EXPRESSION AND RECOGNITION OF HLA-DR/HER-2 COMPLEXES ON CARCINOMA CELLS

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Expression and Recognition of HLA-DR/Her-2 Complexes on Carcinoma Cells

by

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ABSTRACT

Cancer patients frequently generate cellular immune response to Her-2, a protein implicated in promoting tumor growth. While 40% of cancers express HLA class II, the ability of class II^+ tumor cells to process endogenous Her-2 and present immunogenic peptides for $CD4^+$ T cell recognition is unknown. We addressed this question using a $CD4^+$ T cell clone (TCL-6Dn) restricted to Her-2 peptide 883-899 (p883) presented by HLA-DRβ1*0401. We measured the proliferation and cytokine response of TCL-6Dn to p883-loaded and $Her\text{-}2^+$ tumor cells. Our results showed that TCL-6Dn proliferated strongly to p883-loaded DCs, yet TCL-6Dn lysed tumor cells in a p883-specific manner. Cytokine production analysis showed that TCL-6Dn recognized p883 and produced IFN-γ, GM-CSF, TNF-α and IL-4. Tumor cell lysis was mediated by a soluble factor produced by TCL-6Dn, but cell contact increased lysis. Furthermore, we provide evidence that p883 is a naturally processed HLA class II epitope presented by cancer cells.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ag:	Antigen
APC(s):	Antigen presenting cells
BCL(s):	B cell lines
Ca^{2+}:	Calcium
CD40L:	CD40 ligand
cDNA:	Complementary DNA
CIITA:	Class II transactivator
CO_2:	Carbon dioxide
cpm(s):	Counts per minute
Cr:	Chromium
CTLs:	Cytotoxic T lymphocytes
DC(s):	Dendritic cells
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
EBV:	Epstein barr virus
ER:	Endoplasmic reticulum
FACS:	Fluorescence activated cell sorter
FasL:	Fas ligand
Fc:	Constant fragment
FCS:	Fetal calf serum
FITC:	Fluorescein isothiocyanate
FOXP3:	Forkhead/winged helix transcription factor gene
GAD:	Glutamic acid decarboxylase
GAM:	Goat anti-mouse
GM-CSF:	Granulocyte macrophage-colony stimulating factor
HBSS:	Hanks balanced salt solution
Her-2:	Human epidermal growth factor receptor
HIC:	Human investigation committee
HIV:	Human immunodeficiency virus
HLA:	Human leukocyte antigens
IAPs:	Inhibitors of apoptosis proteins
IFN-α:	Interferon alpha
IFN-γ:	Interferon gamma
Ig:	Immunoglubulin
Ii:	Invariant chain
IL-2:	Interleukin-2
IL-4:	Interleukin-4
IL-5:	Interleukin-5
IL-8:	Interleukin 8
IL-10:	Interlekin-10
IL-12:	Interleukin 12
IL-13:	Interleukin-13
IL-15:	Interleukin-15
IL-18:	Interleukin 18
IMDM:	Iscove's modified dulbecco's medium

IAPs:	Inhibitors of apoptosis proteins
IHW:	International histocompatibility workshop
JAM Assay:	Just another method assay
JNK:	Jun N-terminal kinase
kDa:	Kilo Dalton
L cells:	Mouse fibroblast cell lines
mAbs:	Monoclonal antibodies
MAPK:	Mitogen-activated protein kinase
MIIC:	MHC class II compartment
mRNA:	Messenger ribonucleic acid
MHC:	Major histocompatibility complex
NeoR:	Neomycin phosphotransferase II
NK:	Natural killer
OKT3:	anti-CD3 mAb
p883:	Her-2 peptide 883-899
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate buffered saline solution
PCR:	Polymerase chain reaction
PE:	Phycoerythrin
PFA:	Paraformaldehyde
PHA:	Phytohemagglutinin
PI3K:	Phosphatidylinositol 3-kinase
rhIL-2:	Recombinant human IL-2
rhIL-4:	Recombinant human IL-4
rhGM-CSF:	Recombinant human GM-CSF
rhTNF-α:	Recombinant human TNF-α
RNA:	Ribonucleic acid
RT:	Room temperature
RT-PCR:	Reverse transcriptase polymerase chain reaction
SI(s):	Stimulation index
TAP:	Transporter in antigen presentation
TCR:	T cell receptor
TGF-β:	Transforming growth factor-beta
Th0:	T helper-0
Th1:	T helper-1
Th2:	T helper-2
TNF-α:	Tumor necrosis factor alpha
Tr:	Regulatory T cells
TRAIL:	TNF-related apoptosis-inducing ligand
VEGF:	Vascular endothelial growth factor
-/-:	Gene knockout

CHAPTER 1. INTRODUCTION

The immune system responds to tumor cells as tumor antigens are shed from the cell surface, picked up and processed by professional antigen presenting cells (APCs). APCs degrade the protein into peptides and present them in the context of HLA class I and HLA class II molecules for recognition by $CD8^+$ and $CD4^+$ T cells, respectively.

Cancer patients generate cellular immune responses to tumor antigens, but often the response is insufficient to eliminate tumors completely (Coulie et al., 1992, Disis et al., 2000, Rentzsch et al., 2003). These findings have led to various peptide and protein-based immunotherapies. However, a major limitation to peptide-based immunotherapy is that peptide-specific T cells may not recognize naturally processed tumor antigens (Zaks & Rosenberg, 1998).

The anti-tumor immune response could be enhanced if $CD4^+$ T cells directly recognized tumor cells, presenting tumor-derived antigens in the context of class II molecules. Although the intracellular events required for antigen processing and presentation are well described for professional APCs, little is known about these mechanisms in tumor cells. It is known that, approximately 40% of cancers up-regulate HLA-DR and other class II molecules, such as HLA-DM, -DO, Ii and CIITA (Concha et al., 1995, Oldford et al., 2004, Saito et al., 1997, Ishigami et al., 2001, Khalil et al., 2002, Rossi et al., 2002). Potentially, tumor cells themselves may act as antigen presenting cells capable of processing and presenting tumor peptides to activate $CD4^+$ T cells. Her-2, a tumor-associated antigen, is over-expressed in 25-35% of various carcinomas (Afify et al., 1999, Menard et al., 2000, Brabender et al., 2001,

Parton et al., 2004), but the ability of tumor cells to process and present endogenous Her-2 for $CD4^+$ T cell recognition is not well described. The objective of this study was to determine if Her-2^+ and HLA class II^+ tumor cells were able to process and present Her-2-derived epitopes for $CD4^+$ T cell recognition. To address this question a $CD4^+$ T cell clone, TCL-6D, specific for Her-2 peptide 883-899 (p883) and described by Kobayashi et al. (2000) was used. Activation of the T cell clone was measured by cytokine production and proliferation. Therefore, this chapter reviews pertinent literature on HLA class II and cancer, the role of $CD4^+$ T cells in anti-tumor immunity, the role of Her-2 in cancer and the Her-2-specific cellular immune response.

1.1. HLA Structure and Function

Human leukocyte antigens (HLA), also referred to as major histocompatibility complex antigens (MHC), are encoded by genes in three distinct regions of chromosome 6 corresponding to HLA class I, class II and class III molecules (Reviewed in Rees & Mian, 1999, McFarland & Beeson, 2002). The three HLA regions constitute a single HLA haplotype. An individual possesses two co-dominantly expressed haplotypes (one maternal and one paternal).

HLA class I antigens (HLA-A, -B and -C) are highly polymorphic, expressed on all cells and present endogenously derived antigens to $CD8^+$ T cells. Class I molecules are composed of α_1, α_2, α_3 chains and a β_2-microglobulin domains, with the $\alpha 1$ and $\alpha 2$ folding into a peptide binding cleft that is closed on both ends and restricted to binding 8-10 amino acid peptides (Bjorkman et al., 1987). The peptide binding groove of class I molecules contains pockets at the termini of the groove that

secure the peptide's carboxyl and amino groups in a manner independent of the peptide sequence (Bouvier & Wiley, 1994).

HLA class II molecules present peptides generated from exogenous antigens to $CD4^+$ T cells. Analogous to class I, class II molecules (HLA-DR, -DP and -DQ) are polymorphic. However, class II molecules are primarily expressed on professional APCs, such as B cells, macrophages and dendritic cells (DCs). Class II molecules are composed of αβ heterodimers and the $\alpha_1$and β_1 domains form a peptide binding cleft that is open on both ends, allowing longer peptides (13-25 amino acids) to be loaded in the groove (Rudensky et al., 1991, Chicz et al., 1992). X-ray crystallography of class II molecules revealed that several peptide side chains bind to the polymorphic pockets (P1, P4, P6, and P9) that line the peptide-binding groove (Smith et al., 1998, Lee et al., 2001, Fremont et al., 2002, Liu et al., 2002). The class II and peptide complex is stabilized by hydrogen bonds formed between the conserved residues on the MHC and the peptide back-bone carboxyl and amino groups (Reviewed in McFarland & Beeson, 2002).

1.2. HLA Class II and Cancer

1.2.1. HLA Class II Expression

Although class II molecules are normally expressed on APCs, class II expression on normal epithelium is variable. Studies have shown that normal breast, cervical and intestinal tissues do not express class II (Bartek et al., 1987, Fais et al., 1991, Hilders et al., 1994, Concha et al., 1995, Haraldsen et al., 1998, Løvig et al., 2002). Other studies reported low levels of class II on normal intestinal epithelium with enhanced expression in inflammatory conditions, suggesting inflammatory

cytokines up-regulate class II expression (Hershberg et al., 1997, Horie et al., 1998). Indeed, cytokines, such as IFN-γ, IFN-α, and IL-2 up-regulate class II expression on human colon epithelium and melanoma cells, while TNF-α enhances IFN-γ-induced class II expression on human colon epithelium (Nistico et al., 1990, Watanabe and Jacob, 1991, Horie et al., 1998).

Class II molecules have also been detected on various types of cancers (Bartek et al., 1987, Hilders et al., 1994, Cabrera et al., 1992, Concha et al., 1995, Maiorana et al, 1995, Gastl et al., 1996, Tamiolakis et al., 2003). For example, Concha et al. (1995) showed that 34% of breast cancers expressed HLA-DR, whereas DQ and DP were expressed on 18% and 12%, respectively. More recently, Oldford et al. (2004) studied class II protein expression on breast carcinoma and reported that 40% of tumors expressed HLA-DR. Other cancers, such as 80% of cervical, 60% of ovarian and 50% of colon, express HLA-DR (Hilders et al., 1994, Tamiolakis et al., 2003, Løvig et al., 2002).

The biological significance of HLA-DR on cancer cells is controversial. Studies have shown HLA-DR expression on ovarian, gastric and colon cancer is associated with a good prognosis (Ma et al., 1994, Løvig et al., 2002, Tamiolakis et al., 2003). In contrast, Maiorana et al. (1995) showed no such correlation in breast cancer patients. The high degree of polymorphisim of HLA alleles leads to variation in the antigen recognition site. Consequently, specific HLA alleles may lead to resistance or susceptibility to cancer. The association between HLA-DR allelic expression on cancer cells in general and prognosis has been controversial. Carriage of HLA-DRβ1*0401, *11, *1501 and *1601 alleles is associated with an increased risk of cancer development (Odunsi et al., 1996, Beskow et al., 2001, Lee et al., 2002,

Madeleine et al., 2002). Others have shown that HLA-DRβ1*0301, *13, *11 and *0101 alleles may be protective alleles in cancer development (Odunsi et al., 1996, Chaudhuri et al., 2000, Madeleine et al., 2002). However, Gourley et al. (2003) found no association between HLA-DR allelic carriage and prognostic outcome.

1.3. Role of T Cells in Anti-Tumor Immunity

Substantial experimental evidence suggests that an anti-tumor immune response occurs when tumor antigens are shed from the cell surface or released from dying/degrading tumor cells (Chiodoni et al., 1999, Hoffmann et al., 2000). As illustrated in Figure 1.1, tumor antigens are captured by professional APCs, such as DCs, generating peptides that are loaded onto class I or class II molecules. These DCs traffic to the draining lymph node where they mature and interact with $CD8^+$ T cells and $CD4^+$ T cells (Pieters, 1997).

Much attention has been given to $CD8^+$ T cells in the anti-tumor immune response because most tumors are HLA class I positive and a large number of HLA class I-restricted epitopes derived from tumor antigens have been identified (Peoples et al., 1995, Brossart et al., 1999, Nagorsen et al., 2000). Furthermore, cytotoxic $CD8^+$ T cells lyse tumor cells directly and eradicate large tumor masses in-vivo (Townsend & Allison, 1993, Seki et al., 2002).

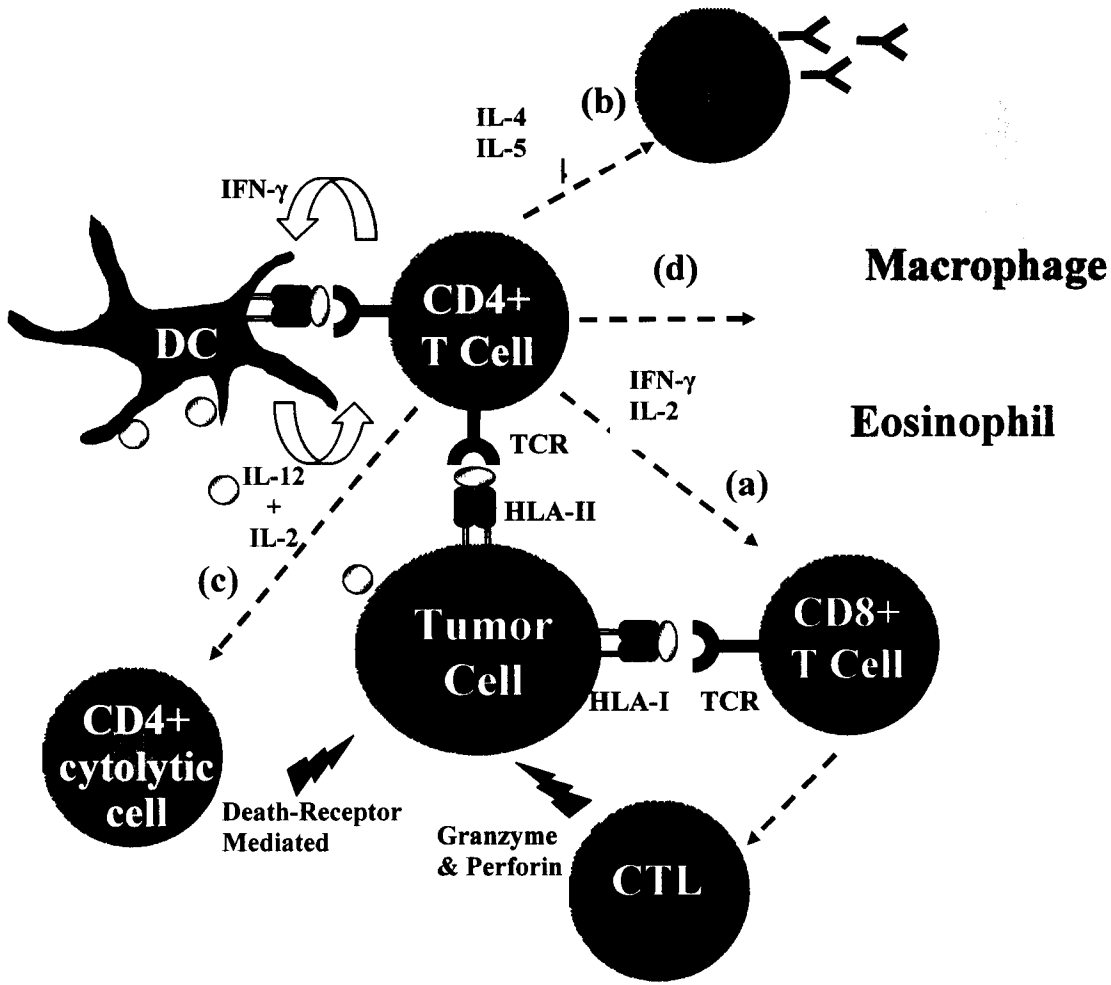

Figure 1.1. The role of $CD4^+$ T cells in mediating anti-tumor immunity. Dendritic cells may capture shed tumor antigens and present antigenic peptides to $CD4^+$ and $CD8^+$ T cells. Activated effector cells may then destroy the tumor cell. Tumor cells also directly present tumor peptides to activate $CD4^+$ and/or $CD8^+$ T cells. $CD4^+$ T cells play a central role in regulating the immune response through the following mechanisms; (a) help $CD8^+$ T cells differentiate into cytolytic anti-tumor CTLs (b) provide help for B cell activation (c) inhibit tumor cell growth directly in a manner similar to CTL killing or generate cytokines which may inhibit tumor growth indirectly (d) recruit tumoricidal effector cells (i.e. macrophages and eosinophils). Adapted from Wang, 2001, Pardoll & Topalian , 1998 and Knutson & Disis, 2005.

1.3.1. Role of $CD4^+$ T cells in Anti-Tumor Immunity

The discovery of HLA class II-restricted epitopes derived from tumor antigens led to re-evaluation of the role $CD4^+$ T cells play in tumor eradication (Reviewed in Pardoll & Topalian, 1998, Toes et al., 1999, Wang, 2001). Naïve $CD4^+$ T (Th0) cells recognize tumor peptides presented by class II molecules on DCs. Depending on the DCs activation state, microenvironment and cytokines present, naïve Th0 cells can differentiate into Th1 or Th2 effector $CD4^+$ T cell subtypes (Reviewed in Rajnavölgyi & Lányi, 2003). Typically Th1 cells produce IL-2, TNF-α and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13 (Bucy et al., 1994, Openshaw et al., 1995, Abbas et al., 1996). Although Th1 and Th2 cells produce different cytokines, both subsets play an essential role in the anti-tumor immune response. Th1 cells provide help to prime the $CD8^+$ T cell response, while Th2 cells activate B cells to become antibody-secreting plasma cells (Reviewed in Wang, 2001).

1.3.1.1. Maintaining the $CD8^+$ T Cell Response

$CD8^+$ T cells are believed to be the predominant anti-tumor effector cells, while $CD4^+$ T cells provide help to DCs for the activation of $CD8^+$ T cells. Indeed, researchers have shown that $CD4^+$ T cells are essential in the induction of a tumor-specific $CD8^+$ T cell response (Hung et al., 1998, Baxevanis et al., 2000, Reilly et al., 2001). $CD4^+$ T cell help is partly mediated by the interaction of CD40 on the DC with CD40L on the $CD4^+$ T cell. This interaction leads to the expression of co-stimulatory molecules, such as CD80 and CD86, enabling the DC to co-stimulate the $CD8^+$ T cell directly (Schoenberger et al., 1998). In addition, $CD4^+$ T cells generate IL-2 to maintain the $CD8^+$ T cell response (Rosenberg et al., 1998).

1.3.1.2. Activation and Recruitment of Effector Cells

Recent evidence suggests tumor specific $CD4^+$ T cells travel to the tumor site where they secrete cytokines, such as TNF-α, IFN-γ and IL-2 (Kagamu & Shu 1998, Wong et al., 1998). Through the release of IFN-γ, IL-2, IL-4 and GM-CSF, $CD4^+$ T cells recruit and activate other effector cells at the tumor site (Tepper et al., 1992). Indeed, mice challenged with B16 tumor cells had high levels of tumor infiltrating macrophages and eosinophils, while these cells were completely absent in the tumor site of CD4 -/- mice (Hung et al., 1998).

Th2 cells enhance the anti-tumor immune response by activating antigen-specific B cells to become plasma cells, which generate tumor-specific antibodies that recognize antigens on cancer cells (Imahayashi et al., 2000, Yasuda et al., 2002, Ichiki et al., 2004). Immunization studies using B cell epitopes derived from Her-2 tumor antigen generated a peptide-specific antibody response that inhibited tumor growth *in-vitro* by complement dependent- and antibody dependent-cell lysis (Jasinska et al., 2003).

1.3.1.3. Direct Elimination by $CD4^+$ T cells

The mechanism by which $CD4^+$ T cells eliminate tumor cells, in the absence of $CD8^+$ T cells, is unclear. Studies have shown that $CD4^+$ T cells kill tumor cells in an HLA-restricted manner (Xiang et al., 1998, Zennadi et al., 2001). For example, Zenaddi et al. (2001) demonstrated that Th2 cells lyse autologous melanoma cells in an HLA-DR-restricted manner via the Fas/FasL pathway. Meanwhile, Thomas et al. (1998a) illustrated that $CD4^+$ T cells kill melanoma cells in an HLA-DR-restricted manner mediated by perforin and granzymes as tumor cell lysis was Ca^{2+}-dependent

in 3 of the 4 $CD4^+$ T cell clones. However, one of the $CD4^+$ T cell clones lysed autologous melanoma cells by a TNF-α-dependent mechanism.

$CD4^+$ T cells also kill tumor cells in a cytokine-mediated manner not requiring cell contact (Mumberg et al., 1999, Brady et al., 2000, Hess et al., 2003). In fact, $CD4^+$ T cells kill class II positive and negative tumor cells in-vitro and in-vivo by the indirect effects of IFN-γ (Mumberg et al., 1999, Brady et al., 2000). Tumor-infiltrating $CD4^+$ T cells also lyse autologous lung carcinoma cells via perforin/granzyme and APO2L/TRAIL-mediated pathways (Dorothée et al., 2002).

1.4. Human Tumor Antigens

Tumor antigens can be divided into two main categories known as tumor-specific and tumor-associated antigens (Reviewed in Platsoucas et al., 2003). Tumor-specific antigens are only expressed on tumor cells. These may be newly-expressed antigens or antigens that contain mutations compared to their normal counterparts, such as β-catenin, cyclin-dependent kinase 4 and Caspase-8 (Wolfel et al., 1995, Robbins & Kawakami, 1996, Mandruzzato et al., 1997). Conversely, tumor-associated antigens are expressed on both normal cells and tumor cells, but are usually over-expressed on tumor cells. Various tumor-associated antigens have been discovered, including MAGE-1, MAGE-2, MAGE-3, BAGE, GAGE, NY-ESO, Mart-1, gp100, tyrosinase, Her-2, mutated p53 and MUC-1 (Jerome et al., 1991, Traversari et al., 1992, Brichard et al., 1993, Coulie et al., 1993, Nagai et al., 1993, Yanuck et al., 1993, Bakker et al., 1994, Kawakami et al., 1994, Boel et al., 1995, Van den Eynde et al., 1995, Chen et al., 1997).

1.5. Her-2 and Cancer

A prototype tumor-associated antigen for immunotherapy is Her-2 (also known as Her2/neu or erbB2), an 185kDa transmembrane protein expressed at low levels on normal tissues and over-expressed on a variety of cancers.

1.5.1. Biological Role of Her-2

Her-2 belongs to the human epidermal growth factor receptor (Her) family, consisting of four receptors known as Her-1, Her-2, Her-3 and Her-4 (Coussens et al., 1985). As illustrated in Figure 1.2, Her-2 receptors are transmembrane proteins with a large extracellular domain (632 amino acids), a short transmembrane domain (22 amino acids) and a cytoplasmic domain (580 amino acids) (Choudhury & Kiessling, 2004). Her-2 receptors are also expressed on a variety of human tissues and have tyrosine kinase activity within an intracellular domain, yet the Her-2 ligand has not been identified (Cochet et al., 1984, Penuel et al., 2001).

Although Her receptors usually exist as monomers, upon ligand binding, heterodimers and homodimers of the Her receptors form, resulting in phosphorylation of the intracellular tyrosine kinase (see Figure 1.3.). The combination of receptors formed and the ligand with which the dimer interacts determines the signal pathway stimulated. Potentially ten different dimers can form between the various Her proteins. However, a hierarchy of inter-receptor relationships exists that favours Her-2 as a dimer partner (Reviewed in Rubin & Yarden, 2001, Yarden, 2001).

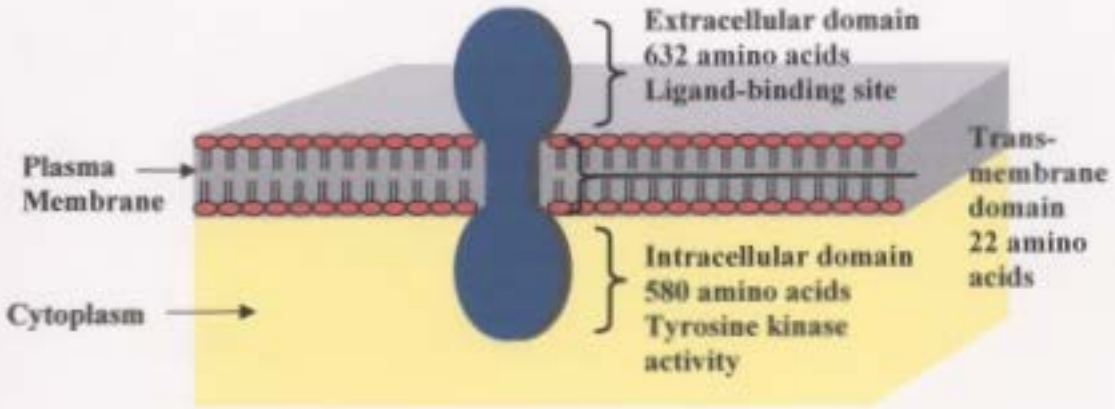

Figure 1.2. Structure of the Her-2 protein. The Her-2 protein has transmembrane topology, containing a ligand binding site on the extracellular domain. Ligand binding to the Her-2 protein results in phosphorylation of the tyrosine kinase in the intracellular domain.

