour site may therefore serve to potentiate a local CD8+ mediated response and be directly involved in anti-tumour protection (Segal *et al.*, 2002).

8.1.9 Conclusions

The functional evaluation of anti-tumour immunity in Pt15392 has demonstrated the simultaneous presence of tumour-specific classes I and II HLA-restricted T cells directed against different TAA determinants derived from gp100, TRP-2 and mutated PTPR-κ protein. It is not clear whether these responses have been activated simultaneously and may have had a synergistic effect on each other. Future studies need to be performed to answer the question as to whether the CD4+ T cell repertoire may have been involved in orchestrating the previously described CD8+ T cell mediated systemic immune response (Castelli *et al.*, 1999).

Moreover, the results presented herein suggest that both CD8+ and CD4+ immune responses have contributed to the favourable clinical outcome in Pt15392. Taking advantage of the availability of the immunogenic epitope defined in the present thesis and of the ELISPOT technique, further immunological monitoring aimed at evaluating the systemic immunity during the subsequent follow up period of Pt15392 will be possible.

The functional characterisation of the T cell response in Pt15392 has contributed to a better understanding of a positive immune response, indicating a possible co-operating CD8+ and CD4+ T cell activity. The current knowledge about CD4+ T cell responses is limited and together with the data presented herein, additional studies of other patients with favourable outcomes may contribute to an increased knowledge about the role of CD4+ T cells in cancer control and allow the application of new strategies for immunotherapy.

In conclusion, the results presented in this thesis demonstrate that CD4+ T cell defined TAAs can be cloned by a novel molecular method and provide evidence for the presence of tumour reactive CD8+ and CD4+ T cells derived from the metastatic lymph node of a melanoma patient with a positive clinical evolution after therapeutic interventions. This

response was accompanied by an unknown cytokine profile whose role in the tumour regression is elusive. Moreover, the data suggest that protein tyrosine phosphatases may constitute a novel group of proteins whose mutation can result in T cell targets. Therefore, a continuous endeavour should be done to identify class II HLA restricted TAAs and characterise CD4+ T cell mediated anti-tumour responses, as such data may provide new insights on *in vivo* T cell/tumour cell interactions situation of cancer patients and hopefully result in new therapeutic strategies.

8.2 FUTURE PLANS

The identification of PTPR-κ/m as a TAA opened several questions regarding its nature and possible roles in the anti-tumour response in Pt15392. The sections below outline future experiments that may be designed to address these issues.

8.2.1 Definition of the shortest epitope/binding motifs for the COOH end

The putative binding anchors at the carboxyl end of the PTPR-κ/m epitope have only been partially defined. In order to more closely characterise the involvement of the N_{678} and L_{679} in the binding between the TCR and the peptide, peptides should be designed where these positions have been replaced with glycine.

8.2.2 Tissue expression of PTPR-κ/m

Although defined as a somatic point mutation and, therefore, considered to be restricted to the melanoma cells of Pt15392, the presence of the PTPR-κ/m gene in additional melanoma samples should be evaluated in order to exclude a wider tumour distribution and role of this particular mutation.

8.2.3 Cloning of the full length PTPR-κ/m gene

The insert of Clone #11, i.e. cDNA #11, corresponded to a 667 bp long region of the 4322 base pair full length *PTPR-κ* gene (Yang *et al.*, 1997). Therefore, to analyse if the *PTPR-κ/m* gene may have a truncated expression in tumour cells and if this may be relevant to a malignant phenotype, the cloning of the full length *PTPR-κ/m* gene could be performed. PCR using genomic DNA extracted from frozen tumour samples will confirm the presence of the identified mutation *in vivo*.

8.2.4 Analysis of the in vivo expressed PTPR-κ protein

As revealed by sequence analysis, the PTPR-κ/m protein may be translated from either or both of two internal ATG codons. Thus, to assess whether both or only one of these proteins (791 or 778 amino acids respectively) are expressed *in vivo*, Western blot can be performed providing specific antibodies are available that can distinguish between the two proteins.

8.2.5 Potential transformation activity

One additional point that remains to be elucidated is the role, if any, of the mutation in modifying the physiological functions of PTPR-κ. In particular the question of whether the mutated gene has acquired transformation capacities still reamins to be addressed. Transfection experiments performed in NH3T3 fibroblasts using the mutated and the wild type gene will represent initial steps for clarifying this issue.

8.2.6 Systemic immune response directed against PTPR-κ/m

The specific T cell response directed against the PTPR-κ/m was detected in the tumour infiltrated lymph nodes. Whether the PTPR-κ/m was also able to induce a systemic response remains to be addressed. To this purpose, the specific response directed against

the mutated or non mutated peptide will be evaluated by ELISPOT in the PBMCs of

Pt15392 obtained during the follow up period.

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