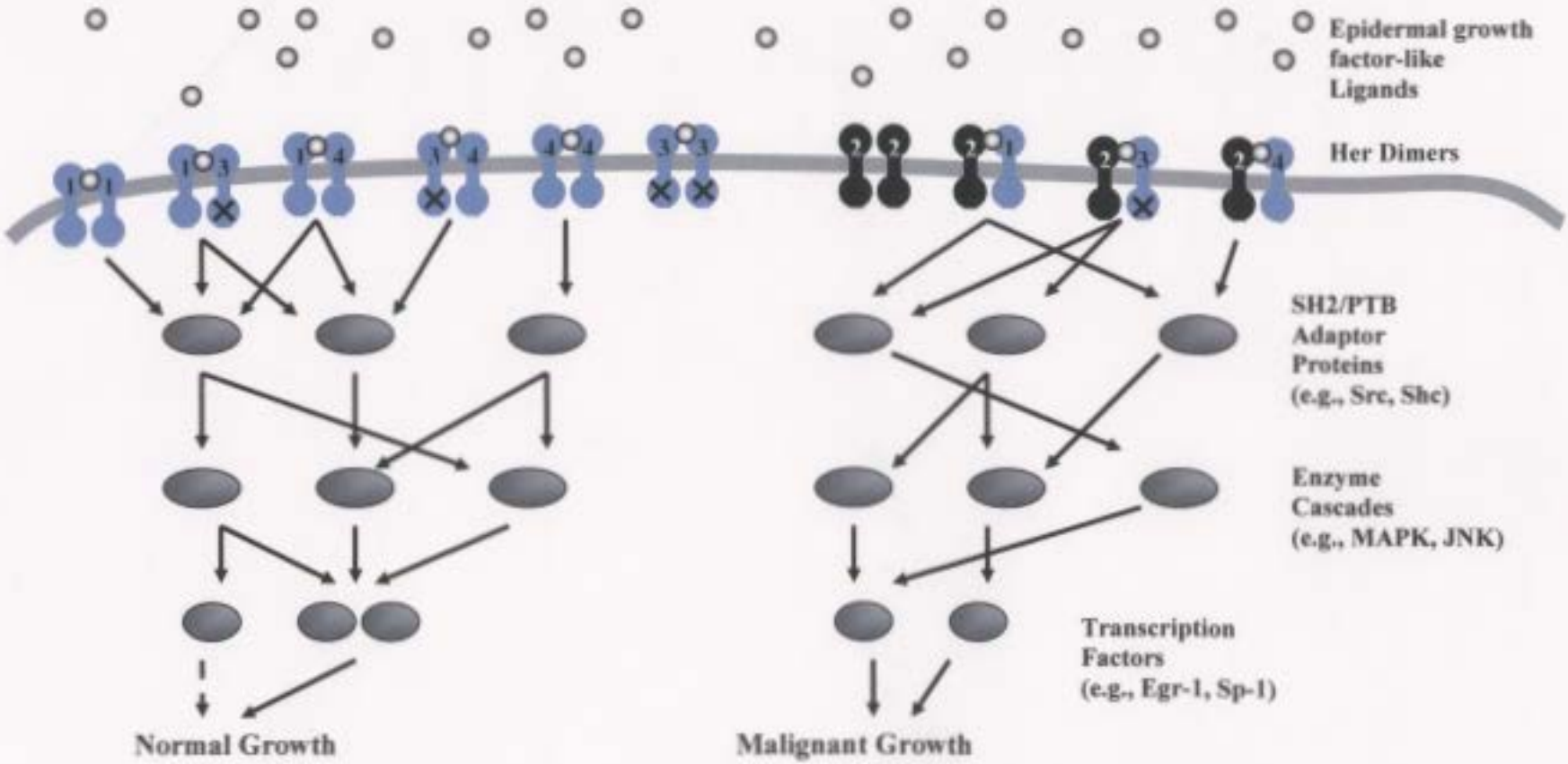

Figure 1.3. The Her signaling network. Numbers refer to the four Her proteins and the inactive catalytic domain is indicated by a X in the intracellular domain. (Adapted from Klapper et al., 2000)

1.5.2. Role of Her-2 in Cancer

There is no published evidence of mutations in the Her-2 protein leading to cancer. Meanwhile, Her-2 is up-regulated in a broad spectrum of human cancers, including 25-30% of breast, 25% of ovarian, 35-45% of pancreatic and 35% of lung cancers (Lei et al., 1995, Afify et al., 1999, Ménard et al., 2000, Brabender et al., 2001, Parton et al., 2004).

In normal cells, activation of Her receptors is highly controlled, resulting in activation of a signalling pathway that promotes normal cell growth, differentiation and development through the mitogen-activated protein kinase pathway (MAPK) (Cousssens et al., 1985, Lee et al., 1995, Threadgill et al., 1995). Normal expression of Her-2 on cells results in few Her-2 combinations and provides weak but essential signals for cell growth (Lin et al., 2000). However, cells that over-express Her-2 show uncontrolled cell growth (Neve et al., 2000, Goebel et al., 2002).

Up-regulation of Her-2 contributes to increased tumor cell growth by various mechanisms. The anti-apoptotic effect of Her-2 has been attributed to activation of the phosphatidylinositol 3-kinase (PI3K) and MAPK pathways, which increase the expression of anti-apoptotic proteins, such as BCL and C-FLIP (Daly et al., 1999, Neve et al., 2000, Bhat-Nakshatri et al., 2002). Up-regulation of Her-2 is associated with increased tumor invasiveness as Her-2 enhances the secretion of matrix metalloproteases, which accelerate the breakdown of normal connective tissue and boost tumor metastasis (Ioachim et al., 1998, Pellikainen et al., 2004). Her-2 is believed to signal via the PI3K pathway, inducing release of phosphoinositides, which contribute to actin re-organization and enhance tumor migration (Feldner & Brandt,

2002). Her-2 is also associated with up-regulation of vascular endothelial growth factor (VEGF), increasing tumor vascularity (Konecny et al., 2004).

Studies have examined the relationship between Her-2 expression and clinical outcome, illustrating that over-expression of Her-2 is associated with disease progression, metastatic phenotype, poor clinical outcome and higher resistance to chemotherapy (Press et al., 1993, Tsai et al., 1996, Menard et al., 2001, Masood & Bui, 2002). Thus, the direct contribution of Her-2 in the development and metastasis of cancer has made this molecule an attractive target for anti-cancer therapy.

1.5.3. Cellular Immune Response to Her-2

Although the immune system is unable to protect against the growth of Her-2^+ tumors, interestingly these patients generate antibody and T cell responses against Her-2 (Rentzsch et al., 2003, Sotiropoulou et al., 2003). Her-2 vaccination studies have shown that this anti-tumor response is enhanced in patients with ovarian, gastric, breast and lung cancers (Fisk et al., 1997, Tuttle et al., 1998, Disis et al., 2002, Kono et al., 2002). Thus, Her-2 may be a promising target for T cell-based immunotherapies.

1.5.3.1. Her-2 Specific $CD4^+$ T Cell Response

Incorporation of both class I and class II-restricted epitopes in vaccines results in a more efficient and longer lasting anti-tumor immune response (Ossendorp et al., 1998, Knutson et al., 2001). Consequently, researchers have attempted to identify class II epitopes from the Her-2 protein. One such class II-restricted peptide (777-798) was presented by HLA-DR4 and recognized by $CD4^+$ T cells in breast cancer patients (Tuttle et al., 1998). Similarly, DCs retrovirally transduced to express Her-2

protein presented Her-2 peptides in the context of HLA-DR and generated an autologous $CD4^+$ T cell response in Her-2 positive breast cancer patients (zum Buschenfelde et al., 2001).

Additional studies have shown that Her-2 contains immunogenic epitopes that bind promiscuously to various HLA-DR molecules. In particular, Her-2 peptide 776-788 was presented by HLA-DRβ5*0101, HLA-DRβ1*0701 and HLA-DRβ1*0405, activating $CD4^+$ T cells (Sotiriadou et al., 2001). Similarly, Kobayashi et al. (2000) identified Her-2 peptides capable of eliciting a $CD4^+$ T cell proliferative response. One of these peptides (883-899) was promiscuously presented in the context of HLA-DR1, DR4, DR52 and DR53. Consequently, Her-2 peptides 776-788 and 883-899 bind to various class II molecules and may offer broad population coverage for immunotherapy.

1.6. HLA Class II Antigen Processing in Tumor Cells

1.6.1. HLA Class II Antigen Processing Pathway

Loading of peptides, generated from exogenous proteins, onto class II molecules is a multi-step process (see Figure 1.4.) and mainly occurs in professional APCs. Initially, HLA class II α and β subunits in the endoplasmic reticulum form an association with the invariant chain (Ii), which inhibits peptide binding by the class II molecules (Roche & Creswell., 1990). Trimerization of the Ii facilitates binding of three αβ class II molecules and forms a nonomeric complex $(\alpha\beta Ii)_3$ that is directed through the endoplasmic reticulum to the Golgi and into the endosomes (Layet et al., 1991, Bikoff et al., 1993, Reviewed in Wang, 2001, Watts, 2004).

Removal of the Ii from class II molecules is achieved in the late endocytic compartment called the MIIC (MHC class II compartment). Aspartyl and cysteinal proteases in the MIIC degrade the Ii leaving a fragment known as CLIP (class II-associated invariant chain protein) in the peptide binding groove (Busch et al., 2000, Bania et al., 2003). HLA-DM, in association with HLA-DO, facilitates the removal of CLIP and stabilizes the empty class II molecule (van Ham et al., 2000, Hsieh et al., 2002). Thereafter, HLA-DM and HLA-DO assist in editing the selection of tightly binding peptides derived from proteins that are also degraded in the endocytic pathway (Reviewed in Kropshofer et al., 1999). Peptide-class II complexes are transported to the cell surface where they are recognized by $CD4^+$ T cells.

1.6.2. Binding of Peptides Derived From Endogenous Proteins onto HLA Class II Molecules

HLA class I molecules generally present peptides generated from endogenous (cytosolic) proteins, whereas class II molecules present peptides from exogenous proteins (Figure 1.4). However, cytosolic antigen-derived peptides have been eluted from class II molecules (Reviewed in Zhou & Blum, 2004). According to Chicz et al (1993), over 85% of the peptides eluted from class II molecules on human B cells were derived from endogenous proteins. Several pathways have been implicated in the presentation of cytosolic protein-derived peptides onto HLA class II molecules. For instance, peptides derived from cytosolic proteins, such as GAD (glutamic acid decarboxylase) and influenza viral proteins require cytoplasmic processing by the proteasome and TAP (Lich et al, 2000, Tewari et al., 2005).

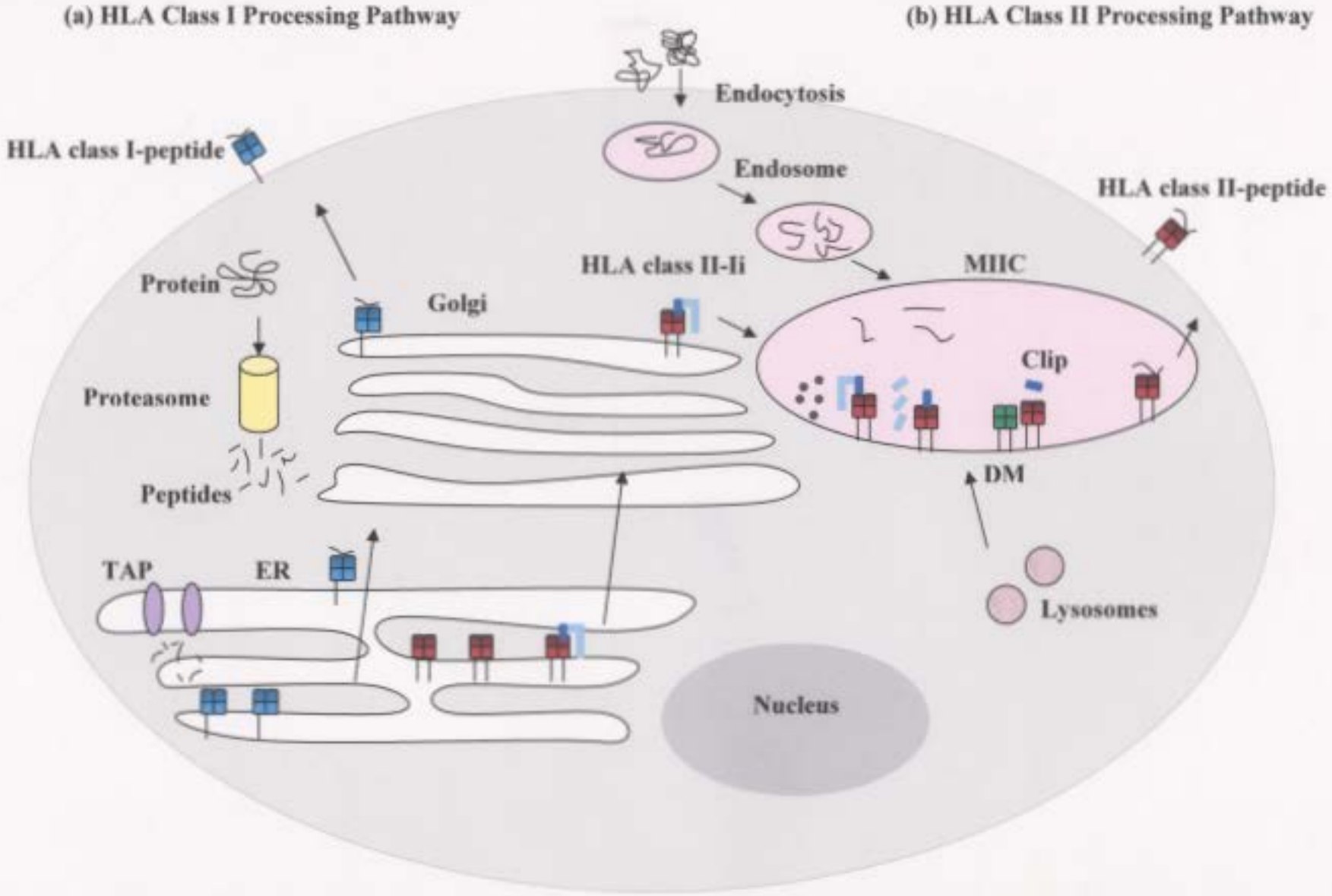

Figure 1.4. HLA class I and class II antigen processing pathways. (a) HLA class I molecules present endogenously processed antigens. Proteins are degraded by the proteasomes, generating peptides that enter the ER and are loaded onto HLA class I molecules. HLA class I-peptide complexes are transported to the cell surface for recognition by $CD8^+$ T cells. (b) HLA class II molecules present peptides generated from exogenous proteins that are taken up by endocytosis. HLA class II-Ii chain complexes are move through the ER, Golgi and into the MIIC where the Ii is degraded and HLA DM facilitates the removal of CLIP and loading of antigenic peptides. Class II-peptide complexes are transported to the cell surface and recognized by $CD4^+$ T cells (Adapted from Wang 2001).

Alternatively, autophagy has been implicated in the presentation of cytosolic antigen-derived peptides by class II molecules. This process involves the sequestration of cytosolic proteins in autophagolysosomes that fuse with the lysosome where antigens are degraded and loaded onto class II molecules. Cytosolic proteins acquired through autophagy, such as the Epstein bar virus nuclear antigen and the neomycin phosphotransferase II (NeoR) antigen, are processed by acidic proteases in the lysosomal compartments of the class II processing pathway (Nimmerjahn et al., 2003, Paludan et al., 2005). In contrast, Dörfel et al., 2005 have shown that proteasomal and endosomal compartments are required for the presentation of class II epitopes from cytosolic proteins in DCs.

1.6.3. Expression of HLA Class II Antigen Processing Molecules in Tumor Cells

Class II antigen processing molecules (Ii, HLA-DM and HLA-DO) are mainly expressed in professional APCs and can be up-regulated by cytokines, such as IFN-γ, in other cell types (Chang & Flavell, 1995, Boss & Jensen, 2003). Although the expression of class II processing molecules in tumor cells is not well resolved, studies using immunocytochemistry have shown that 15% of colorectal, 38% of gastric and 54% of breast tumors express invariant chain (Ishigami et al., 2001, Rossi et al., 2002, Oldford et al., 2004). Until recently, HLA DO expression was only documented in lymphoid cells (Alfonso & Karlsson, 2000). However, recent studies demonstrated that myeloid leukemia cells express HLA class II/CLIP complexes (Khalil et al., 2002, Chamuleau et al., 2004). Thus, tumor cells may be capable of processing tumor antigens and presenting peptides.

1.6.4. Role of HLA Class II Antigen Processing Molecules in Tumor Cells

Transfection of the class II transactivator (CIITA) into murine mammary adenocarcinoma has led to expression of class II molecules and anti-tumor immunity (Meazza et al., 2003). Activation of the CIITA gene upreguates HLA class II and HLA class II-associated accessory molecules, such as HLA-DM and Ii (Chang & Flavell, 1997). However, expression of class II and class II-accessory molecules on tumor cells does not necessarily confer antigen processing capabilities. Given that the role of the Ii chain is to protect the peptide binding groove of class II molecules, one would expect that tumor cells expressing HLA-class II molecules in the absence of class II-associated molecules would be better suited to endogenously process tumor antigens. Indeed, mouse-transfectant studies demonstrated that tumor cells expressing class II without co-expression of the Ii or Ii and DM are immunogenic and present endogenous antigens, while class II positive tumor cells co-expressing the Ii or Ii and DM are not immunogenic (Armstrong et al., 1997). Meanwhile, suppression of the Ii chain expression in class II-positive murine tumors can result in increased tumor clearance (Qiu et al., 1999).

1.6.5. Antigen Presenting Capacity of Tumor Cells

Tumor cells generally lack the co-stimulatory molecules, CD80 and CD86, necessary to activate naïve T cells (Staveley-O'Carroll et al., 1998, reviewed in Schwartz et al., 2002). However, manipulation of tumor cells to express co-stimulatory molecules can confer antigen presenting capabilities to the tumor cell (Reviewed in Allison et al., 1995, Hurwitz et al., 2000). Recent evidence suggests that tumor specific $CD4^+$ T cells activated by professional APCs travel to the tumor

site where they secrete cytokines, such as TNF-α, IFN-γ and IL-2 (Kagamu and Shu 1998, Wong et al., 1998). Production of IFN-γ can up-regulate HLA-class II molecules on tumor cells, possibly resulting in tumor antigen processing, presentation and recognition by tumor infiltrating effector $CD4^+$ cells .

1.7. Hypothesis

Previously, Kobayashi et al. (2000) showed that TCL-6D recognized the Her2 peptide p883 presented by HLA-DRβ1*0401 molecules. In particular, TCL-6D proliferated strongly in response to p883-pulsed $DR\beta1*0401^+$ DCs, PBMCs and L cells. However, in this study, Kobayashi et al. (2000) did not assess the cytokine profile generated by activated TCL-6D, but showed that another p883-specific $CD4^+$ T cell clone, TCL-7C, produced GM-CSF in response to p883-pulsed DCs. Consequently, an important aim was to determine the cytokines produced by TCL-6D upon recognition of p883-pulsed APCs.

TCL-6D recognized p883 presented by $DR\beta1*0401^+$ L cells, which lack co-stimulatory molecules CD80 and CD86 (Kobayashi et al., 2000). Therefore, we hypothesized that HLA-DR^+ tumor cells, also lacking co-stimulatory molecules, might present p883 to activate TCL-6D. Generally, tumor cells do not express class II molecules, but studies have shown that class II molecules are up-regulated by various cytokines (Nistico et al., 1990, Watanabe & Jacob, 1991, Horie et al., 1998). Thus, we predicted that HLA-DR^+ tumors would present exogenous p883 to activate TCL-6D.

Although expression of class II antigen processing molecules on tumor cells is not well resolved, tumor cells can up-regulate HLA class II processing molecules (Ishigami et al., 2001, Khalil et al., 2002, Rossi et al., 2002, Chamuleau et al., 2004,

Oldford et al., 2004). Therefore, we asked whether Her-2^+ and class II^+ tumor cells were able to process endogenous Her-2 protein, presenting p883 to activate TCL-6D. Studies have shown that HLA-DR^+ tumor cells are capable of presenting tumor peptides, eliciting a $CD4^+$ T cell tumor-specific response (Reviewed in Ostrand-Rosenberg, 1994, Fisk et al., 1995, Armstrong et al., 1997, Armstrong et al., 1998a, Armstrong et al., 1998b). In addition, Her-2 epitopes 776-788 and 884-899 are naturally processed epitopes presented on tumor cells that are recognized by $CD4^+$ T cell clones (Sotiriadou et al., 2001, Perez et al., 2002). Consequently, we predicted that tumor cells expressing HLA class II antigen processing molecules and Her-2 protein would endogenously process and present Her-2 peptides, activating the Her-2 p883-specific $CD4^+$ T cell clone.

1.8. Objectives

i. To determine the cytokines produced by TCL-6D upon recognition of p883-pulsed APCs.

ii. To investigate the ability of TCL-6D to respond to exogenous p883-pulsed HLA-DR^+ tumor cells.

iii. To examine the ability of HLA class II^+ and Her-2^+ tumor cells to process endogenous Her-2 and present the 883-899 epitope to activate TCL-6D.

CHAPTER 2. METHODOLOGY

2.1. Cell Culture

2.1.1. Culture and Maintenance of Cell Lines

Tumor cell lines and mouse fibroblasts cell lines (L cells) (Table 2.1) were grown as adherent cell cultures in 25 cm^2 tissue culture-treated flasks (Corning Incorporated, Corning, NY, USA). EBV-transformed B cell lines (BCLs), T cell line, Jurkat, and NK cell line (C10MJ) were grown as suspension cultures. C10MJ was grown in RPMI 1640 (GibcoBRL, Grand Island, NY, USA) while other cell lines were cultured in Iscove's Modified Dulbeco's Medium (IMDM). Both RPMI and IMDM media were enriched with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) and antimycotic amphotericin B (0.25 μg/ml; all obtained from GibcoBRL).

Adherent cells were harvested and sub-cultured when cell growth was 90-100% confluent. Medium was aspirated from the culture flask and adherent cells were detached using 5 ml of 0.25% trypsin diluted in sterile phosphate buffered saline solution (PBS) for 3-5 min at 37°C. Five millilitres of enriched IMDM was added to the flask, to inactivate the trypsin. Cells were transferred to sterile 15 ml polypropylene tubes, centrifuged at 300 g for 7 min and washed in 5 ml of enriched IMDM. Cell pellets were resuspended in 5 ml of enriched IMDM and 3 x 10^5 cells were re-seeded into culture flasks containing 7 ml of enriched IMDM.

BCLs (see Table 2.2) and C10MJ were maintained at 3 x 10^5 cells/ml and 6 x 10^5 cells/ml, respectively and Jurkat was maintained at 2 x 10^5 cells/ml. Cells were cultured at 37°C, in a 6.5% CO_2 humid chamber.

Table 2.1. Description of tumor cell lines and L cell lines used in this study

Tumor Cell Line	Source	Description	Transfected HLA-DR	Constitutive HLA-DR	IFN-γ induced HLA-DR Type
HT29	R. Hershberg	Colon Carcinoma	None	None	DRβ1*0402, *0701, DRβ4
HT29-*0401	R. Hershberg	Colon Carcinoma	DRβ1*0401	None	DRβ1*0402, *0701, DRβ4
T47D	J. Blum	Breast Carcinoma	None	DRβ1*0102	DRβ1*0102
T47D- *0401	J. Blum	Breast Carcinoma	DRβ1*0401	DRβ1*0102	DRβ1*0102
MDA MB 435	J. Blum	Breast Carcinoma**	None	DRβ1*1320	DRβ1*0404, DRβ1*13, DRβ3, DRβ4
MDA MB 435-*0401	J. Blum	Breast Carcinoma**	DRβ1*0401	DRβ1*1320	DRβ1*0405, DRβ1*1320, DRβ3, DRβ4
L243.6	R. Karr	Mouse Fibrobalst	DRβ1*0401	None	None
L164.11	R. Karr	Mouse Fibroblast	DRβ1*0401	None	None

American Type Culture Collection (Manassas, VA)

**Using DNA microarrays, MDA MB 435 was found to express melanoma associated genes (Ross et al., 2000).

Table 2.2. Human B cell lines used as feeder cells to expand TCL-6Dn or as control APCs in this study.

B Cell Lines	**IHW** Identification**	**HLA-DR Type**
MGAR	IHW 9014	DRB1*1501, DRB5*0101
YAR	IHW 9026	DRB1*0402, DRB4*0101
JESTHOM	IHW 9004	DRB1*0101
SAVC	IHW 9034	DRB1*0401, DRB4*0101
PLH	IHW 9047	DRB1*0702, DRB4*0101
IBW9	IHW 9049	DRB1*0701, DRB4*0101
MT14B	IHW 9098	DRB1*0404, DRB4*0101
SWEIG007	IHW 9037	DRB1*1101, DRB3*0202
DBB	IHW 9052	DRB1*0701, DRB4*0101
MZO70782	IHW 9002	DRB1*0102

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2.1.2. Expansion and Culture of p883-Specific T Cell Clone

TCL-6D, a $CD4^+$ T cell clone restricted to Her-2 peptide 883-899 (p883) presented in the context of HLA-DRβ1*0401 molecules was a gift from Dr. E. Celis (Mayo Clinic, Minnesota, USA). TCL-6D was expanded, now called TCL-6Dn, in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/ml gentamicin, 25 μM 2-ME and antibiotic/antimycotic containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (all obtained from GibcoBRL). On day 0 of the expansion, 2×10^4 T cells/ml were combined with 30 ng/ml of anti-CD3 (OKT3), 2×10^5 irradiated (γ8000 rads) mixed human BCLs/ml and 2.5×10^7 irradiated (γ4200 rads) human PBMCs/ml that were collected from various healthy donors and pooled. Twenty-five ml of T cell expansion mixture was placed in a 50 ml tissue culture flask and incubated for 18 days.

On day one, 50 units/ml of recombinant human interleukin-2 (IL-2) was added to each flask. On days 5, 8, 11 and 14, 12.5 ml of supernatant was gently removed from each flask and replaced with fresh T cell medium supplemented with 50 units/ml of rhIL-2 (Hoffmann-LaRoche, Nutley, NJ, USA). On day 18, T cells were counted and cultures were centrifuged at 300 g for 7 min. Cell pellets were resuspended at 5×10^6/ml in freezing solution containing 10% dimethyl sulfoxide (DMSO; Sigma, Saint Louis, Missouri, USA) in FCS. Aliquots of T cells were placed in a minus 70°C freezer for a minimum of 24 h, prior to being stored in liquid nitrogen.

2.2. Interferon-γ Treatment of Tumor Cell Lines

To up-regulate MHC class II and class II co-chaperone molecules, tumor cells were treated with recombinant human interferon gamma (rhIFN-γ; BD Pharmingen, San Diego, CA, USA). Prior to rhIFN-γ treatment, tumor cell lines were sub-cultured (as described in Section 2.1.1) and rested overnight at 37°C. On day 0, one culture was treated with 500 units/ml rhIFN-γ and a second culture was left un-treated in order to determine constitutive expression of class II molecules. Cells were incubated for 96 h.

2.3. Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by density gradient centrifugation. Blood was collected in tubes containing anticoagulant acid citrate dextrose solution (BD Biosciences, Rutherford, NJ, USA) and centrifuged at 700 g, 18°C for 15 min. The PBMC layer was collected and diluted 1:2 with 0.9% NaCl solution (Baxter, Toronto, ON, Canada). Ten ml of diluted blood was layered over 5 ml of lymphoprep (Nycomed, Oslo, Norway) in a 15 ml polypropylene tube and centrifuged at 550 g, 18°C for 35 min. The PBMC layer was sterilely collected, washed twice in Hanks balanced salt solution (HBSS; GibcoBRL) and centrifuged at 400 g, 18°C for 10 min. This was followed by two washes in enriched IMDM for 7 min at 400 g, 18°C and a final 5 min wash at 150 g to remove platelets. Cell pellets were resuspended in 5 ml of enriched IMDM. One-hundred microliters of cell suspension was removed, diluted 1:2 with 3% acetic acid to lyse any residual erythrocytes and counted, using a hemocytometer counting chamber (Fisher Scientific, Nepean, ON, Canada).

2.4. In Vitro Generation of Dendritic Cells

DCs were isolated from the adherent fraction of freshly isolated PBMCs obtained from healthy volunteers with the Memorial University's Human Investigation Committee's (HIC) approval. PBMCs were isolated as described above and were resuspended at 2 x 10^6/ml in enriched IMDM. Thereafter, 2 ml of DC culture was dispensed into each well of a six-well plate (Fisher Scientific). Plates were incubated in a 37°C, 6.5% CO_2 humidified chamber for 2 h, after which, non-adherent cells were removed by aspirating the medium and washing each well four times with 2 ml of HBSS. Remaining adherent cells were cultured in 2 ml of enriched IMDM supplemented with 1000 units/ml rhGM-CSF (BD Pharmingen) and 1000 units/ml rhIL-4 (BD Pharmingen) for 7 days. On days two and four, 2 ml of enriched IMDM supplemented with 1000 units/ml rhGM-CSF and 1000 units/ml rhIL-4 was added to each well.

TNF-α induces DCs maturation and up-regulation of CD80, CD86 and CD83 (Tang et al., 2005). To induce maturation of DCs, on day 6 an additional 4 ml of enriched IMDM, containing 100 ng of rhTNF-α (BD Pharmingen) was added to half of the wells. Remaining wells received enriched IMDM without TNF-α, causing DCs to retain the immature phenotype. On day 7, mature and immature DCs were collected in 50 ml polypropylene tubes, on ice, and centrifuged at 300 g for 7 mins at 4°C. Pellets were resuspended in enriched IMDM, counted and used as APCs or phenotyped by flow cytofluorometry.

2.5. Flow Cytofluorometry

2.5.1. Primary Antibodies

A description of the mouse monoclonal antibodies (mAbs) used to detect cell surface proteins on TCL-6Dn is presented in Table 2.3. Negative isotype controls were PE-conjugated mIgGs (DAKO, Carpinteria, CA, USA) and FITC-conjugated mIgG1 (BD Pharmingen). Primary mAbs used to detect intracellular cytokines are described in Table 2.4. Negative isotype controls were FITC-conjugated rat IgG2a, rat IgG1, mIgG1 (Caltag Laboratories, Hornby, ON, Canada) and un-conjugated mIgG1 (BD Pharmingen). Primary mAbs used to detect cell surface antigens on *in-vitro* generated DCs are described in Table 2.5. Primary antibodies used to detect HLA-DR allelic products (L243, NFLD.D1 and NFLD.D11) were obtained locally. All other antibodies were obtained commercially. Negative isotype controls were mIgG1 and mIgG2a and mIgM (BD Pharmingen).

2.5.2. Secondary Antibodies

To detect binding of un-conjugated mouse mAbs, PE-conjugated affinipure $F(ab')_2$ fragment goat-anti-mouse (GAM) IgG, Fc fragment specific (Jackson ImmunoResearch, West Grove, PA) was used. For some experiments, FITC-conjugated GAM IgG, γ and L chain specific (Caltag Laboratories) was used, while FITC-conjugated affinipure $F(ab')_2$ fragment donkey anti rat IgG, heavy and light chain specific (Jackson ImmunoResearch) was used to detect binding of rat mAbs.

Table 2.3. Primary antibodies used to detect expression of cell surface proteins on TCL-6Dn by flow cytofluorometry

Antibody	Isotype	Directly Conjugated Fluorochrome	Concentration	Reference/ Source
CD2 Clone S5.2	Mouse IgG2a	PE	5 µl/test	BD Pharmingen
CD3 Clone SK7	Mouse IgG1	PE	5 µl/test	BD Pharmingen
CD4 Clone SK3	Mouse IgG1	PE	5 µl/test	BD Pharmingen
CD8 Clone SK1	Mouse IgG1	PE	5 µl/test	BD Pharmingen
CD16 Clone B73.1	Mouse IgG1	PE	16 µl/test	BD Pharmingen
CD19 Clone SJ25C1	Mouse IgG1	PE	5 µl/test	BD Pharmingen
CD25 Clone 2A3	Mouse IgG1	PE	5 µl/test	BD Pharmingen
CD38 Clone HB7	Mouse IgG1	PE	5 µl/test	BD Pharmingen
CD45 Clone 2D1	Mouse IgG1	PE	5 µl/test	BD Pharmingen
CD45RA Clone L48	Mouse IgG1	FITC	5 µl/test	BD Pharmingen
CD45RO Clone UCHL1	Mouse IgG2a	PE	5 µl/test	BD Pharmingen
CD56 Clone MY31	Mouse IgG1	PE	16 µl/test	BD Pharmingen
CD69 Clone FN50	Mouse IgG1	FITC	5 µl/test	BD Pharmingen
CD95 Clone DX2	Mouse IgG1	Un-conjugated	1.0 µg/ml	BD Pharmingen
CD95L Clone NOK-1	Mouse IgG1	Un-conjugated	0.2 µg/ml	Caltag Laboratories
HLA-DR Clone TU36	Mouse IgG2b	PE	5 µl/test	BD Pharmingen

Table 2.4. Primary antibodies used to detect CD4 expression and intracellular cytokines produced by TCL-6Dn measured by flow cytofluorometry

Antibody	Isotype	Directly Conjugated Fluorochrome	Concentration	Reference/ Source
IL-2 Clone MQ1-17H12	Rat IgG2a	FITC	8 µl/test	Caltag Laboratories
IL-4 Clone MP4-25D2	Rat IgG1	FITC	8 µl/test	Caltag Laboratories
IL-10 Clone JES3-9D7	Rat IgG1	FITC	8 µl/test	Caltag Laboratories
IFN-γ B27	Mouse IgG1	FITC	8 µl/test	Caltag Laboratories
TGF-β Clone A75-2.1	Rat IgG2a	Unconjugated	10 µg/ml	BD Pharmingen
TNF-α Clone J1D9 + J2D10	Mouse IgG1	Unconjugated	10 µg/ml	Neomarkers (Union City, CA, USA)
GM-CSF Clone BVD2-21C11	Rat IgG2a	Unconjugated	5 µl/test	Caltag Laboratories
CD4	Mouse IgG2a	PE	5 µl/test	Caltag Laboratories

Table 2.5. Primary antibodies used to determine the phenotype of *in-vitro*-generated dendritic cells by flow cytofluorometry

Antibody	Isotype	Specificity	Concentration	Reference/ Source
CD1a Clone HI149	Mouse IgG1	CD1a	5 μg/ml	BD Pharmingen
CD3 CloneUCHT1	Mouse IgG1	CD3	5 μg/ml	BD Pharmingen
CD14 Clone M5E2	Mouse IgG1	CD14	5 μg/ml	BD Pharmingen
CD19 Clone FMC63	Mouse IgG2a	CD19	2 μg/ml	Neomarkers
CD40 Clone 5C3	Mouse IgG1	CD40	5 μg/ml	BD Pharmingen
CD68 Clone EMB11	Mouse IgG1	CD68	20 μg/ml	DAKO
CD80 Clone DAL-1	Mouse IgG1	CD80	2 μg/ml	Caltag Laboratories
CD83 Clone HB15e	Mouse IgG1	CD83	5 μg/ml	BD Pharmingen
CD86 Clone BU63	Mouse IgG1	CD86	10 μg/ml	BD Pharmingen
L243 (Supernatant)	Mouse IgG2a	All HLA-DR	5 μg/ml	Lampson & Levy, 1980
NFLD.D1 (purified)	Mouse IgG1	HLA-DR4	20 μg/ml	Drover et al., 1994
NFLD.D11 (Supernatant)	Mouse IgM	HLA-DRβ1*0401	Neat	Drover et al, 1998

2.5.3. Extracellular Flow cytofluorometry

2.5.3.1. Utilizing Fluorochrome-Conjugated mAbs

To detect expression of cell surface antigens, a pre-determined amount (as per Tables 2.3 and 2.4) of the fluorochrome-conjugated mAb was added to each 12 x 75 mm polystyrene tube (Fisher Scientific). Cells were washed once in PBS, once in FACS buffer (see appendix 1) and resuspended at 2 x 10^6/ml in FACS buffer. Fifty µl of cell suspension was mixed with the mAb and incubated for 20 min in the dark at 4°C. Cells were washed twice in 2 ml of ice-cold FACS buffer, centrifuged at 300 g for 5 min at 4°C and cell pellets were re-suspended in 200 µl of 1% paraformaldehyde (PFA) (see appendix A). All tubes were protected from light and stored at 4°C for approximately 18 h. Ten thousand events were acquired using the FACScan (Becton-Dickinson, San Jose, CA). Data was analyzed using Cell Quest pro analysis software or WINMIDI 2.8 software.

2.5.3.2. Utilizing Un-Conjugated mAbs

Twenty five µl of the un-conjugated mAb, diluted to an optimal concentration (Tables 2.3, 2.4 and 2.5) using FACS buffer was added to each 12 x 75 mm tube. Fifty µl of cell suspension was mixed with the mAbs and incubated for 30 min in the dark at 4°C. Cells were washed twice in ice-cold FACS buffer and incubated with 25 µl of PE-conjugated GAM IgG (Jackson ImmunoResearch), optimally diluted to 25 µg/ml in FACS buffer, for 30 minutes at 4°C protected from light. Cells were washed twice in ice-cold FACS buffer, fixed and analyzed as described in section 2.5.3.1.

2.5.4. Intracellular Flow Cytofluorometry

Detection of invariant chain (Ii), HLA-DM and Her-2 required fixation and permeabilization of the cells. Cells were washed once in ice-cold FACS buffer, fixed in 5 ml of 2% PFA for 15 min at 4°C and washed once in enriched IMDM. Cells were permeabilized in 5 ml of 0.2% saponin (Polysciences, Warrington, PA, USA) in PBS for 15 min at 4°C and resuspended to 1.5 x 10^6/ml in permeabilization buffer (1x PBS + 0.5% BSA + 0.2% saponin). Primary mAbs specific for Ii (10 μg/ml; clone LN2, BD Pharmingen), HLA-DM (5 μg/ml; clone MaP.DM11, BD Pharmingen) and Her-2 (2.5 μg/ml; clone CB11, NeoMarkers) were diluted in permeabilization buffer. Isotype controls included mIgG1, mIgG2a (BD Pharmingen) and mIgG2b (Southern Biotech Associates, Birmingham, AL, USA) mAbs.

Twenty five μl of mAb was mixed with 50 μl of cell suspension and incubated on ice for 30 min. Cells were washed twice with ice-cold permeabilization buffer and incubated with 25 μl of PE-conjugated GAM-IgG (25 μg/ml) for 30 min on ice protected from light. Cells were washed twice in permeabilization buffer, fixed and analyzed as described in section 2.5.3.1.

2.6. Reverse Transcriptase-Polymerase Chain Reaction (RTPCR)

The presence of FOXP3 transcripts in TCL-6Dn was assessed by RT-PCR. An aliquot of TCL-6Dn was thawed, washed twice in T cell medium and once in PBS. Thereafter, RNA was prepared from TCL-6Dn using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions and treated with DNAase to remove any contaminating DNA. cDNA was prepared from 1 μg of RNA using the first strand cDNA synthesis kit from Pharmacia Biotech (Baie d'Urfe,

Quebec, Canada) according to the manufacturer's instructions. Expression of FOXP3 was measured using the FOXP3 sense (5' CAG CTG CCC ACA CTG CCC CTA 3') and anti-sense (5' CAT TTG CCA G CA GTG GGT AG 3') primers at a final concentration of 20 pM (Takahata et al., 2004). The housekeeping gene β-actin was included as a positive control to ensure the quality of cDNA in the samples. The β-actin sense (5' ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG 3') and anti-sense (5' CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC 3') primers (Paterno et al., 1998) were used at a final concentration of 20 pM.

The PCR amplification was performed in a total volume of 50 µl and the PCR mixture contained 5 µl 10X buffer, 1 µl dNTP, 1.5 µl $MgCl_2$, 1 µl cDNA, 1 µl sense primer and 1 µl anti-sense primer. The PCR protocol used included 1 min at 94°C, 1 min at 65°C, 1 min at 72°C repeated for 35 cycles and held at 4°C until the amplified products were observed.

2.7. Antigen-Specific Response of Her-2 p883-Specific TCL-6Dn

2.7.1. γ-Irradiation of the APCs

Adherent cells were harvested using 0.25% trypsin (see section 2.1.1.) and counted, whereas, non-adherent cell lines were removed from culture. Approximately 1 x 10^6 of each cell line was transferred to separate 15 ml polypropylene tubes, centrifuged at 300 g for 7 min and pellets were resuspended in 15 ml of enriched IMDM.

Irradiation of the APCs was conducted at the J.H. Bliss Murphy Cancer Center. L-cells, BCLs, DCs and PBMCs received γ4200 rads, whereas tumor cells received γ20000 rads. Following irradiation, cells were centrifuged at 300 g for 7 min,

washed twice in 7 ml of enriched IMDM and cell pellets were resuspended in the appropriate medium, depending on the intended use for the irradiated APCs.

2.7.2. Synthetic Peptides

The synthetic peptide 883-899 (p883; KVPIKWMALESILRRRF) derived from Her-2 was synthesized by Dr. Wang (Banting Institute, Toronto Hospital of Sick Kids, Toronto, ON). An irrelevant peptide derived from HIV Gag p24 SILDIRQGPKEPFRDYVDRF, a kind gift from Dr. M. Grant, was used as a control peptide. This HIV peptide was predicted to bind to HLA-DRβ1*0401 molecules using a peptide binding prediction algorithm (Venturini et al., 2002).

2.7.3. Proliferative Response of TCL-6Dn

Irradiated APCs were resuspended in T cell medium, containing RPMI 1640 medium supplemented with 10% heat-inactivated human male AB serum (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 ug/ml gentamicin, 0.1 mM MEM non-essential amino acids, 100 U penicillin/ml, 100 μg streptomycin/ml, 0.25 μg amphotericin B/ml. APCs were resuspended at the following concentrations; PBMCs 1 x 10^6/ml, DCs 5 x 10^4/ml, tumor cells 1 x 10^5/ml, BCLs 1 x 10^5/ml and L cells, 1 x 10^5/ml. One hundred μl of APCs were plated in triplicates in a 96-well u-bottom plate (Fisher Scientific). APCs were pulsed with 1 μg/ml of peptide or un-pulsed for 2 h.

During the peptide-pulsing period an aliquot of TCL-6Dn was thawed, washed twice and viable cells were counted by trypan blue staining. TCL-6Dn was resuspended in T cell assay medium at 3 x 10^5/ml. One hundred μl of TCL-6Dn was co-cultured with triplicates of the peptide-pulsed or un-pulsed APCs. In addition,

triplicates of TCL-6Dn was added to wells containing media which received either no treatment, 30 ηg/ml OKT3 with 50 U/ml rhIL-2 or 1 μg/ml of peptide. All cultures were incubated at 37°C in a 6.5% CO_2 humid chamber for 72 h. During the last 16 h of co-culture, each well was pulsed with 1.0 μ Ci/well of tritiated thymidine (^{3}H-thymidine, 6.7 ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ, USA).

After 72 h co-culture 0.25% trypsin was added to each well for 3-5 min, to remove adherent cells, cultures were harvested onto 90 x 120 mm printed filtermatA glass fiber filters (Wallac, Turku, Finland), using the Harvester 96 Mach III M (Tometec, Hamden, CT, USA) and dried in the microwave for 2 min. Filtermats were placed on OmniFilter holders (Packard Bioscience Company, Meridian, CT, USA), 35 μl of Microscint (Packard Bioscience Company) was added to each well and ^{3}H-thymidine incorporated into dividing cells was measured using the TopCount NXT Microplate Scintillation and Luminescence Counter (Packard Bioscience Company). Results were expressed as mean cpm values or stimulation indexes (SI). The SI was calculated as follows; SI= Mean cpm of TCL-6Dn + APC + peptide/Mean cpm of TCL-6Dn + APC.

2.7.4. Cytokine Response of TCL-6Dn

APCs were diluted in T cell assay medium to attain 5.6 x 10^4/ml, 1 ml was plated in a 24-well plate (Fisher Scientific) and pulsed with 1 μg/ml peptide or un-pulsed for 2 h. During the peptide-pulsing period, an aliquot of TCL-6Dn was thawed, washed twice, counted and resuspended in T cell assay medium to attain 1.7 x 10^5 cells/ml. One ml of TCL-6Dn was co-cultured with peptide-pulsed or un-pulsed APCs for 18 h.

In addition, cultures of TCL-6Dn (5 x 10^5/ml) were either un-stimulated, stimulated with a T cell mitogen (PHA; 5 μg/ml, Sigma) or peptide-pulsed (10 μg/ml). Positive control cells for each cytokine included C10MJ, for IFN-γ, TNF-α and GM-CSF and PHA-stimulated (5 μg/ml) PBMCs for IL-2, IL-4, IL-10 and TGF-β. Newly synthesized cytokines produced by the cells were trapped intracellularly using a Golgi stop, Brefeldin A (10 μg/ml; Sigma), which was present for the full 18 h incubation.

2.7.4.1. Intracellular Cytokine Detection by Flow Cytofluorometry

To assess production of cytokines, cells were fixed and permeabilized using a Fix & Perm kit (Caltag Laboratories). Cells were collected from the twenty-four well plates (See section 2.7.4) and placed in 15 ml polypropylene centrifuge tubes, which were centrifuged at 300 g, 4°C for 7 min. Next, they were washed in 7 ml of ice-cold wash buffer containing 0.1% sodium azide (BDH Biochemicals, Toronto, ON, Canada) and 5% FCS in PBS and resuspended at 2 x 10^6/ml in wash buffer.

Fifty μl of cell suspension was mixed with a pre-determined amount (Table 2.5) of PE-conjugated mAb, anti-CD4 or mIgG2a isotype control, in 12 x 75 mm tubes, vortexed gently and incubated in the dark for 15 min at room temperature (RT). Cells were fixed in 100 μl of fixation medium, incubated for 15 min at RT in the dark and washed twice in 3 ml of ice-cold wash buffer at 300 g for 5 min at 4°C. Cells were permeabilized with 100 μl of permeabilization medium containing a pre-determined amount (Table 2.4) of the relevant anti-cytokine or isotype control FITC-conjugated mAb. Un-conjugated mAbs were optimally diluted in 100 μl of permeabilization medium. Each sample was vortexed gently and incubated in the dark

for 20 min at 4°C. Cells were washed twice in 3 ml of ice-cold wash buffer as described above, fixed in 1% PFA and analyzed as described in section 2.5.3.1.

2.7.5. Cytotoxic Response of TCL-6Dn

2.7.5.1. ^{51}Chromium Release Assay

Tumor cells were harvested, washed and resuspended in T cell assay medium to attain 1 x 10^6/ml. Thereafter, tumor cell pellets (1 x 10^6 cells) were incubated with 100 µCi (25 µl) of $Na_2{}^{51}CrO_4$ (sodium chromate; Amersham Biosciences 250 mCi/mg) at 37°C with 6.5% CO_2 for 90 min. ^{51}Cr-labelled target cells were washed four times (one 10 ml wash and three 5 ml washes) with PBS containing 1% FCS. Target cell pellets were resuspended in T cell assay media to attain 1.0 x 10^5 targets/ml and 50 µl of tumor targets were added to wells of a 96-well u-bottom plate, containing 30 µl of p883 peptide (at 1 µg/ml) or 30 µl of assay medium and incubated at 37°C with 6.5% CO_2 for 2 h. TCL-6Dn was thawed, washed and resuspended in assay medium to attain 1.2 x 10^6 cells/ml. Effector cells (TCL-6Dn) were cultured with ^{51}Cr-labelled tumor cells at effector:target ratios of 12:1, 6:1 3:1 and 0:1. Thus, 50 µl, 25 µl or 12.5 µl of effector T cells were added to wells containing the p883-pulsed or un-pulsed tumor targets. To determine the spontaneous and maximum ^{51}Cr-release, target cells were cultured with either 250 µl of assay medium or 250 µl of 1M HCl, respectively. Effector T cells and ^{51}Cr-labelled tumor cells were co-cultured for 5 h at 37°C with 6.5% CO_2. A 125 µl aliquot of supernatant was then removed from each well and ^{51}Cr release was measured using a gamma counter (Wallac 1480 Wizard, Perkin Elmer). Percent specific lysis was calculated using the following

formula: (experimental ^{51}Cr-release – spontaneous ^{51}Cr-release / maximum ^{51}Cr-release – spontaneous ^{51}Cr-release) x 100.

2.7.5.2. JAM Assay (Thymidine Release Assay)

Tumor cells were removed from culture and pre-incubated with 5.0 μCi/ml of ^{3}H-thymidine in a 15 ml polypropylene tube (1 x 10^{6} cells/10ml) for 18 h. Cells were washed three times in enriched IMDM and resuspended in T cell assay medium to attain 1 x 10^{5}/ml. One hundred μl of APCs were pulsed with peptide (1 μg/ml) or un-pulsed for 2h. During the peptide-pulsing period, an aliquot of TCL-6Dn was thawed, washed twice, counted and resuspended in T cell assay medium to attain 3.0 x 10^{5} cells/ml. Peptide-pulsed and un-pulsed APCs were co-cultured with 100 μl of TCL-6Dn or 100 μl of medium for 48 h. All cultures were performed in triplicates in a 96 well u-bottom plate.

After 48h co-culture, 0.25% trypsin was added to each well for 3-5 min, to ensure all adherent cells were detached from the plastic, and target cell lysis was evaluated by measuring the amount of ^{3}H-thymidine remaining in the target cells (as described in section 2.7.3). Specific lysis due to TCL recognition of exogenous p883 was calculated as [(cpm p883-pulsed APC) – (cpm p883-pulsed APC + TCL) / (cpm p883-pulsed APC)] x 100%. Specific lysis due to recognition of endogenously produced Her-2 p883 was calculated as [(cpm APC) – (cpm APC + TCL) / (cpm APC)] x 100%.

2.7.6. Tumor Cell Proliferation Assay

Un-irradiated tumor cells were resuspended in T cell medium at 1 x 10^{5}/ml. One hundred μl of tumor cells were plated in wells of a 96-well u-bottom plate and

pulsed with 1 μg/ml of peptide or un-pulsed for 2 h. During the peptide-pulsing period an aliquot of TCL-6Dn was thawed, washed, counted and resuspended in T cell assay medium to attain 3 x 10^5 cells/ml. One hundred μl of TCL-6Dn was co-cultured with peptide-pulsed or un-pulsed tumor cells for 72 h. During the last 16 h of co-culture, each well was pulsed with 1.0 μCi/well of ^{3}H-thymidine. Cell cultures were trypsinized, harvested and ^{3}H-thymidine incorporated into tumor cells was measured, as described in section 2.7.3. Results were expressed as mean cpm values of triplicate wells. Percent inhibition was calculated as 100 minus [(cpm APC + TCL-6Dn / cpm APC) x 100] or 100 minus [(cpm p883-pulsed APC + TCL-6Dn / cpm p883-pulsed APC) x 100].

2.7.7. Transwell Assay

Non-irradiated tumor cells were resuspended in T cell assay medium at 7.0 x 10^4/ml. Eight hundred μl of tumor cells (5.6 x 10^4 cells) were plated in a 24-well flat-bottom plate, designed for use with cell culture inserts (Falcon, Becton Dickinson, Franklin Lakes, NJ) and pulsed with 1 μg/ml of peptide or un-pulsed for 2 h. Duplicate wells of tumor cells were set up to allow culture of tumor cells and TCL-6Dn without and with a 0.4 μm cell culture insert (Falcon) present. During the 2 h peptide-pulsing period an aliquot of TCL-6Dn was thawed, washed, counted and resuspended in assay medium to attain 5.6 x 10^5/ml. Three hundred μl of TCL-6Dn (1.68 x 10^5 cells) was co-cultured, without a cell culture insert present, with tumor cells. In addition, TCL-6Dn was placed in a cell culture insert and cultured with tumor cells, preventing cell contact between tumor cells and TCL-6Dn. As a control, 300 μl of T cell assay media was added directly to tumor cells or placed in the cell culture

insert. During the last 16 h of a 72 h culture period, each well was pulsed with 1.0 μCi/well of ^{3}H-thymidine. For wells containing the cell culture insert, ^{3}H-thymidine was added below the insert. Thereafter, cell culture inserts were removed, cells were trypsinized and equally distributed into five wells of a 96 well u-bottom plate. Cells were harvested and ^{3}H-thymidine incorporated into cells was measured, as described in section 2.7.3. Results were expressed as mean cpm values of five wells. Percent Inhibition was calculated as 100 minus [(cpm APC + TCL-6Dn / cpm APC) x 100] or 100 minus [(cpm p883-pulsed APC + TCL-6Dn / cpm p883-pulsed APC) x 100].

CHAPTER 3. RESULTS

3.1. Determination of TCL-6Dn specificity

TCL-6D was previously shown to proliferate in response to 2.5 μg/ml of Her-2 p883 presented by DRβ1*0401$^+$ DCs, PBMCs and L cells (Kobayashi et al., 2000). To confirm the specificity of expanded TCL-6D, now called TCL-6Dn, and the optimal peptide concentration required for its activation, we performed proliferation assays using DRβ1*0401$^+$ and DRβ1*0401$^-$ APCs and p883 from 0.01 μg/ml to 10 μg/ml. As shown in Figure 3.1A, the highest proliferative responses of TCL-6Dn were attained with p883 at 1 μg/ml using DRβ1*0401$^+$ and *0402$^+$ L cells. Meanwhile, TCL-6Dn responded poorly to homozygous DR4$^+$ B-cell lines at all peptide concentrations. Indeed, the SI values were only slightly higher than those attained using DRβ1*1501$^+$ APCs, which were not expected to stimulate TCL-6Dn. The low SI values were not due to poor HLA-DR expression as all cell lines had abundant levels of HLA-DR (Fig 3.1B). Since these data suggested that the affinity and possibly the specificity of TCL-6Dn may be different from those of the original TCL-6D clone, we performed several experiments using DCs as APCs and phenotyped TCL-6Dn.

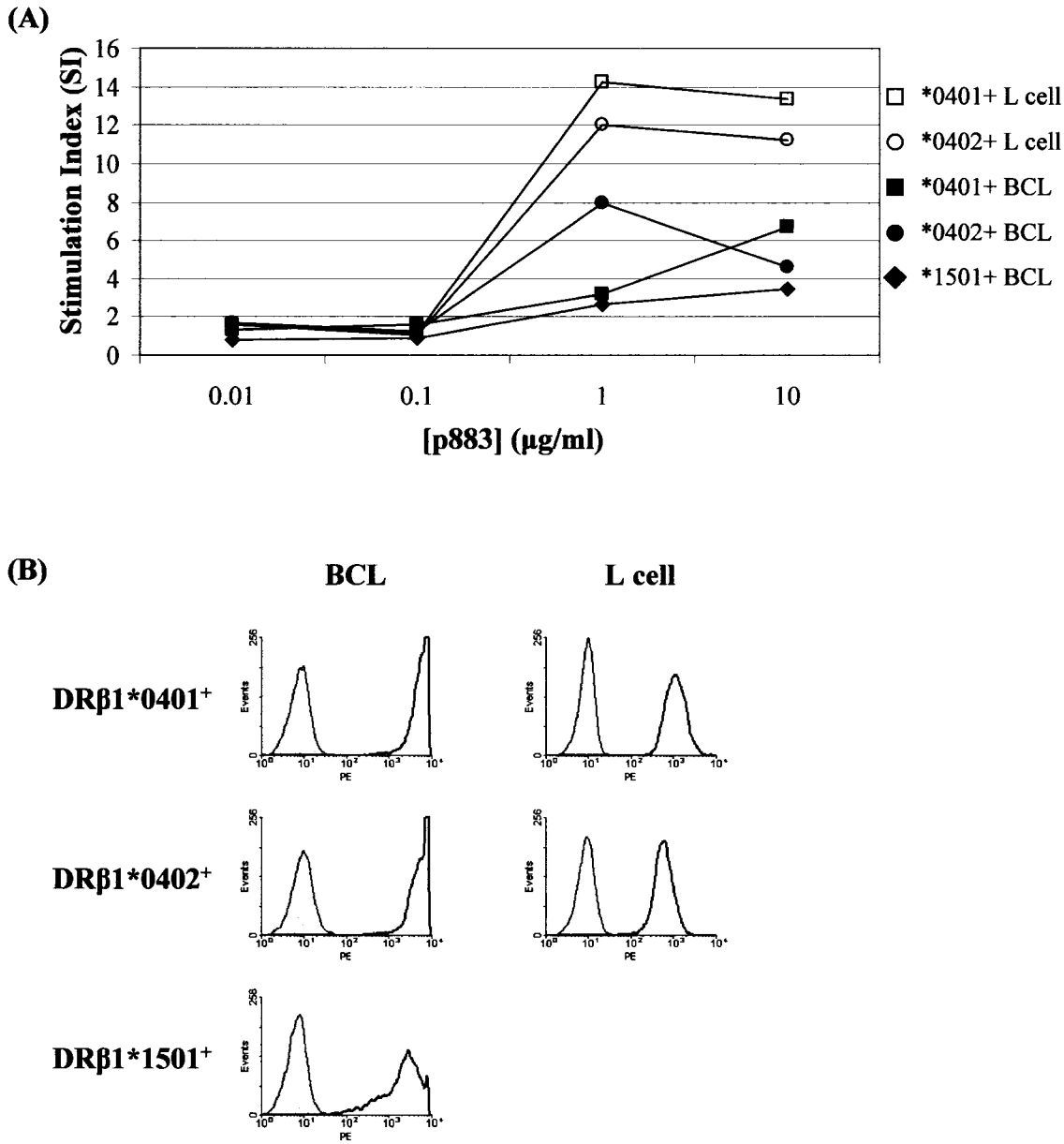


Figure 3.1. TCL-6Dn proliferation in response to APCs pulsed with various concentrations of p883. (A) Irradiated APCs were pulsed with concentrations of p883 from 0.01 μg/ml to 10 μg/ml and cultured with TCL-6Dn. Stimulation indexes were calculated based on the mean cpm of triplicate wells. (B) HLA-DR (mAb L243; open histogram) expression on BCLs and L cells was detected by flow cytofluorometry. The negative isotype (mIgG2a) control is gray. Data shown are representative of two experiments.

3.2. Phenotype of TCL-6Dn

Expression of cell surface markers on un-stimulated TCL-6Dn was detected by flow cytofluorometry. PBMCs were included as a positive control cell population. As shown in Figure 3.2, TCL-6Dn expressed CD2, CD3, CD4, CD56 and an α:β T-cell receptor (TCR). The presence of CD25, CD38, CD69, CD45RO and CD28 indicated an activated T cell phenotype. In addition, TCL-6Dn expressed the HLA class II molecules, HLA-DR, -DP, -DQ, and co-stimulatory molecules CD80 and CD86. Further assays performed in Dr. Drover's laboratory showed TCL-6Dn also expressed intracellular HLA-DM and Ii (data not shown). The absence of CD8, γ:δ TCR, CD16 and CD45RA expression on TCL-6Dn were not due to poor antibody binding as cell populations expressing these antigens were present in the PBMCs positive control.

Due to CD25 expression on TCL-6Dn, we also tested for FOXP3 and showed that TCL-6Dn expressed mRNA for FOXP3 (Figure 3.3). However, Yagi et al. (2004) have shown memory T cells in human PBMCs express low but detectable levels of FOXP3 mRNA, which can be up-regulated by anti-CD3 and anti-CD28 stimulation. Altogether, TCL-6Dn was considered to be an activated $CD4^+$ T cell clone.

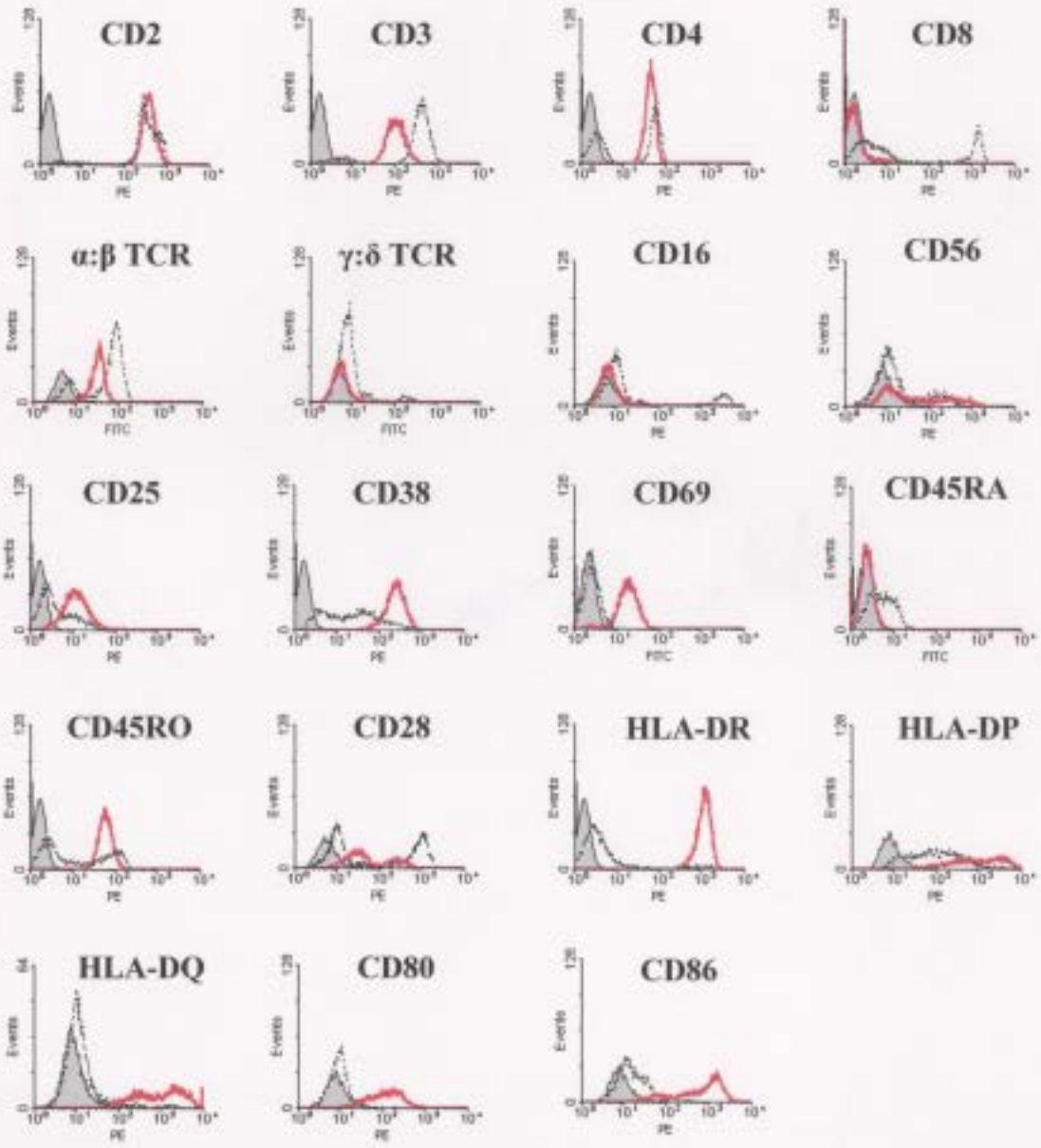


Figure 3.2. TCL-6Dn expressed an activated $CD4^+$ T cell phenotype. Cell surface markers expressed on TCL-6Dn (red line) and PBMCs (dotted-black line) were examined by flow cytofluorometry. Negative control (non-specific mIgG with TCL-6Dn) is shown in gray. Histograms show fluorescence values of gated viable cells. Data shown are representative of two experiments.

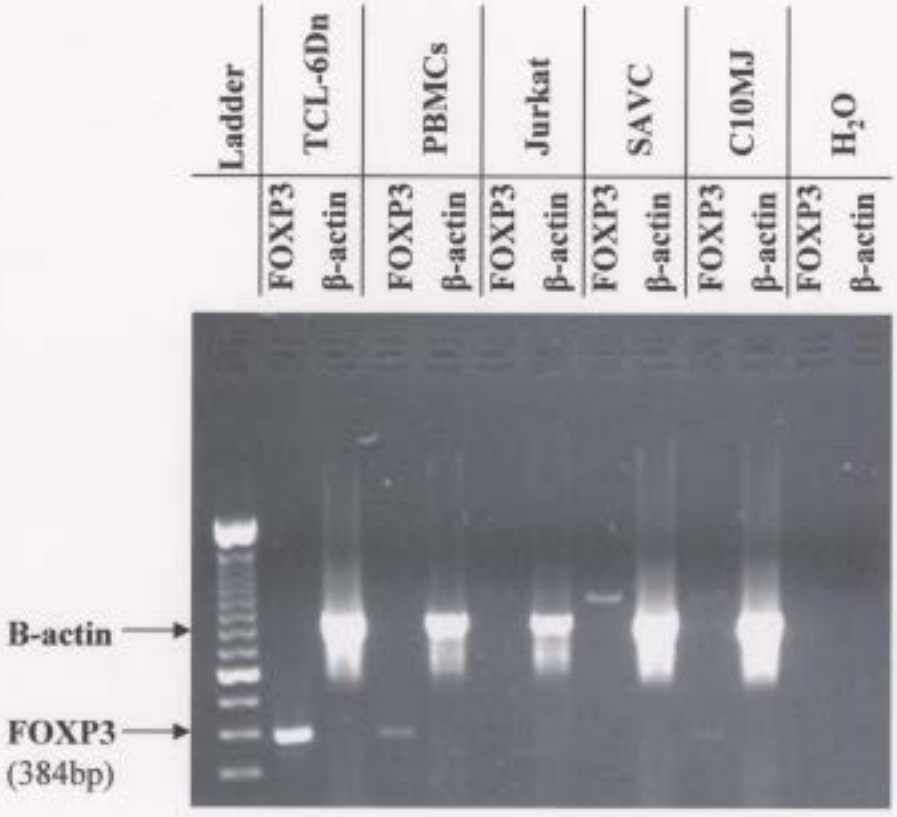


Figure 3.3. TCL-6Dn expressed FOXP3 mRNA. FOXP3 transcripts were detected by RT-PCR in cDNAs prepared from purified mRNAs from TCL-6Dn, PBMCs, Jurkat, SAVC and C10MJ as described in section 2.6. Sterile H_2O was used as a negative control and β-actin was included as a housekeeping gene.

3.3. HLA-DR Restriction and Peptide Specificity of TCL-6Dn

Although TCL-6D was previously shown to recognize p883 presented by DRβ1*0401 molecules (Kobayashi et al., 2000), our preliminary experiment (Figure 3.1) suggested TCL-6Dn had a more complex specificity. To clarify the HLA-DR restriction of TCL-6Dn, proliferation assays were performed using p883 and immature or mature DCs from DRβ1*0401^{+} and DRβ1*0401^{-} healthy donors. Using flow cytofluorometry, we confirmed that immature DCs expressed moderate levels of CD40 and HLA-DR and low levels of CD83 and CD86, while mature DCs expressed higher levels of CD40, CD83, CD86 and HLA-DR (Figure 3.4).

Surprisingly, TCL-6Dn recognized p883 presented by mature and immature DCs from both DRβ1*0401,07;DR53 and DRβ1*1501,1301; DR51,DR52 donors (Figure 3.5). Although TCL-6Dn proliferation was higher with p883-pulsed DRβ1*0401^{+} DCs, p883-pulsed DRβ1*0401^{-} DCs were not expected to stimulate TCL-6Dn. Since these donors did not share common HLA-DR molecules, the results suggested that TCL-6Dn displayed degenerate HLA-DR restriction. Alternatively, since TCL-6Dn expressed abundant HLA-DR (Figure 3.2) TCL-6Dn may act as an APC.

To further evaluate TCL-6Dn's specificity, we performed proliferation assays using mature DRβ1*0401^{+} DCs pulsed with p883 or an irrelevant peptide. As shown in Figure 3.6, TCL-6Dn did not respond to un-pulsed or irrelevant peptide-pulsed DCs, while moderate proliferation responses were attained with p883-pulsed DCs. However, due to lower background cpms, our calculated SI values were similar to those attained by Kobayashi et al. (2000).

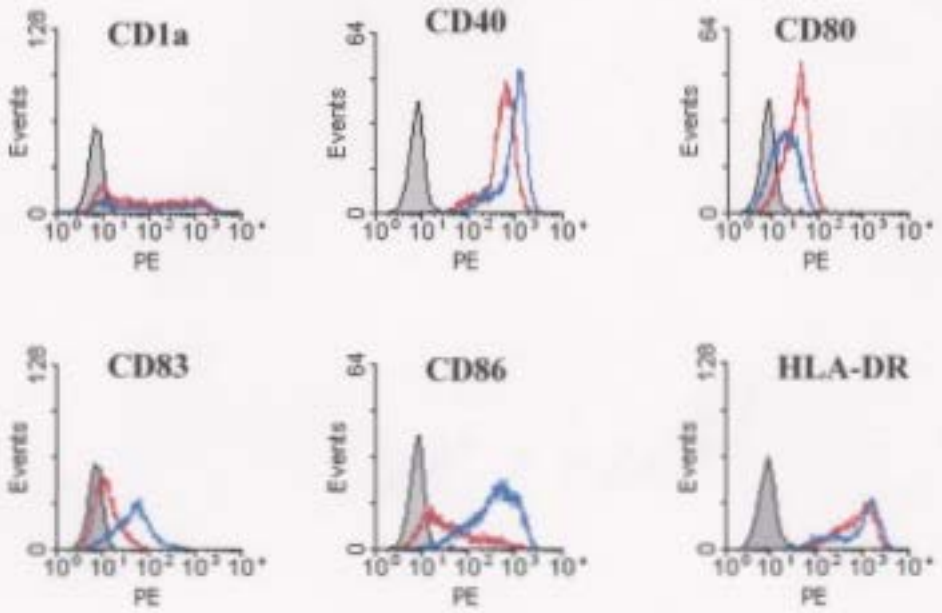


Figure 3.4. Phenotype of *in-vitro*-generated DCs. The phenotype of immature (red line) and mature (blue line) DCs (generated as described in section 2.4) were examined by flow cytofluorometry. Binding of unlabelled mAbs specific for CD1a, CD40, CD80, CD83, CD86 and HLA-DR were detected using PE-conjugated GAM. Negative controls, mIgG1 or mIgG2a, are shown in gray. Histograms show fluorescence values of gated viable cells.

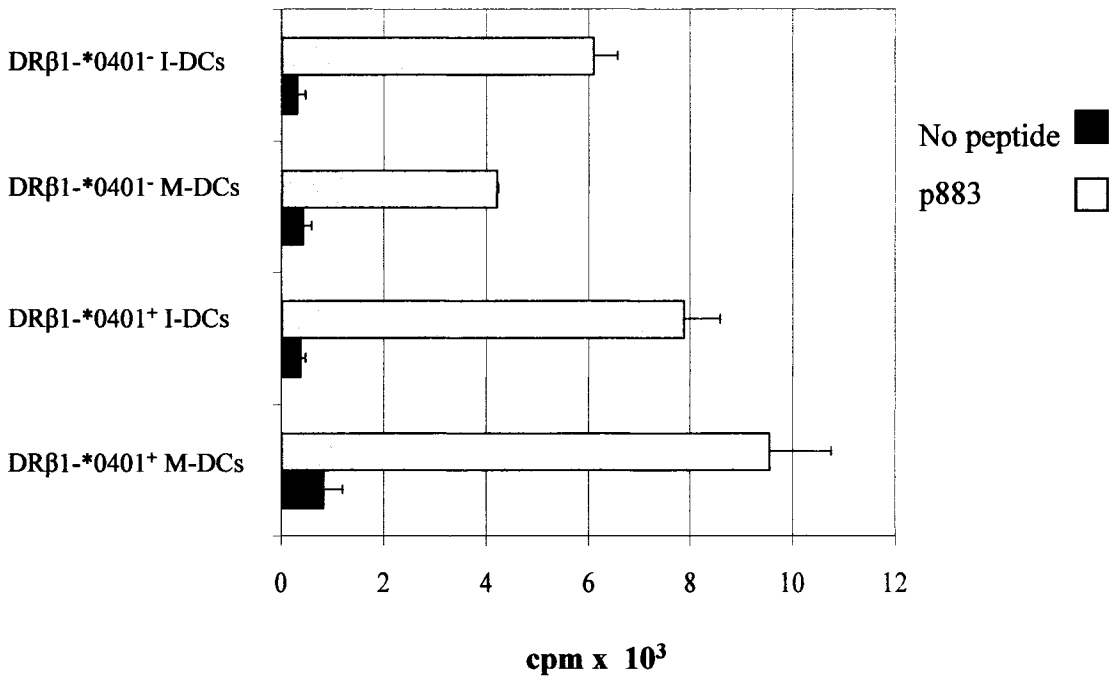


Figure 3.5. TCL-6Dn proliferated in response to p883 presented by DRβ1*0401$^+$ (DRβ1*0401,07;DR53) and DRβ1*0401$^-$ (DRβ1*1501,1301;DR51,DR52) DCs. Mature (M) and immature (I) DCs from healthy donors were mitomycin C treated, pulsed with p883 (1 μg/ml) and cultured with TCL-6Dn for 72 h. Proliferation of DCs and TCL-6Dn in the absence of p883 (black bars) or presence of p883 (gray bars) was measured as described in section 2.7.3. Each bar represents the mean and SD of triplicate wells.

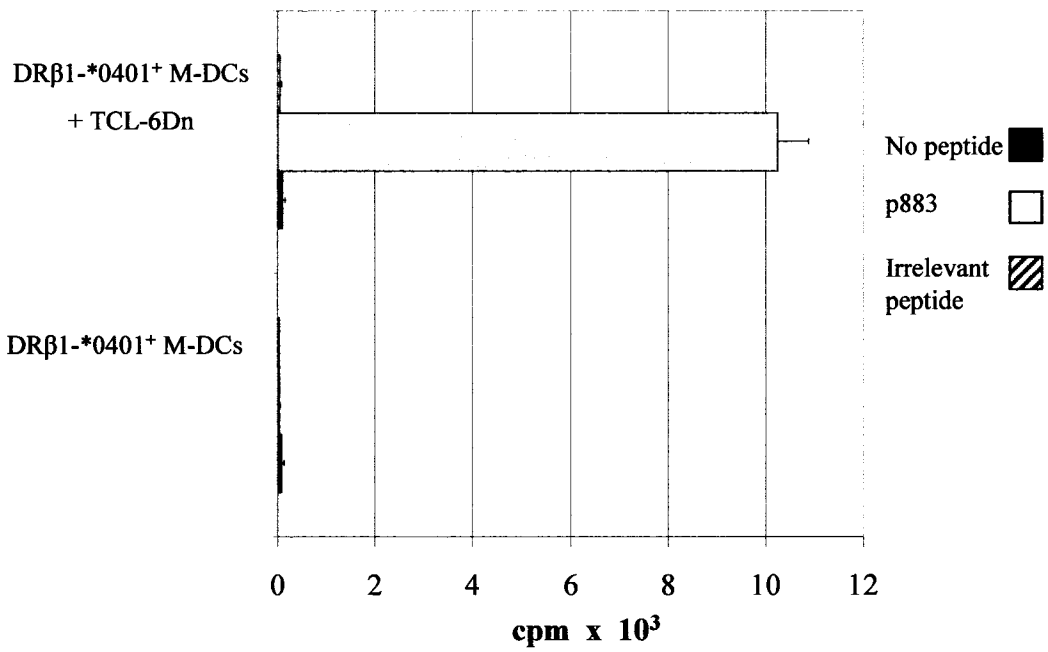


Figure 3.6. TCL-6Dn proliferation in response to DRβ1*0401$^+$ DCs was p883-specific. Mature DCs from a DRβ1*0401$^+$ healthy donor were irradiated, pulsed with p883 or an irrelevant peptide and co-cultured with TCL-6Dn for 72 h. Proliferation of TCL-6Dn in response to un-pulsed DCs (black bars) p883-pulsed DCs (1 μg/ml; gray bars) or irrelevant peptide-pulsed DCs (1 μg/ml; striped bars) was measured as described in section 2.7.3 Each bar represents the mean and SD of triplicate wells.

Meanwhile, since TCL-6Dn retained its specificity for p883, we attempted to determine if TCL-6Dn also recognized p883 presented by HLA-DR4$^+$ tumor cells.

3.4. Characterization of Human Tumor Cell Lines used as APCs

3.4.1. Her-2 Expression on Tumor Cell Lines

TCL-6Dn was tested for its ability to recognize tumor cell lines naturally expressing the Her-2 protein. For this purpose, Her-2$^+$ (T47D and HT29) and Her-2$^-$ (MDA MB 435) tumor cell lines were chosen. As shown in Figure 3.7, surface expression of Her-2 was undetectable on all cell lines tested. However, intracellular Her-2 was detected in HT29 and T47D whereas Her-2 was undetectable in MDA MB 435. These results were confirmed by immunocytochemistry (data not shown).

3.4.2. HLA-Class II Expression on Tumor Cell Lines

Generally, tumor cell lines do not constitutively express HLA-DR or express very low levels (see Table 2.1). Therefore, up-regulation of HLA-class II molecules was induced with rhIFN-γ. Un-transfected and DRβ1*0401-transfected human tumor cell lines, were tested for constitutive (NI) and induced (I) expression of HLA-DR, HLA-DR4, HLA-DM and invariant chain (Ii).

As shown in Table 3.1, HT29 and T47D did not constitutively express HLA-DR, while MDA MB 435 expressed low levels of DR molecules. HLA-DR was up-regulated on the three cell lines with IFN-γ treatment; as expected HLA-DR4 was also up-regulated on the DR4$^+$ lines, MDA MB 435 and HT29.

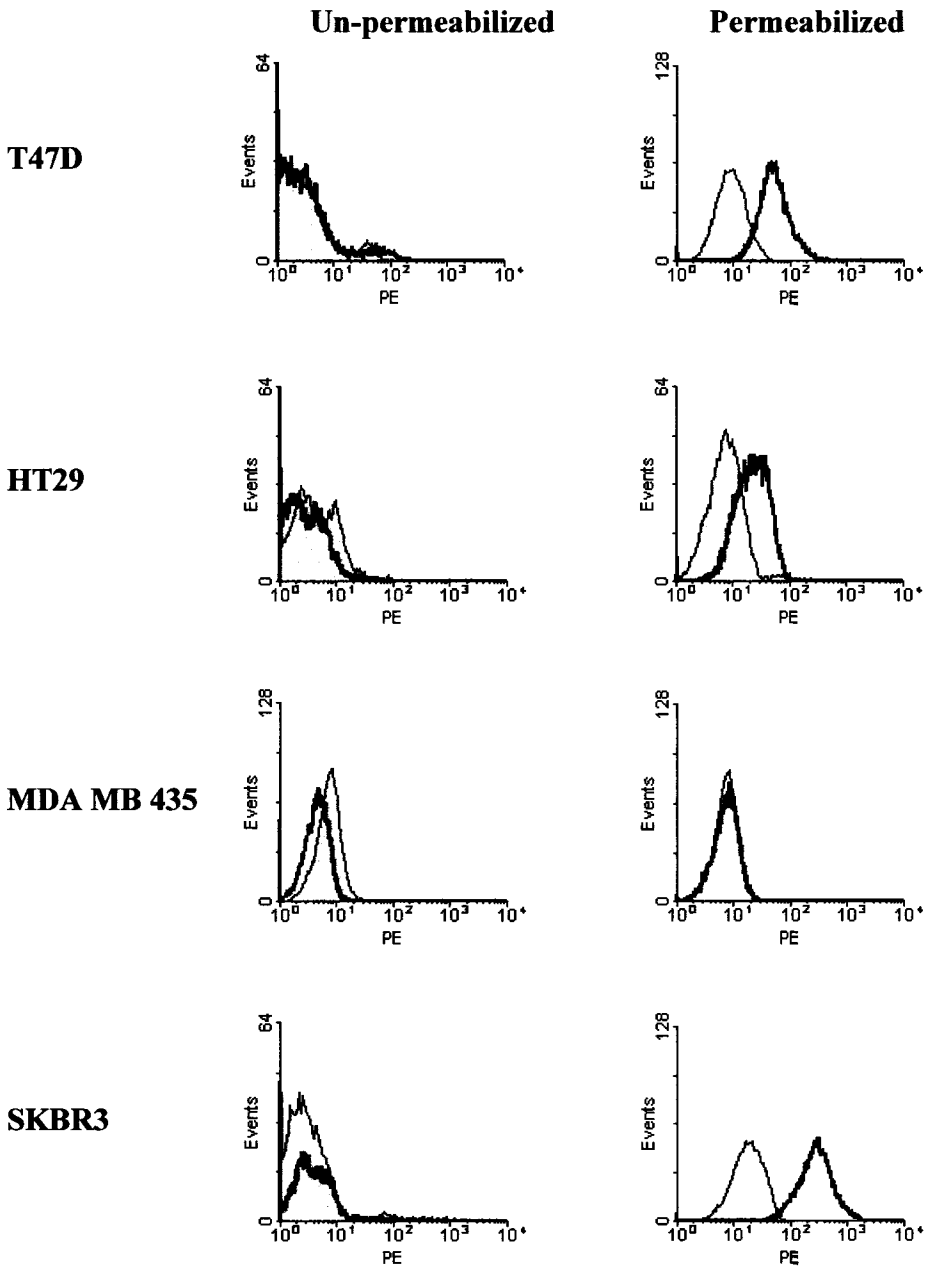


Figure 3.7. Intracellular and surface expression of Her-2 in tumor cell lines. Her-2 expression was detected by flow cytofluorometry in non-induced T47D, HT29 and MDA MB 435. Histograms show Her-2 expression (in black) and the negative control mIgG1 (in gray). SKBR3 is the positive control tumor cell line for Her-2 expression.

Table 3.1. Constitutive (NI) and IFN-γ induced (I) HLA-DR, HLA-DR4, HLA-DM and Ii expression on human tumor cell lines[d].

Cell Line[a]	HLA-DR[b]	HLA-DR4[b]	HLA-DM[b]	Invariant Chain[b]
HT29 (NI)	0	0	1.8	13
HT29 (I)	4,354	2,031	145	230
HT29-*0401 (NI)	1,283	472	0	34
HT29-*0401 (I)	4,614	915	77	288
T47D (NI)	9.4	0	0	41
T47D (I)	6,842	83	143	396
T47D-*0401 (NI)	2,425	1,771	0	17
T47D-*0401 (I)	5,298	2,142	66	251
MDA MB 435 (NI)	682	22	10	101
MDA MB 435 (I)	5,604	2,232	158	240
MDA MB 435-*0401 (NI)	5,707	4,936	20	64
MDA MB 435-*0401 (I)	6,696	8,061	174	180
SAVC[c]	2,945	2,160	114	309

[a] Flow cytofluorometric analysis was performed on un-transfected and HLA-DRβ1*0401 transfected tumor cell lines (T47D, HT29, MDA MB 435).

[b] The mean fluorescence intensity (MFI) of the negative control mAbs, non-specific IgG1 and IgG2a, were subtracted from the MFI of the antigen specific mAbs.

[c] Control human B cell line was SAVC.

[d] Data shown represents one of two experiments performed.

Results also confirmed the DRβ1*0401-transfected tumor cell lines (NI and I) expressed the DRβ1*0401 molecules.

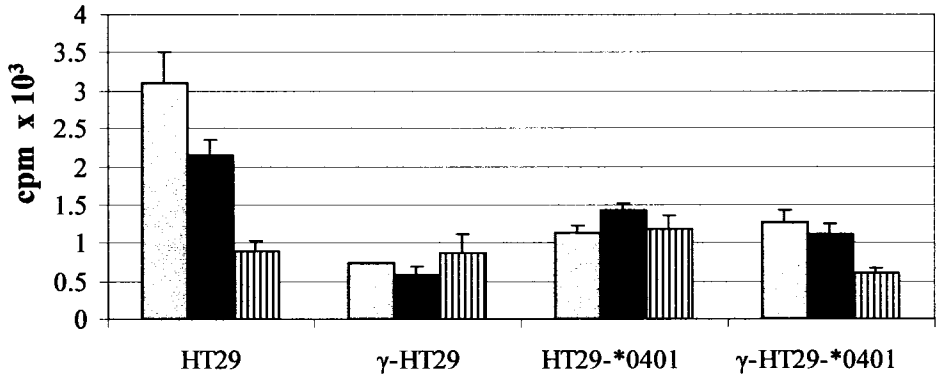
Constitutive and induced expression of class II co-chaperones, HLA-DM and Ii, were assessed by intracellular flow cytofluorometry. Low levels of Ii were expressed constitutively by all tumor cell lines (Table 3.1). However, constitutive Ii expression was higher in T47D and MDA MB 435 compared to HT29. IFN-γ treatment of all tumor cell lines resulted in up-regulation of this co-chaperone. HLA-DM was not constitutively expressed by HT29 and T47D, but MDA MB 435 expressed low levels of HLA-DM (Table 3.1). Meanwhile, HLA-DM expression was up-regulated by all IFN-γ-treated tumor cells.

3.5. Non-Proliferative Response of TCL-6Dn to p883 Presented by Tumor Cell Lines

TCL-6Dn was tested for its ability to recognize p883-pulsed or un-pulsed Her-2^+ and Her-2^- tumor cell lines. All tumor cells (NI and I) were irradiated and cultured with TCL-6Dn with or without exogenous p883. Although the tumor cell lines were exposed to 20,000 rads prior to co-culture with TCL-6D, we were unable to completely inhibit tumor cell proliferation. We tested the ability of mitomycin C and various doses of gamma irradiation (γ4200, γ8400, γ12600 and γ20000 rads) to inhibit tumor cell proliferation and the lowest proliferation was observed when tumor cell lines were exposed to 20,000 rads of gamma irradiation (data not shown). In addition, we also examined the effect of cell density at the time of irradiation whereby 5×10^5 or 1×10^6 tumor cells/tube were exposed to γ20,000 rads. We have not shown this data, but the cell density did not affect tumor cell irradiation.

We continued to examine whether TCL-6Dn proliferated in response to p883-pulsed or un-pulsed Her-2^+ and Her-2^- tumor cell lines. Our results in Figure 3.8, showed that TCL-6Dn failed to proliferate to p883 presented by DRβ1*0401^+ and DRβ1*0401^- tumor cells. Indeed, co-cultures of TCL-6Dn with p883-pulsed HLA-DR^- [HT29 (NI) and T47D (NI)] and HLA-DR^+ tumor cell lines [HT29-*0401 (I), T47D (I), T47D-*0401 (NI and I) and MDA MB 435-*0401 (NI and I)] had lower cpm values than tumor cells cultured alone. The lowest proliferation was observed when IFN-γ-treated DRβ1*0401-transfected tumor cells were cultured with TCL-6Dn, suggesting TCL-6Dn prevented tumor cell growth.

(A)


cpm x 10³
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3
2.5
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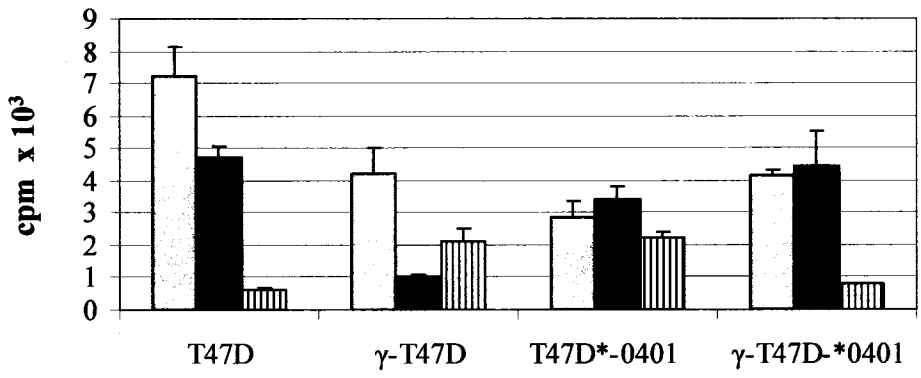
1
0.5
0
HT29
γ-HT29
HT29-*0401
γ-HT29-*0401


cpm x 10³
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T47D
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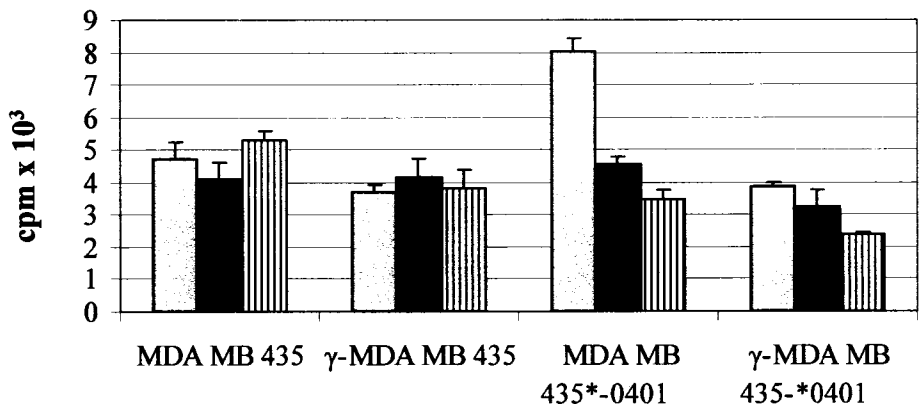
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MDA MB 435
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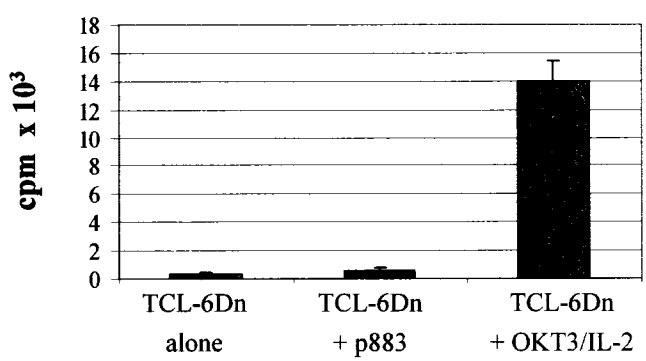
(B)


cpm x 10³
18
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14
12
10
8
6
4
2
0
TCL-6Dn alone
TCL-6Dn + p883
TCL-6Dn + OKT3/IL-2

Assay controls were included in all proliferation assays, showing that TCL-6Dn proliferated with anti-CD3 and IL-2 treatment, but did not proliferate with p883 alone. Consequently, these results may address why TCL-6Dn proliferated when DCs were used as APCs. Activated DCs generate IL-12, which may send a survival signal to TCL-6Dn, resulting in IL-2 production. However, our data showed that p883-loaded and PHA-stimulated TCL-6Dn did not generate IL-2 (data not shown) and were unable to stimulate TCL-6Dn to proliferate.

When p883-loaded tumor cell lines and TCL-6Dn were co-cultured together we know that TCL-6Dn failed to proliferate. Since both TCL-6Dn and tumor cells expressed HLA-DR, TCL-6Dn may recognize p883 presented by either the peptide-loaded tumor cells or peptide-loaded TCL-6Dn. Meanwhile, our data suggested that TCL-6Dn recognized the p883 and inhibited tumor cell growth or initiated tumor cell death. Tumor cells used in this study were resistant to irradiation and required at least 12600 rads to inhibit cell growth (data not shown). Even at 12600 rads, we still detected tumor cell proliferation. Consequently, we decided to test the affect of TCL-6Dn on tumor cell growth by co-culturing TCL-6Dn with non-irradiated tumor cell lines.

3.6. Inhibition of Tumor Cell Proliferation by TCL-6Dn

To confirm that TCL-6Dn inhibited tumor cell growth we performed proliferation assays using non-irradiated tumor cells as APCs. As shown in Figure 3.9, nearly all p883-pulsed tumor cells had significantly lower proliferation when cultured with TCL-6Dn. Unexpectedly, TCL-6Dn decreased the proliferation of p883-pulsed HLA-DR$^-$ cell lines HT29 and T47D, but inhibition of proliferation was most notable with HLA-DR$^+$ tumor cells. Indeed, DR$^+$ cell lines (non-DRβ1*0401) HT29 (I) and T47D (I) growth was inhibited by 95% and 52%, respectively, while MDA MB 453 (I) was slightly inhibited by 24%. All DRβ1*0401$^+$ cell lines (NI and I) were significantly inhibited by TCL-6Dn, though inhibition was higher in HT29-*0401 (I) and T47D-*0401 (I). In addition, except for MDA MB 435, the inhibition of DRβ*0401$^+$ tumor cells was more pronounced in IFN-γ induced cell lines compared to their non-induced counterparts, suggesting that IFN-γ may pre-condition the tumor cells to undergo cell death or inhibit cell growth.

Un-pulsed HLA-DR$^-$ tumor cell lines (HT29, T47D and MDA MB 435) were not affected by TCL-6Dn; showing that TCL-6D did not inhibit tumor cell growth non-specifically. Similarly, unpulsed DR$^+$ (*0401$^-$) tumor cells showed no significant reduction in growth when cultured with TCL-6Dn. Proliferation of un-pulsed, DRβ1*0401$^+$, non-induced tumor cells were slightly inhibited by TCL-6Dn. However, TCL-6Dn significantly inhibited the proliferation of un-pulsed DRβ1*0401$^+$, IFN-γ induced cell lines HT29 and MDA MB 435. These results suggested HT29 and MDA MB 435, but not T47D, processed endogenous Her-2 protein and presented the p883 epitope by DRβ1*0401 molecules to activate TCL-

6Dn. Inhibition of MDA MB 435 cell growth by TCL-6Dn was unexpected as this cell line was Her-2 negative.

All tumor cell lines proliferated strongly in the absence of TCL-6Dn. Meanwhile, trumor cells that were cultured with IFN-γ displayed reduced cell growth compared to the non-IFN-γ-induced tumor cells as determined by microscopic examination and cell counts (data not shown). This is likely due to the inhibitory effect of IFN-γ on tumor cell growth.

TCL-6Dn appeared to recognize p883, causing tumor cell lysis or inhibition of tumor cell growth. However, proliferation of tumor cells cultured with p883 did not differ compared to tumor cells cultured alone, indicating the peptide was not cytotoxic (data not shown). Thus far, our data has shown that TCL-6Dn proliferated strongly to p883 presented by DCs, but in the presence of p883-loaded APCs TCL-6Dn inhibited tumor cell proliferation.

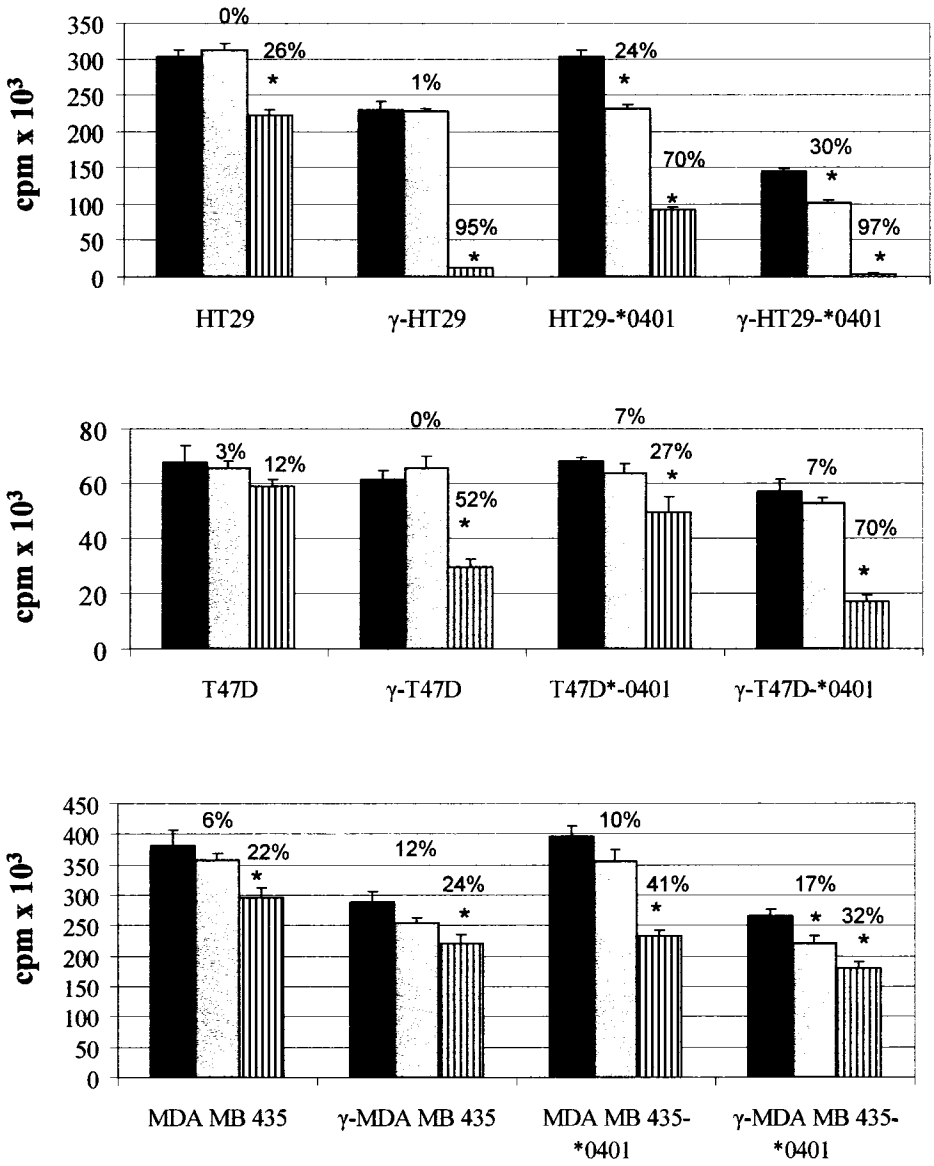


Figure 3.9. TCL-6Dn inhibits proliferation of non-irradiated, p883-pulsed tumor cells. Tumor cell lines were treated or not with IFN-γ and used as APCs. Proliferation of APCs alone (black bars), APCs and TCL-6Dn in the absence of p883 (gray bars) or presence of p883 (1 μg/ml; striped bars) was measured as described in section 2.7.6. Each bar represents the mean and standard deviation of triplicate wells. Statistical significance (*), ($p < 0.05$) was determined using the paired t-test. % inhibition is shown above each bar and calculated as described in section 2.7.6. Data shown represents one of two experiments performed.

3.6.1. Increasing the concentration of p883 augments the effect of TCL-6Dn on tumor cell growth

To determine the concentration of peptide required for TCL-6Dn to inhibit tumor cell proliferation, proliferation assays using non-irradiated tumor cells and p883 from 0.1 μg/ml to 5.0 μg/ml were performed. As shown in Figure 3.10, as the concentration of p883 increased, proliferation of tumor cells decreased when cultured with TCL-6Dn. However, greatest inhibition (97%) was observed when TCL-6Dn was cultured with HT29 (I) and HT29-*0401 (NI and I) and 0.5, 1.0 or 5.0 μg/ml of p883. TCL-6Dn also inhibited proliferation of p883-pulsed HLA-DR$^-$ HT29, indicating presentation of p883 to TCL-6Dn does not involve HLA-DR on the tumor cells. However, we cannot rule out the involvement of HLA-DR in antigen presentation of the p883 peptide as TCL-6Dn cells may be acting as APCs and presenting p883 to other TCL-6Dn cells in the context of HLA-DR molecules.

In summary, these results led us to ask whether TCL-6Dn lysed tumor cells and whether this was cytokine mediated or contact-dependent. Therefore, as a first step we examined the cytokines produced by activated TCL-6Dn.

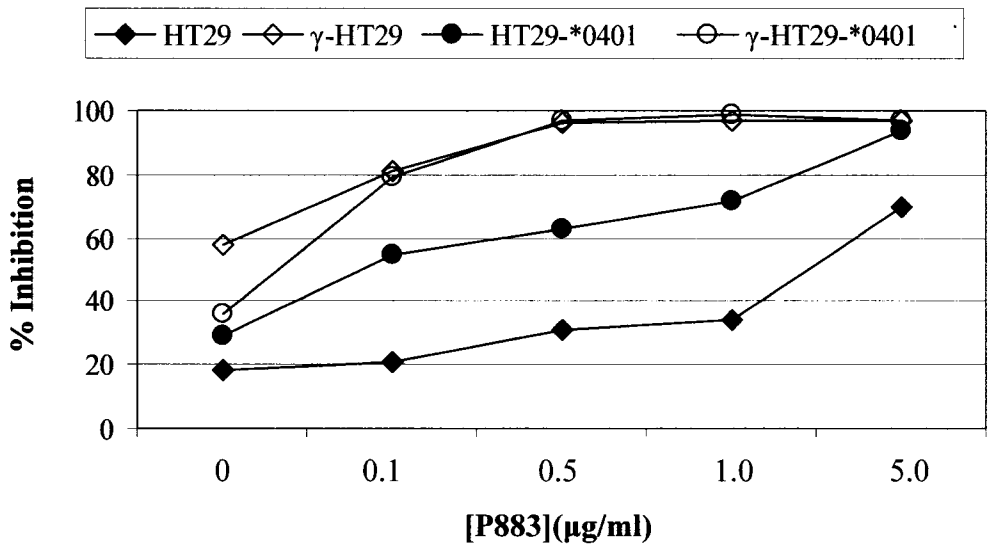


Figure 3.10. Increasing the concentration of p883 increases the inhibition of tumor cell growth by TCL-6Dn. Tumor cell lines (non-induced and IFN-γ-induced) were cultured alone or cultured with TCL-6Dn and [p883] from 0 µg/ml to 5 µg/ml. The % inhibition was calculated as described in section 2.7.6. Data shown are representative of two experiments.

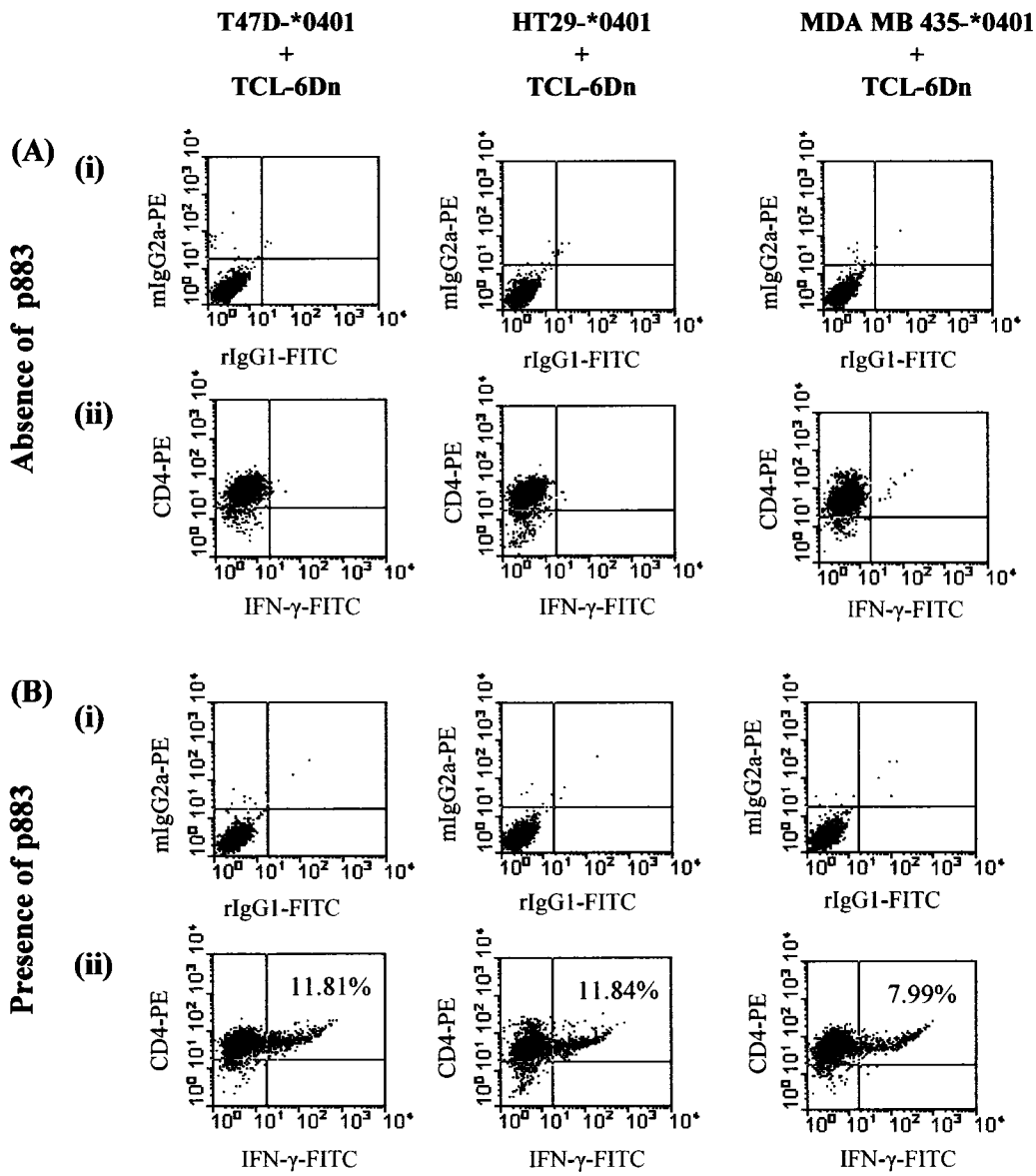
3.7. Cytokines Produced by Activated TCL-6Dn

Cytokines produced by TCL-6Dn were detected by intracellular flow cytofluorometry. For these assays TCL-6Dn was cultured with DRβ1*0401$^+$ tumor cells and p883 for 18 h in media containing brefeldin A (10 μg/ml; BFA). Thereafter, cytokine producing T cells were detected using PE-conjugated anti-CD4 and FITC-conjugated cytokine-specific mAbs. As a control, TCL-6Dn was also cultured with and without a T cell mitogen (PHA; 5 μg/ml).

3.7.1. TCL-6Dn Produces IFN-γ in Response to p883-pulsed Tumor Cells

To determine the cytokine profile, TCL-6Dn was cultured with p883-pulsed and un-pulsed tumor cell lines (T47D*0401, HT29*0401, and MDA MB 435*0401). Cytokine-producing T cells were detected using CD4- and IFN-γ-specific mAbs. As shown in Figure 3.11A, IFN-γ was undetected when TCL-6Dn was cultured with un-pulsed tumor cells, but TCL-6Dn responded to p883-pulsed tumor cells, resulting in IFN-γ production (Figure 3.11B). Although the % IFN-γ$^+$ T cells was low (8% to 12%) when cultured with p883-pulsed tumor cells, assay controls showed 48.8% of PHA-stimulated TCL-6Dn produced IFN-γ, but without stimulation TCL-6Dn did not produce IFN-γ (Figure 3.11C).

IFN-γ generated by TCL-6Dn in response to p883-pulsed HT29-*0401 and MDA MB 435-*0401 was tested once, but this assay was repeated with p883-pulsed T47D*0401 and similar results were obtained. In addition, this assay included TCL-6Dn cultured with and without p883 in the absence of tumor cells. p883-pulsed TCL-6Dn produced IFN-γ (data not shown), suggesting that TCL-6Dn presented p883 in the context of HLA-DR molecules resulting in activation of other TCL-6Dn cells.


T47D-*0401
+
TCL-6Dn
HT29-*0401
+
TCL-6Dn
MDA MB 435-*0401
+
TCL-6Dn
(A)
Absence of p883
(i)
(ii)
(B)
Presence of p883
mIgG2a-PE
rIgG1-FITC
CD4-PE
IFN-γ-FITC
11.81%
11.84%
7.99%

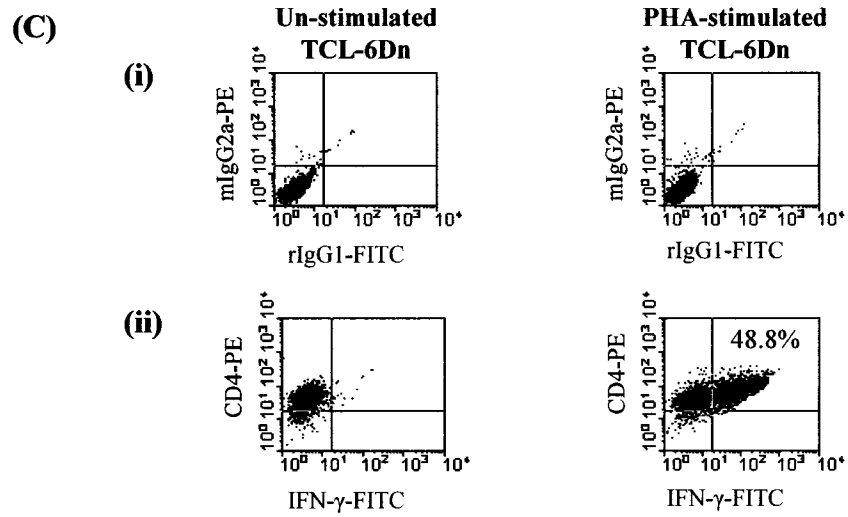


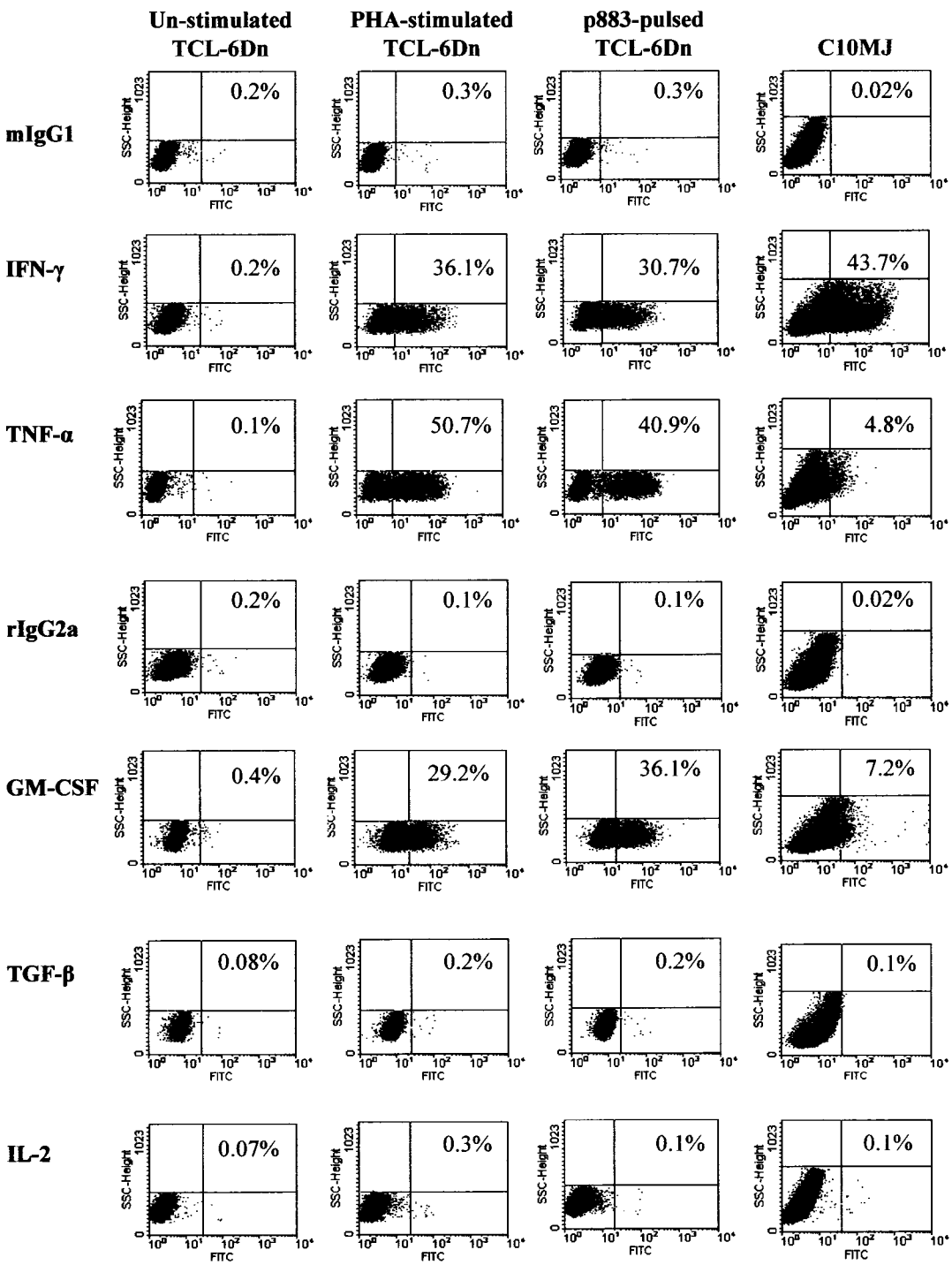
Figure 3.11. TCL-6Dn produced IFN-γ in response to p883-pulsed tumor cell lines. Un-pulsed (A) and p883-pulsed (B) tumor cells were cultured with TCL-6Dn in media containing BFA (10 μg/ml) for 18h. (C) Un-stimulated and PHA-stimulated (5 μg/ml) TCL-6Dn were used as controls. Cells were stained with (i) mIgG2a-PE & mIgG1-FITC isotype controls mAbs or (ii) CD4-PE & IFN-γ-FITC antigen-specific mAbs. 10,000 events were acquired. Dot plots show fluorescence of gated cells and percentage of $FITC^{+}PE^{+}$ cells. Quadrant locations were determined based on the appropriate negative controls.

3.7.2. Cytokines Produced by TCL-6Dn in Response to p883-Activation

Since TCL-6Dn expressed abundant levels of class II molecules and based on our previous data, we asked whether TCL-6Dn was auto-activated by p883 to produce cytokines capable of inhibiting tumor cell growth. TCL-6Dn was PHA-stimulated or p883-pulsed, in the absence of APCs, and tested for intracellular IFN-γ, IL-2, IL-4, IL-10, GM-CSF, TNF-α and TGF-β. As shown in Figure 3.12, un-stimulated TCL-6Dn did not produce cytokines, but PHA and p883 stimulated a high number of TCL-6Dn cells to produce IFN-γ, TNF-α and GM-CSF while a low number of TCL-6Dn cells produced IL-4. C10MJ was a positive control cell line in this assay and constitutively produced IFN-γ, TNF-α and GM-CSF. Although a positive control for IL-2, IL-10 and TGF-β was not shown, prior to this assay these cytokines were detected in PHA-stimulated PBMCs, proving the mAbs were capable of binding to the specific cytokines.

To ensure that activation of TCL-6Dn and production of cytokines was p883 specific, we examined cytokines produced by TCL-6Dn when cultured with an irrelevant HIV peptide. As shown in Figure 3.13, TCL-6Dn did not respond to the irrelevant peptide, but was stimulated by p883. These results showed TCL-6Dn activation was p883-specific and may involve auto-activation with p883 loaded onto HLA class II molecules on TCL-6Dn.

In summary, TCL-6Dn proliferated in response to p883-pulsed DCs, but not p883 alone or p883-pulsed tumor cells. Possibly IL-12 generated by the DCs, but not other APCs, may stimulate activated T cells to produce IL-2 and proliferate. Meanwhile, p883-pulsed TCL-6Dn produces Th1 cytokines, IFN-γ, TNF-α and GM-CSF, which may inbibit tumor cell growth or causes tumor cell lysis.


Un-stimulated TCL-6Dn
PHA-stimulated TCL-6Dn
p883-pulsed TCL-6Dn
C10MJ
mIgG1
IFN-γ
TNF-α
rIgG2a
GM-CSF
TGF-β
IL-2
0.2%
0.3%
0.3%
0.02%
0.2%
36.1%
30.7%
43.7%
0.1%
50.7%
40.9%
4.8%
0.2%
0.1%
0.1%
0.02%
0.4%
29.2%
36.1%
7.2%
0.08%
0.2%
0.2%
0.1%
0.07%
0.3%
0.1%
0.1%
SSC-Height
FITC

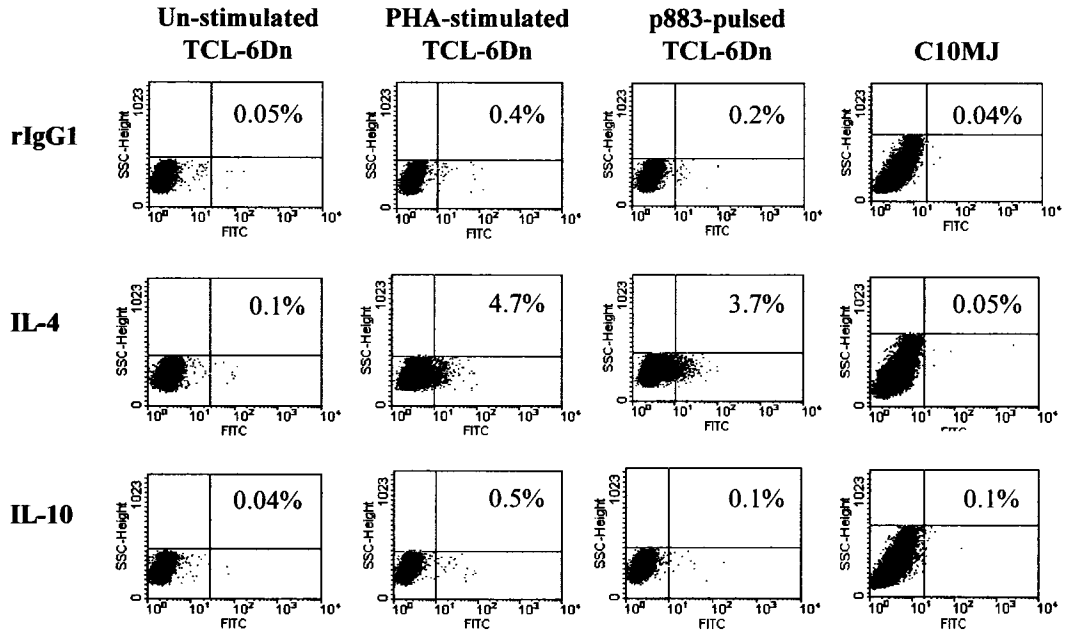


Figure 3.12. p883 activated TCL-6Dn to produce IFN-γ, TNF-α, GM-CSF and IL-4. TCL-6Dn was cultured in media containing BFA (10 μg/ml) and either un-stimulated, PHA-stimulated (5 μg/ml) or p883-pulsed (1 μg/ml). C10MJ was used as a positive control in this assay. T cells were tested for intracellular cytokines following 18h culture using FITC-conjugated cytokine-specific mAbs. 20,000 events were acquired and values show the % of FITC positive cells in the gated cell population. Quadrant locations are determined based on the appropriate negative control shown above each cytokine-specific mAb.

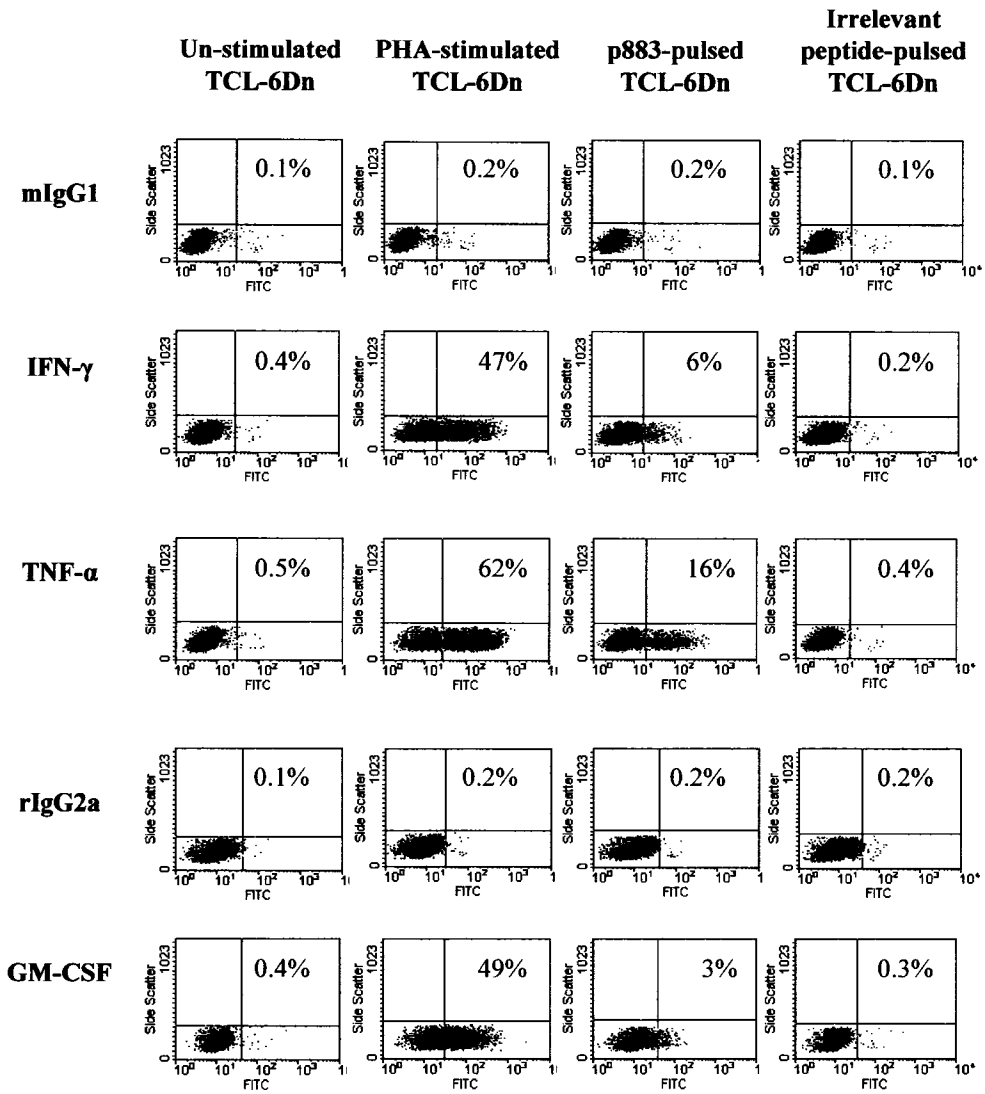


Figure 3.13. TCL-6Dn was not stimulated by an irrelevant peptide to produce cytokines. TCL-6Dn was cultured in media containing BFA (10 μg/ml) and either un-stimulated, PHA stimulated (5 μg/ml), p883 pulsed (1 μg/ml) or irrelevant peptide pulsed (1 μg/ml). T cells were tested for intracellular IFN-γ, TNF-α and GM-CSF following 18h culture using FITC-conjugated cytokine-specific mAbs. 15,000 events were acquired and values show the percent of FITC positive cells in the gated cell population. Data shown are representative of two experiments. Quadrant locations are determined based on the appropriate negative control.

3.8. Antibody Blocking Studies to Determine the Involvement of Class II Molecules in TCL-6Dn Activation

Kobayashi et al (2000) showed TCL-6D recognized p883 presented by HLA-DRβ1*0401 molecules, whereas our data suggested that TCL-6Dn displayed promiscuous HLA-DR restriction. Potentially, TCL-6Dn may recognize p883 presented by HLA-DR, -DP or -DQ molecules. Therefore, various assays were performed using blocking antibodies specific for HLA class I and class II molecules to determine how TCL-6Dn recognized p883. These assays were performed using 10 μg/ml of mAbs, as performed by Kobayashi et al (2000).

3.8.1. TCL-6Dn Proliferation in Response to p883-pulsed PBMCs was not inhibited by HLA maAbs

To determine the involvement of HLA-DR in TCL-6Dn activation, we performed proliferation assays using p883 and DRβ1*0401$^+$ PBMCs treated with mAbs specific for HLA class I, HLA-DP, -DQ, -DR, -DR4 and -DRβ1*0401. Non-specific mIgG1 and mIgG2a were used as isotype control mAbs.

As shown in Figure 3.14, in the absence of a mAb, TCL-6Dn responded strongly to p883-pulsed DRβ1*0401$^+$ PBMCs. Meanwhile, only M67 (anti-HLA-DP mAb) decreased TCL-6Dn proliferation compared to the mIgG1 control mAb, but this was not significant. In contrast, L243 (anti-HLA-DR mAb) increased TCL-6Dn proliferation compared to the mIgG2a isotype control. Although a clear conclusion could not be drawn from these results because both the mIgG1 and mIgG2a control

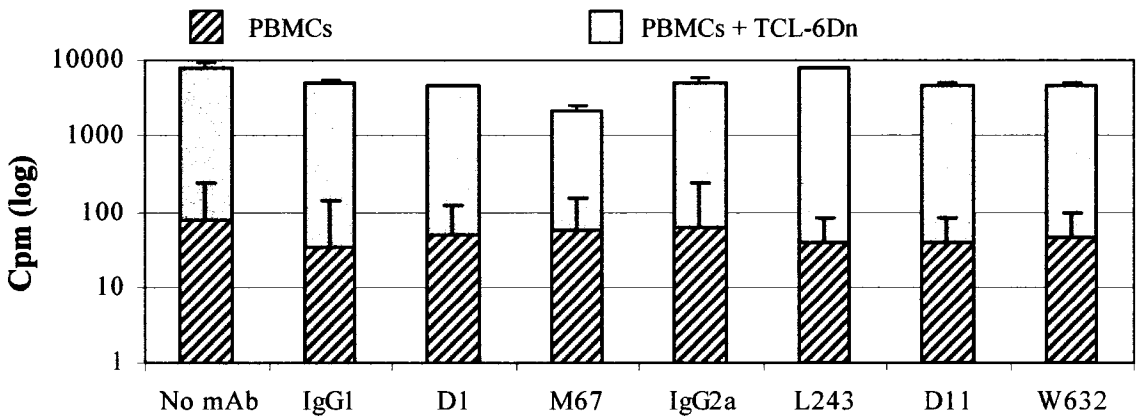


Figure 3.14. Inhibition of TCL-6Dn proliferation in response to p883-pulsed DRβ1*0401^{+} PBMCs. P883-pulsed PBMCs were cultured without (striped bars) or with TCL-6Dn (gray bars) for 72 h. Prior to peptide pulsing PBMCs were incubated with various mAbs at 10 μg/ml including, non-specific mIgG1, anti-HLA-DR4 (NFLD.D1), anti-HLA-DP (M67), non-specific mIgG2a, anti-HLA-DR (L243), anti-HLA-DRβ1*0401 (NFLD.D11) or anti-HLA-Class I (W632) for 30 min. Each bar represents the mean and standard deviation of triplicate wells. Data shown are representative of two experiments.

mAbs decreased TCL-6Dn proliferation, there is no evidence that the HLA class II mAbs significantly modulated TCL-6Dn proliferation.

3.8.2. TCL-6Dn-mediated inhibition of tumor cell proliferation was not blocked by anti-HLA-DR mAb

To determine the involvement of HLA-DR in the inhibition of tumor cell proliferation, we cultured TCL-6Dn with p883 and HT29-*0401 treated with anti-HLA-DR (L243), mIgG2a or without blocking mAb. As shown in Figure 3.15, TCL-6Dn inhibited proliferation of un-pulsed and p883-pulsed HT29-*0401. Treatment of these tumor cells with either mIgG2a or L243 enhanced the response of TCL-6Dn. Indeed, up to 94% of p883-pulsed tumor cell proliferation was inhibited when tumor cells were treated with L243. In the absence of TCL-6Dn, addition of mAbs, particularly L243, decreased proliferation of p883-pulsed HT29-*0401, but increased proliferation of un-pulsed HT29-*0401. A plausible explanation is the anti-HLA-DR mAb may bind to the p883:HLA-DRβ1*0401 complexes and stimulate the production of TNF-α or up-regulate death-inducing molecules on the tumor cell lines. An alternative explanation is that the blocking mAbs may have bound to the Fc receptors on TCL-6Dn, resulting in T cell activation.

Altogether, we were unable to block the interaction between TCL-6Dn and tumor cells with L243. Thus, TCL-6Dn may recognize p883 presented by tumor cells in an HLA-DR-independent manner. However, we are uncertain whether L243 completely blocked the T cell and tumor cell interaction, since the mAb would have also bound to HLA-DR molecules on TCL-6Dn.

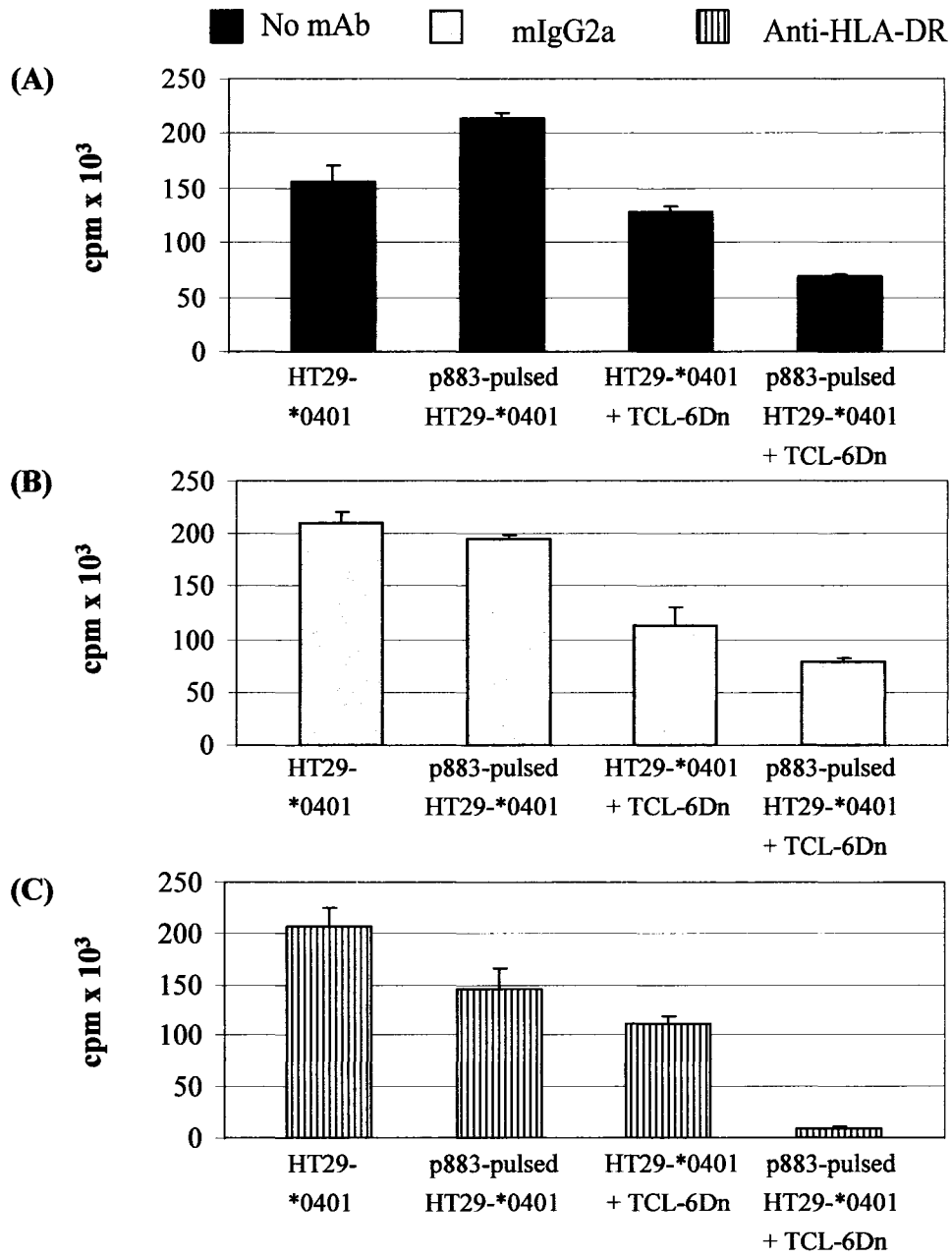


Figure 3.15. Treatment of tumor cells with anti-HLA-DR mAb did not affect the ability of TCL-6Dn to inhibit tumor cell proliferation. Non-irradiated HT29-*0401 was un-pulsed or p883-pulsed (1 μg/ml) and cultured with or without TCL-6Dn for 72h. Prior to peptide pulsing or co-culture with TCL-6Dn, tumor cells were incubated (A) without mAb present, (B) with mIgG2a (azide free; 10 μg/ml) or (C) with anti-HLA-DR (azide free L243; 10 μg/ml) for 30 min. Values above each bar represent the % inhibition calculated was calculated as 100 minus [(cpm APC + TCL-6Dn / cpm APC) x 100] or 100 minus [(cpm p883-pulsed APC + TCL-6Dn / cpm p883-pulsed APC) x 100]. Data shown are representative of two experiments.

3.8.3. Treatment of TCL-6Dn with anti-HLA-DR mAb prior to p883-pulsing did not decrease cytokine production.

To determine whether HLA-DR is involved in p883-activation of TCL-6Dn, T cells were treated with anti-HLA-DR mAb (azide free; L243) or mIgG2a isotype control (azide free) for 30 min prior to adding p883. Blocking mAbs were left in throughout the culture period. Thereafter, intracellular IFN-γ, GM-CSF and TNF-α generated by TCL-6Dn were detected by flow cytofluorometry.

TCL-6Dn alone did not produce cytokines, but p883 stimulated TCL-6Dn to produce IFN-γ, TNF-α and GM-CSF (Figure 3.16). TCL-6Dn cultured with mIgG2a and p883 resulted in slightly higher levels of IFN-γ compared to TCL-6Dn cultured with p883 alone. However, the percentage of IFN-γ, TNF-α and GM-CSF producing cells increased dramatically when TCL-6Dn was cultured with p883 and anti-HLA-DR. Additional assays showed that mAbs in the absence of p883 did not activate TCL-6Dn to produce cytokines (data not shown). The increased cytokine production in the presence of peptide and blocking mAbs may have occurred due to antibody binding via the Fc receptors, activating TCL-6Dn. Alternatively, anti-HLA-DR mAb may bind to DR molecules on TCL-6Dn causing activation and increased cytokine production. Altogether, these data show that p883 activated TCL-6Dn, yet the mechanism of anti-HLA-DR in the activation of TCL-6Dn remained unclear.

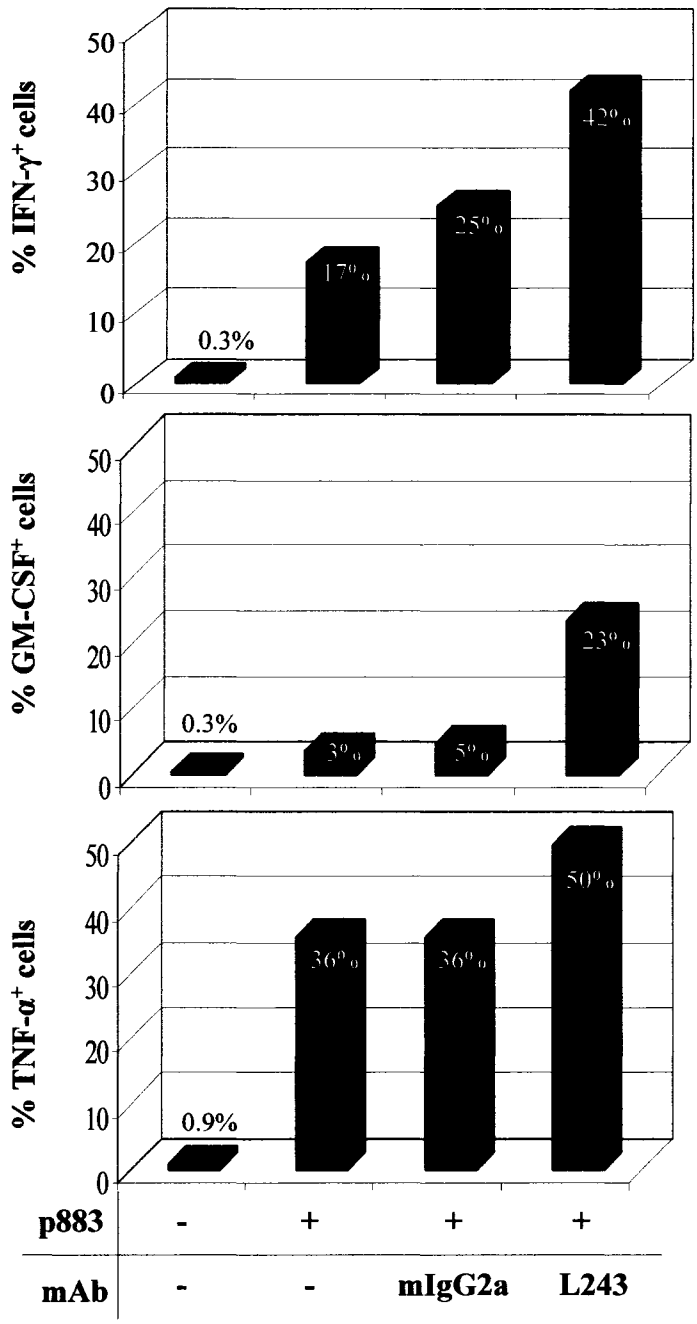


Figure 3.16. Treatment of TCL-6Dn with anti-HLA-DR mAb prior to p883-pulsing did not decrease cytokine production. TCL-6Dn was treated with purified mIgG2a mAb (10 μg/ml) or anti-HLA-DR mAb (L243; 10 μg/ml) for 30 min. Thereafter, TCL-6Dn was cultured with or without p883 (1 μg/ml). TCL-6Dn was cultured in media containing BFA (10 μg/ml) for 18h after which intracellular IFN-γ, GM-CSF and TNF-α were analyzed by flow cytofluorometry. Values show the percent of FITC positive cells in the gated viable cell population. Data shown are representative of two experiments.

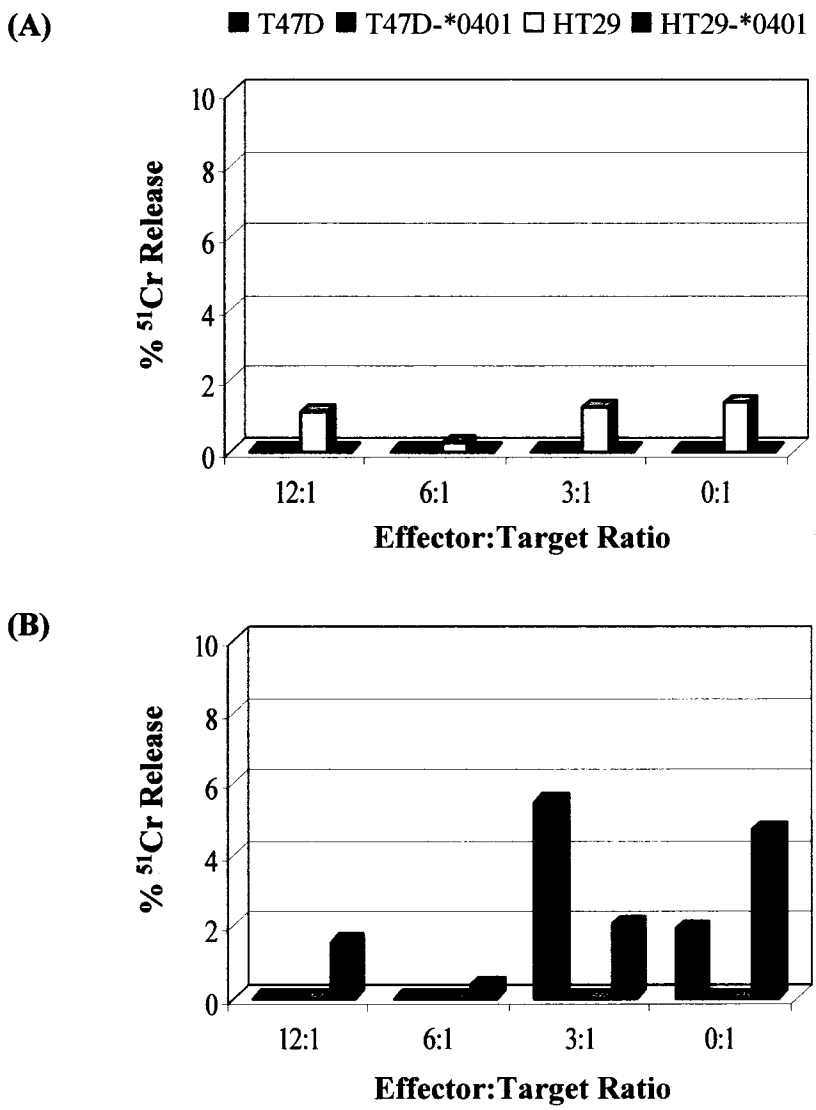
3.9. Detection of Tumor Cell Lysis

Thus far, our data had shown TCL-6Dn inhibited proliferation of p883-pulsed tumor cells, but did not distinguish inhibition of tumor cell growth from tumor cell lysis. Therefore, ^{51}chromium release and JAM assays were performed to clarify whether TCL-6Dn lysed p883-pulsed tumor cells.

3.9.1. Detection of Tumor Cell Lysis by ^{51}Chromium Release Assays

HLA-DR*0401^{+} tumor cell lines were labelled with ^{51}Cr, p883-pulsed and cultured with TCL-6Dn at effector to target ratios of 12:1, 6:1 and 3:1, which are similar to tumor cell and T cell ratios used in proliferation assays. The amount of ^{51}Cr-released from target cells was measured after 5 h co-culture. As shown in Figure 3.17, tumor cell lysis by TCL-6Dn was not above 2%. Additional assays were performed using higher effector:target ratios (50:1, 25:1 and 12.5:1) and lysis of p883-pulsed tumor cells by TCL-6Dn remained below 2% (data not shown).

From these results we know that TCL-6Dn did not lyse p883-pulsed tumor cells within a 5 h co-culture period. We cannot conclude that TCL-6Dn was unable to lyse p883-pulsed tumor targets altogether, as TCL-6Dn may use a cytotoxic mechanism that requires a longer co-culture period. However, increasing the culture period for a ^{51}Cr-release assay can cause inaccurate results due to increased background values or spontaneous ^{51}Cr-release. Consequently, we also examined tumor cell lysis using the JAM assay, which allowed a longer co-culture period.



3.17. Detection of tumor cell lysis caused by TCL-6Dn using ^{51}chromium release assays. ^{51}Cr-labelled tumor cell targets were (A) un-pulsed or (B) p883-pulsed (1 μg/ml) and co-cultured with TCL-6Dn for 5 h. The % ^{51}Cr released from target cells was calculated as described in section 2.7.5.1.

3.9.2. Detection of Tumor cell Lysis by JAM Assays

Tumor cell lysis was also measured using the JAM assay (Matzinger, 1991), which is based on DNA-fragmentation of the target cells. The fragmented DNA is washed through the glass fiber filter during cell harvesting using vacuum aspiration, while intact DNA in viable cells is trapped in the filter and reflects the amount of viable cells remaining. Tumor cells were labelled with ^{3}H-thymidine for 18h, and initial amounts of ^{3}H-thymidine incorporated into tumor cells was measured (see appendix 1). Thereafter, ^{3}H-thymidine-labelled tumor cells were pulsed with p883 and cultured with TCL-6Dn. Radioactivity remaining in the viable tumor cells was measured and used to calculate the percentage of tumor cell lysis.

3.9.2.1. Lysis of Tumor Cells at 24, 48 and 72h

Using the JAM assay we examined whether TCL-6Dn lysed p883-pulsed tumor cells and the approximate time required for lysis to occur. Therefore, TCL-6Dn was cultured with un-pulsed and p883-pulsed ^{3}H-labelled tumor cell lines and the percent tumor cell lysis was calculated at 24, 48 and 72 h. As shown in Figure 3.18, TCL-6Dn did not lyse p883-pulsed T47D at 24 or 48h, while lysis increased to 31% at 72 h. Since T47D is an HLA-DR^{-} tumor cell line, the increased lysis at 72h may be due to bystander effect, as p883 activates TCL-6Dn to produce IFN-γ, TNF-α and GM-CSF (see *Section 3.7.2*).

Lysis of p883-pulsed T47D (I) and T47D-*0401 (I) occurred as early as 24 h, resulting in 55% and 72% tumor cell lysis, respectively. At 48 h the % lysis of these tumor cell lines increased, while after 72 h co-culture the % lysis remained constant. In the absence of p883, lysis of T47D (NI) and T47D-*0401 (NI) was less than 20%

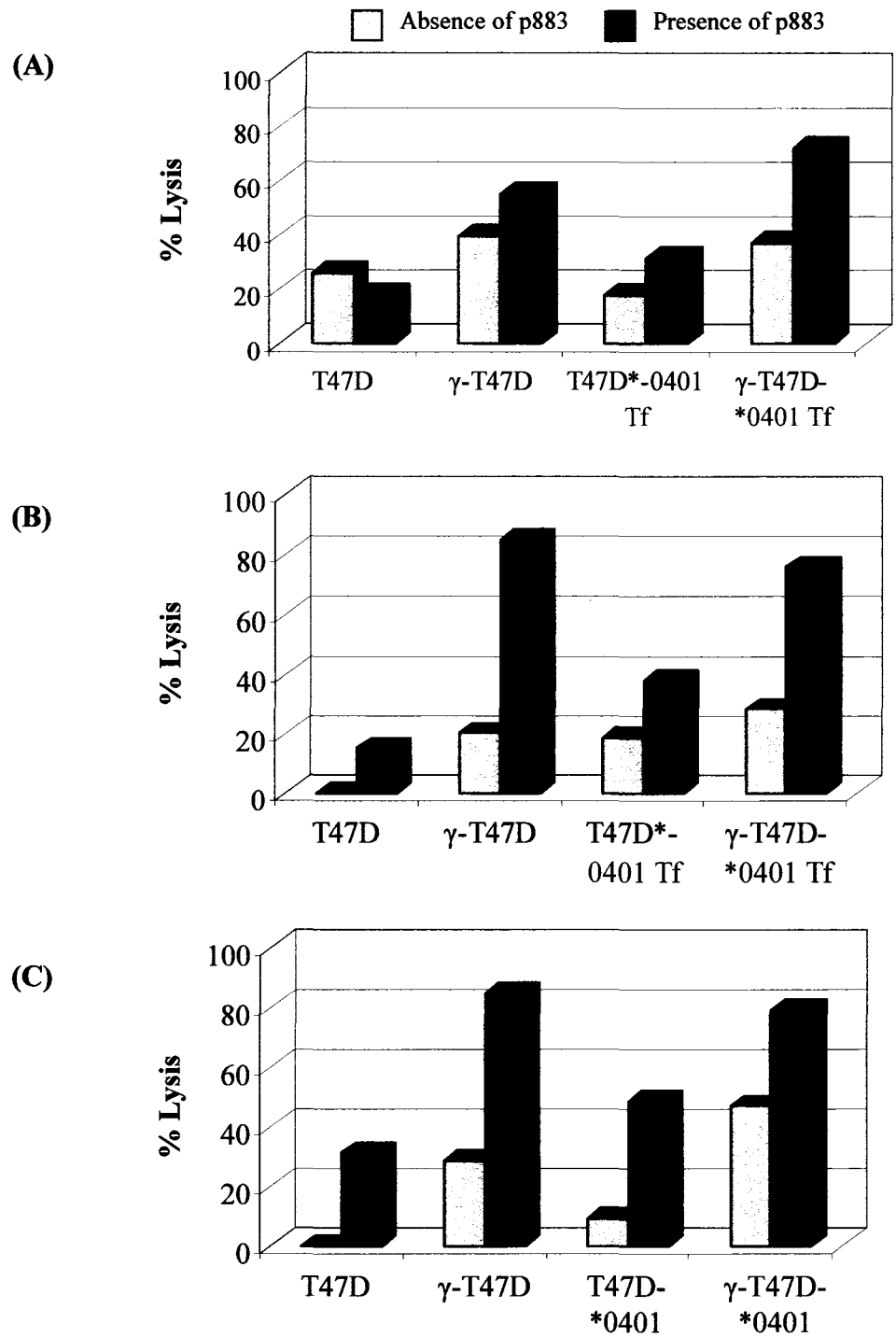


Figure 3.18. Detection of tumor cell lysis caused by TCL-6Dn at (A) 24h, (B) 48h and (C) 72h using JAM assays. Tumor cells were treated or not with IFN-γ and labelled with ^{3}H-thymidine. See Table 6.1 in Appendix 1 for the amount of ^{3}H-thymidine-incorporated into each cell line after 18 h. ^{3}H-thymidine labelled tumor cells were cultured with TCL-6Dn in the absence (gray bars) or presence (black bars) of p883 (1 μg/ml). Percent tumor cell lysis was calculated as described in section 2.7.5.2.

at 24 h, 48 h and 72 h. Lysis of un-pulsed T47D (I) and T47D-*0401 (I) increased up to 29% and 47% at 72 h, respectively. Notably, lysis of all IFN-γ-induced tumor cell lines was higher compared to their non-induced counterparts. One plausible explanation is that IFN-γ up-regulates molecules on the tumor cells, such as TNF receptors, making them more susceptible to lysis by TCL-6Dn. Alternatively, IFN-γ treatment may pre-condition the tumor cells to undergo cell death.

This time-course assay revealed that TCL-6Dn lysed p883-pulsed tumor cells after 24 h co-culture, which significantly increased at 48h, but remained stable at 72 h co-culture. Thus, the % tumor cell lysis was measured at 48 h for further JAM assays.

3.9.2.2. Her-2 Specific Lysis of Tumor Cells

To determine if TCL-6Dn lysed tumor cells in a p883-specific manner, tumor cells were labelled with ^{3}H-thymidine, pulsed with p883 or an irrelevent peptide and cultured with TCL-6Dn. The percentage of tumor cell lysis was measured after 48 h co-culture. As shown in Figure 3.19, TCL-6D lysed p883-pulsed, but not un-pulsed or irrelevant peptide-pulsed T47D and T47D-*0401 (NI and I).

Similarly, TCL-6D lysed p883-pulsed HT29 (I) and HT29-*0401 (NI and I). Lysis of un-pulsed or irrelevant peptide-pulsed tumor cells may be due to recognition of the naturally processed p883 epitope, as IFN-γ treatment of Her-2^{+} HT29 may induce natural processing and presentation of endogenous Her-2.

MDA MB 435 and MDA MB 435-*0401 (NI and I) in the absence of peptide, presence of p883 or an irrelevant peptide was not lysed by TCL-6D. The percentage of tumor cell lysis was not higher than 20%, except when TCL-6D was cultured with

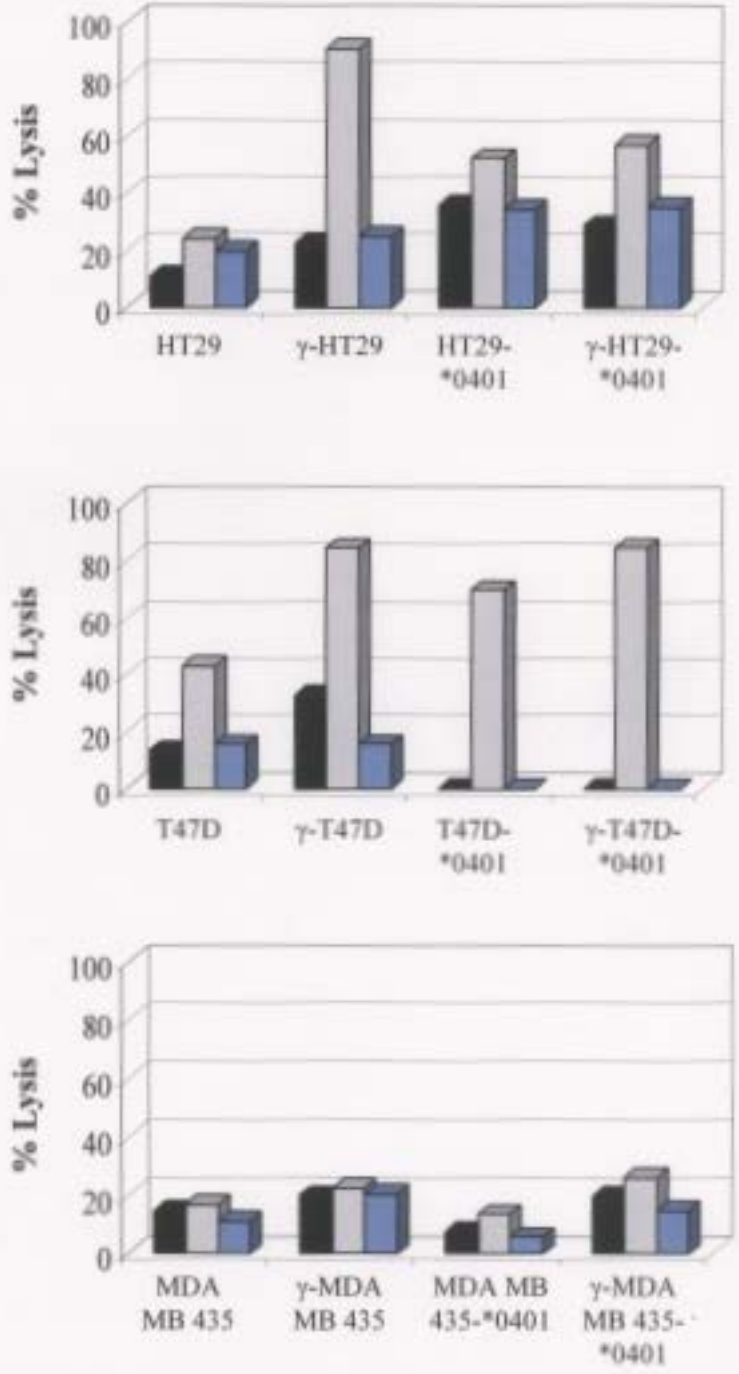


Figure 3.19. TCL-6Dn lysed tumor cells in a p883-specific manner. Tumor cells were treated or not with IFN-γ and labelled with ^{3}H-thymidine. See Table 6.2 in Appendix 1 for the amount of ^{3}H-thymidine incorporated into each cell line following 18 h. ^{3}H-thymidine-labelled tumor cells were co-cultured with TCL-6Dn in the absence of peptide (black bars), presence of p883 (1 μg/ml; gray bars) or an irrelevant peptide (1 μg/ml; blue bars). Percent tumor cell lysis was calculated after 48h co-culture as described in section 2.7.5.2

p883-pulsed MDA MB 435 and MDA MB 435-*0401 the percentage of tumor cell lysis was 22% and 26%, respectively.

In summary, tumor cell lysis occurred mainly when TCL-6Dn was co-cultured with p883-pulsed tumor cells. It was unclear from these data whether TCL-6Dn was activated by p883-presenting tumor cells or TCL-6Dn cells. However, lysis of the un-pulsed or irrelevant peptide-pulsed Her-2 positive cell line HT29 suggested that TCL-6Dn recognized the naturally processed p883 epitope generated by the tumor cell. Meanwhile, our results showed that TCL-6Dn recognized the p883 peptide causing tumor cell lysis, which led us to examine the exact mechanism used by TCL-6Dn to lyse tumor targets.

3.10. Elucidating the Mechanism Used by TCL-6D to Lyse Tumor Cells

3.10.1. Requirement for Tumor Cell and T Cell Contact

Requirement for TCL-6Dn and tumor cell contact was assessed using both the JAM and tumor cell proliferation assays. HT29 and HT29-*0401 (NI and I) were labelled with ^{3}H-thymidine and cultured in supernatant collected from un-stimulated, PHA-stimulated or p883-pulsed TCL-6Dn for 48h. In these assays, TCL-6Dn was stimulated for 48h in media without BFA present before the supernatants were collected and added to the ^{3}H-labelled tumor cells, ensuring the cytokines were released into the supernatant. To confirm the T cells were stimulated to produce cytokines, TCL-6Dn was also cultured with PHA (5 μg/ml) or p883 (1 μg/ml) in media containing BFA. Intracellular cytokines analysis confirmed that TCL-6Dn produced IFN-γ, TNF-α and GM-CSF (data not shown). Thus, supernatants collected from activated TCL-6Dn after 48 h should have also contained these cytokines.

As shown in Figure 3.20, tumor cell lysis may be mediated by a soluble factor produced by TCL-6Dn. Up to 54% of tumor cells cultured in supernatant collected from PHA-stimulated TCL-6Dn and up to 37% of tumor cells cultured in supernatant collected from p883-pulsed TCL-6Dn were lysed. Although tumor cell lysis in this assay was significantly lower than those attained when TCL-6Dn was co-cultured with tumor cells, these results indicated cell contact between TCL-6Dn and tumor cells was not essential for tumor cell lysis to occur. Since TCL-6Dn produced high levels of IFN-γ, TNF-α, and GM-CSF, we suspect that tumor cell lysis may be cytokine-mediated. However, tumor cells cultured in various concentrations of TNF-α from 0.1 ng/ml to 100 ng/ml showed no difference in cell growth (data not shown). Therefore, the combination of IFN-γ, TNF-α or GM-CSF may be required for tumor cell lysis to occur.

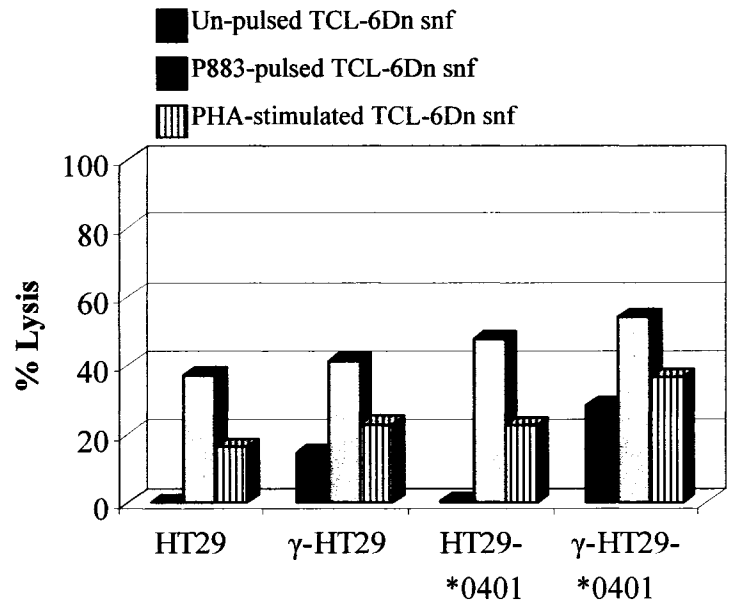


Figure 3.20. Lysis of tumor cells cultured in supernatant collected from stimulated TCL-6Dn. ^{3}H-thymidine-labelled tumor cells were cultured in supernatant collected from either un-stimulated (black bars), PHA-stimulated (5 μg/ml; gray bars) or p883-pulsed TCL-6Dn (1 μg/ml; striped bars) for 48 h. See Table 6.3 in Appendix 1 for the amount of ^{3}H-thymidine incorporated into each cell line following the 18 h labelling period. % lysis was calculated as cpm of tumor cell in T cell media - cpm of tumor cells in supernatant from TCL-6D / cpm of tumor cells in T cell media. Data shown are representative of two experiments.

To confirm that cell contact was not required for TCL-6Dn to lyse tumor cells, a transwell tumor cell proliferation assay was performed. Non-irradiated HT29-*0401(I) was un-pulsed or pulsed with p883 and cultured with TCL-6Dn for 72 h. In addition, TCL-6Dn was cultured with tumor cells in the presence of a 0.4 μm membrane cell culture insert, which allowed peptide and soluble factors to freely pass though the membrane, but prevented tumor cell and T cell interaction.

In the absence of TCL-6Dn, un-pulsed and p883-pulsed tumor cells showed strong proliferation in both the absence and presence of a cell culture insert (Figure 3.21). In the presence of TCL-6Dn, proliferation of un-pulsed and p883-pulsed HT29-*0401 (I) was significantly reduced in both the transwell and co-culture conditions. However, tumor cell proliferation was most drastically reduced in co-culture conditions. Although TCL-6Dn reduced proliferation of the un-pulsed tumor cells, these tumor cells were Her-2^{+} and IFN-γ treated and possibly processing and presenting endogenous p883. Previously, in Figure 3.11, we showed that TCL-6Dn when co-cultured with p883-pulsed HT29-*0401 does not produce IFN-γ. Therefore, TCL-6Dn may lyse the p883-pulsed tumor targets in a cytokine-independent manner.

Altogether, these results suggested that TCL-6Dn was able to lyse tumor cells in the absence of cell contact, but for tumor cells to be completely inhibited by TCL-6Dn cell both contact and p883 were required. In addition, these results indicate that TCL-6Dn used both a cytokine-dependent and independent mechanism to lyse p883-pulsed tumor targets.

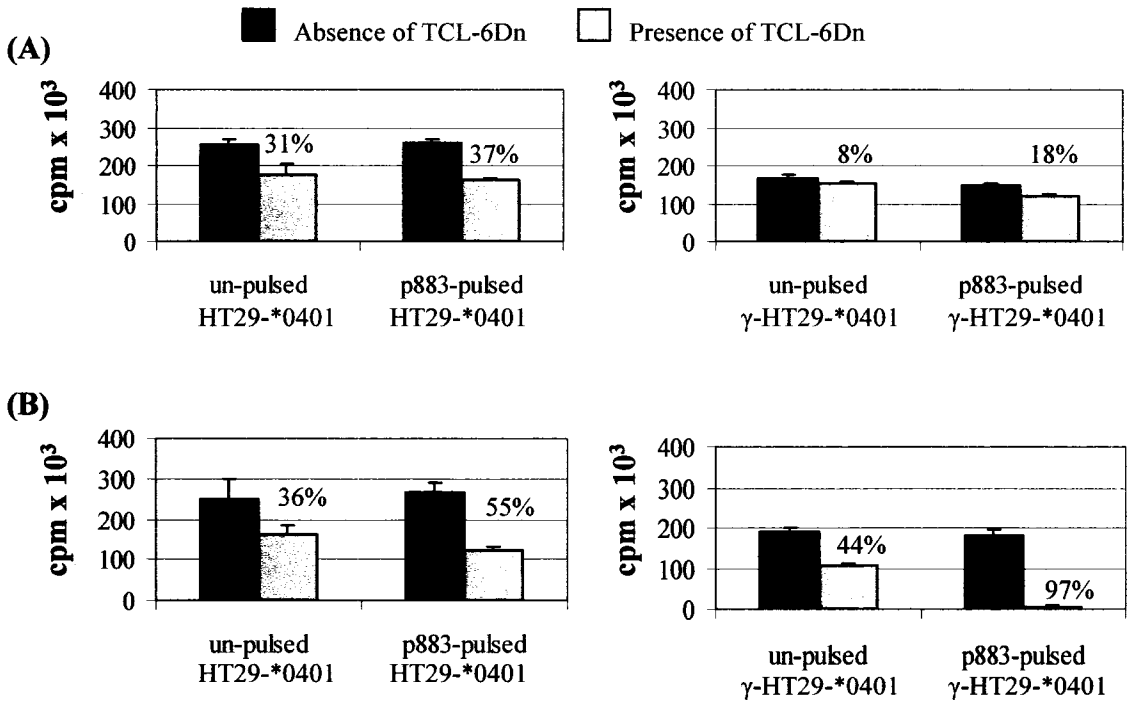


Figure 3.21. TCL-6Dn decreased the proliferation of Her2$^+$ and p883-pulsed tumor cells in a contact-dependent and -independent manner. Untreated or IFN-γ-treated, HT29-*0401 was un-pulsed or p883-pulsed (1 μg/ml) and cultured alone (black bars) or with TCL-6Dn (gray bars). Tumor cells and TCL-6Dn were either (A) separated by a transwell insert or (B) co-cultured for 72 h. Values represent the mean cpm and standard deviation of quadruplicate wells. Values above each bar represent the % inhibition and was calculated as described in section 2.7.6.

CHAPTER 4. DISCUSSION

4.1. Summary of Findings

Although TCL-6Dn proliferated in response to both HLA-DRβ1*0401^{+}and HLA-DRβ1*0401^{-} p883-pulsed DCs, we do not believe TCL-6Dn promiscuously responded to p883 presented by various HLA-DR alleles. Rather, it is probable that TCL-6Dn is auto-reactive to p883 presented by self HLA-DRβ1*0401 molecules. The high proliferation of TCL-6Dn in response to Ag-loaded DCs, but not Ag-loaded B cell lines, could be due to the presence of IL-12 or IL-10, depending on the APC present. TCL-6Dn may receive a survival signal, likely IL-12, from the DCs that it does not receive from other APCs. In turn, IL-12 may stimulate activated T cells to produce IL-2 and proliferate, independent of the HLA class II allele expressed on the DC. Meanwhile, engagement of CD40 on the B cell line and CD40L on the TCL-6Dn, in the absence of antigen recognition, may cause B cells to produce IL-10, suppressing TCL-6Dn activation.

TCL-6Dn lysed various tumor cells, in particular HT29-*0401^{+} cells, that were not loaded with exogenous p883. It is possible that Her-2 protein was shed from the tumor cell surface and processed by TCL-6Dn, resulting in auto-activation of the T cells. However, this is unlikely since TCL-6Dn lysed un-pulsed DRβ1*0401^{+}, but not DRβ1*0401^{-}, Her-2^{+} tumor cell lines. Thus, the p883 epitope may be naturally processed and presented by HLA-DRβ1*0401 molecules on the tumor cells.

Although we have shown that TCL-6Dn lysed p883-loaded tumor targets, we have not determined the exact cytotoxic mechanism used by the T cell clone. We know that activated TCL-6Dn secreted IFN-γ and TNF-α and we believe that the anti-

tumor cytotoxic effect of TCL-6Dn is partly due to a synergistic affect of both cytokines. Tumor cell lysis was mediated by a soluble factor produced by TCL-6Dn, but cell contact increased lysis. Therefore, TCL-6Dn possibly lysed tumor target cells via multiple mechanisms that were both contact-dependent and independent.

4.2. HLA-DR Restriction ofTCL-6Dn

Various antigen-derived epitopes that promiscuously bind to multiple HLA-DR and HLA-DP molecules exist (Neumann et al., 2004A, Neumann et al., 2004B, Mandic et al., 2005, Kobayashi et al., 2005). In particular, Her-2 p883 was shown to be presented by HLA-DR1, -DR4, -DR52 and -DR53 (Kobayashi et al., 2000). Southwood et al. (1998), suggested a motif for promiscuous binding peptides that is characterized by a large aromatic or hydrophobic residue at position one (P1) and a small non-charged residue at position 6 (P6). Her-2 p883 fills this criterion as it possesses tryptophan and phenylalanine, either of which could be a potential candidate for P1 and serine or isoleucine that could fit at P6. As such, the synthetic Her-2 p883 peptide may promiscuously bind to various HLA-DR molecules.

TCL-6D responded equally well to p883 presented by HLA-DRβ1*0401^{+} PBMCs and DR-transfected L cells (Kobayashi et al., 2000). This T cell clone was believed to be restricted by the DRβ1*0401 molecule based on its proliferation in response to the L cell transfectant. Kobayashi et al. (2000) did not report whether TCL-6D proliferated in response to a non-DRβ1*0401 APC. However, the T cell line that we expanded responded to p883 presented by mature DCs from DRβ1*0401,07;DR53 and DRβ1*1501,1301; DR51,DR52 donors. Despite the high degree of specificity of ligand recognition by TCRs (Reviewed in McFarland &

Beeson, 2002), antigen-specific T cells have been known to respond to peptide presented by various HLA-DR molecules (Doherty et al., 1998, Hemmer et al., 2000, Sotiriadou et al., 2001). Hypervariable regions of the HLA class II molecule encompassing residues 67-71, in particular residue 71, and residue 86 have been known to affect peptide binding and recognition by the TCR (Demotz et al., 1993, McKinney et al., 1994). As shown in Table 4.1, both DRβ1*0401 and DR52 alleles possess amino acids lysine and glycine at positions 71 and 86, respectively, while DR51 and DRβ1*0701 posses amino acids arginine and glycine at positions 71 and 86, respectively. Therefore, overlapping amino acid sequences at these positions may allow TCL-6Dn to recognize p883 presented by various HLA class II molecules. We have not presented evidence to show that TCL-6Dn responds to p883 presented by HLA-DR52. Therefore, we should have addressed this issue by testing the proliferative response of TCL-6Dn to p883-pulsed DCs from DRβ1*0401^{+}, DR52^{-}, DRβ1*0401^{-}, DR52^{-} and DRβ1*0401^{+}, DR52^{+} individuals.

Altogether, we do not believe that TCL-6Dn promiscuously responded to p883 presented by multiple HLA-DR alleles. An alternative, and more plausible, explanation is that TCL-6Dn is auto-reactive to p883 presented by self HLA-β1*0401 molecules. TCL-6Dn did not proliferate when cultured with peptide in the absence of DCs as TCL-6Dn may receive a survival signal, likely IL-12, from the DCs that it does not receive from other TCL-6Dn cells. In accordance, other studies have shown that IL-12 acts on activated Th cells resulting in T cell proliferation and increased cytokine production (Dredge et al., 2002, Knutson & Disis, 2004)

Table 4.1. Amino acid sequence of HLA-DR molecules at positions 67 to 71 and position 86.

HLA-DR Allele	Position 67 – 71 Amino Acids**	Position 86 Amino Acid**
DRβ1*0401	L L E Q K	G
DRβ1*0402	I L E D E	V
DRβ1*0701	I L E D R	G
DRβ1*1301	I L E D E	V
DRβ1*1501	I L E Q A	V
DRβ5 (DR51)	F L E D R	G
DRβ3 (DR52)	L L E Q K	G
DRβ4 (DR53)	L L E R R	V

**Major Histocompatibility Complex Data Base www.ncbi.nlm.nih.gov/mhc

4.3. Differential Response of TCL-6Dn to Various p883-Presenting APCs

Interaction between the TCR and peptide:HLA complexes, without a second co-stimulatory signal, is sufficient to activate memory and effector T cells (London et al., 2000). Consequently, we expected TCL-6Dn to be activated by all APCs presenting p883 in the context of HLA-DRβ1*0401, yet TCL-6Dn responded differently to the various p883-loaded APCs.

TCL-6Dn proliferated strongly to antigen when DCs were used as APCs. This result was expected as DCs are considered professional APCs. Upon antigen presentation to TCL-6Dn, CD40 on the DC may bind with CD40L on the T cell. Ligation of CD40/CD40L up-regulates HLA class II and co-stimulatory molecules, such as CD80 and CD86, improving the ability of DCs to present antigen and activate T cells (Caux et al., 1994). CD40/CD40L ligation also triggers DCs to produce cytokines such as IL-8, IL-12, TNF-α and IL-18 (Caux et al., 1994, Cella et al., 1996, Koch et al., 1996, Gardella et al., 1999). IL-12 has been shown to augment the proliferation and cytokine production of antigen-specific Th cells (Trinchieri et al., 1994, Cella et al., 1996, Knutson & Disis, 2004). Therefore, DCs may provide a co-stimulatory signal and generate IL-12, resulting in higher proliferation of TCL-6Dn. An alternative explanation is that TCL-6Dn may be activated indirectly by the DCs as p883-activated TCL-6Dn generated TNF-α, which is known to trigger DC maturation and IL-12 production (Sallusto et al., 1995, Morrison et al., 2003).

A major counter argument that TCL-6Dn required co-stimulation, is that B cell lines also express high amounts of CD40, CD80 and CD86, yet TCL-6Dn failed to proliferate when B cell lines were used as APCs. In addition, Kobayashi et al. (2000) showed that TCL-6D did not require co-stimulation for activation, as the $CD4^+$

T cell clone responded equally well to antigen presented by PBMCs and DR-transfected L cells. Although the B cell lines used in this study expressed the relevant co-stimulatory molecules, these B cell lines were EBV-transformed and likely produced IL-10, which may suppress TCL-6Dn activation. Engagement of CD40 on the B cell and CD40L on the T cell may cause B cells to produce higher amounts of IL-10, suppressing TCL-6Dn activation. In fact, Duddy et al. (2004) have shown that engagement of CD40 on the B cell with CD40L on the T cell, in the absence of Ag recognition, increases the amount of IL-10 generated by the B cell.

The differences in TCL-6Dn activation observed with antigen-loaded B cells and L cells may be due to the number of p883:HLA complexes present on the APC. Although B cell lines expressed higher amounts of HLA-DR, these class II molecules may be tightly bound to self peptides that can not be easily displaced to allow loading of exogenous p883. Thus, L cells may possess a higher number of p883:HLA complexes. However, a more plausible explanation is that B cells, but not L cells, generate IL-10 which may suppress activation of TCL-6Dn.

In summary, it is most likely that TCL-6Dn proliferated strongly in response to p883-pulsed DCs because TCL-6Dn received a survival signal, possibly IL-12, which was absent when TCL-6Dn was cultured with other APCs. Indeed, the poor response to p883-pulsed B cell lines may be due to the production of IL-10, which may suppress TCL-6Dn activation. We cannot conclude that TCL-6Dn proliferation is dependent on the presence of IL-12, yet this could be addressed by measuring TCL-6Dn proliferation in the presence of IL-12 alone, IL-12 and IL-10 or IL-10 alone.

4.4. Tumor Cell Lysis

Since TCL-6Dn was activated by p883-loaded DCs, PBMCs and DR-transfected L cells, we expected that p883-loaded HLA-DR^+ tumor cells would also activate TCL-6Dn. In contrast, TCL-6Dn appeared to be cytotoxic toward Ag-loaded tumor cell lines. Anti-tumor cytotoxic $CD4^+$ T have also been described by others (Chen & Hersey, 1992, Takahashi et al., 1995, Thomas & Hersey, 1998a, Manici et al., 1999, Schultz et al., 2000). In particular, one $CD4^+$ T cell proliferated in response to Ag-loaded PBMCs, while the T cell was cytotoxic toward Ag-presenting DR^+ tumor cells (Manici et al., 1999).

The Ag dose used to stimulate *in vitro*-generated T cells is an important factor in determining their phenotype and function (Ise et al., 2002). High doses of Ag and repetitive stimulation of T cells *in vitro* can induce cytotoxic properties (Janssens et al., 2003). Possibly, TCL-6Dn possessed cytotoxic properties due to high dose antigen stimulation of the naïve $CD4^+$ T cells, cloned by Kobayashi et al. (2000). Meanwhile, the cytotoxic properties of TCL-6Dn may have increased with further clonal expansions of the T cell clone.

JAM assays performed using adherent ^{3}H-thymidine-labelled target cells could lead to falsely high killing values due incomplete harvesting of the adherent radioactive-labelled cells. Although, we do not think this occurred in our assays as we collected all adherent radioactive-labelled target cells by adding trypsin to all wells and three rounds of washing were performed during cell harvesting. Others have shown that at least three rounds of washing is required during cell harvesting to effectively transfer all radioactive DNA from adherent cells onto the filter mat (Böhm et al., 1998, Atarashi et al., 2000).

Despite limitations of the JAM assay, it was useful in distinguishing tumor cell lysis from inhibition of cell growth. TCL-6Dn lysed the ^{3}H-labelled tumor cell targets, as tumor cells underwent DNA-fragmentation, which is characteristic of the early stages of cell death, but not growth inhibition (Weis et al., 1995). Yet, we did not perform electrophoresis to confirm that DNA fragmentation occurred in the ^{3}H-labelled target cells. We cannot exclude the possibility that ^{3}H-thymidine labelling enhanced tumor cell death, but tumor cell lysis was significantly higher when tumor cells were p883-loaded and cultured with TCL-6Dn compared to tumor cells cultured alone.

4.4.1. Cytotoxic Response of TCL-6Dn toward various p883-pulsed HLA-DR^{+} Tumor Cell Lines

TCL-6Dn lysis of p883-pulsed HLA-DR expressing breast and colon cancer cell lines (T47D and HT29, respectively), but not p883-loaded breast cancer cell line (MDA MB 435) shown in figure 3.19 is difficult to explain. Our data in figure 3.9 showed that growth of p883-pulsed MDA MB 435 cells was significantly inhibited by TCL-6Dn, while figure 3.19 showed that TCL-6Dn did not lyse peptide-pulsed MDA MB 435 cells. On the contrary, our data has shown that TCL-6Dn can lyse and inhibit growth of both cell lines T47D and HT29.

Potentially, IFN-γ produced by TCL-6Dn may inhibit MDA MB 435 cell growth, as IFN-γ can arrest the cell cycle at the G_0/G_1 stage (Garbe & Krasagakis, 1993, Kortylewski et al., 2004). Our data showed that IFN-γ-treatment, used to induce HLA class II expression, decreased tumor cell proliferation. This was slightly evident with T47D and HT29 cell lines, but IFN-γ drastically affected the proliferation of

MDA MB 435. This supports our hypothesis that IFN-γ produced by p883-pulsed TCL-6Dn could inhibit the proliferation of the tumor cell lines, particularly MDA MB 435. However, lysis of MDA MB 435 cells may have been undetected as non-proliferating MDA MB 435 cells would not incorporate the ^{3}H-thymidine used in the JAM assay.

On the contrary, MDA MB 435 may not be susceptible to the death-inducing mechanism used by TCL-6Dn to lyse T47D and HT29. Although we have referred to MDA MB 435 as a breast cancer cell line, Ross et al. (2000) showed that MDA MB 435 cells express melanoma associated genes. Therefore, MDA MB 435 may be a melanoma cell line. Other researchers have shown that some melanoma cells are resistant to killing by FasL and TNF-α, but susceptible to TRAIL-induced killing mediated by $CD4^{+}$ T cells (Thomas & Hersey, 1998a, Thomas & Hersey, 1998b). It is not clear why melanoma cells would be resistant to TNF-α and FasL, but susceptible to TRAIL, as the receptors for these ligands belong to the TNF family and their mechanism of inducing cell death is thought to be similar (Reviewed in Baker & Reddy, 1998, Özören & El-Deiry, 2003). Perhaps, MDA MB 435 may not express, or express a mutated form, of the receptors for the death-inducing ligand used by TCL-6Dn. Similarly, Pan et al. (1997) showed that TRAIL-resistant tumor cells expressed TRAIL receptors that lacked the intracellular domain.

4.4.2. Increased Lysis of IFN-γ Treated Tumor Cell Lines

IFN-γ is a well known modulator of HLA class II expression on tumor cells *in-vitro*, and *in-vivo* (Chang & Flavell, 1995, Muczynski et al., 1998, Walter et al., 2000, Boss & Jensen, 2003, Propper et al., 2003, Zehbe et al., 2005). Since we tested

whether HLA class II^+ tumor cells processed and presented epitopes generated from endogenous Her-2 protein, tumor cells were pre-treated with rhIFN-γ to up-regulate class II expression. We detected higher killing of peptide-loaded tumor cells that were pre-treated with IFN-γ compared to their un-treated counterparts. One explanation for these results is that IFN-γ up-regulated the expression of HLA class II and class II accessory molecules in the tumor cells. As a result, IFN-γ-treated tumor cell lines may process endogenous Her-2 protein and present the naturally processed Her-2 883-899 epitope, increasing the number of peptide:HLA class II complexes recognized by TCL-6Dn. Indeed, studies have shown that an increase in the number of peptide:class II complexes can enhance T cell activation (Cochran et al., 2000, Reay et al., 2000). However, this explanation is unlikely as TCL-6Dn lysed $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ tumor cell lines.

A more probable explanation is that IFN-γ treatment of the tumor cells, prior to co-culture with TCL-6Dn, up-regulated cell death receptors on tumor cells. IFN-γ has been shown to increase expression of several apoptotic-related molecules including death receptors, such as members of the Bcl-2 family or caspases (Ruiz-Ruiz et al., 2000, Fulda & Debatin, 2002, Ossina et al., 1997, Ugurel et al., 1999). As a consequence, tumor cell lines in this study may be highly susceptible to apoptosis due to IFN-γ modulation of apoptotic-related proteins.

4.5. Mechanisms Potentially Responsible for Tumor Cell Lysis

Our data suggested that TCL-6Dn lysed tumor cells via multiple pathways that were both contact-dependent and -independent. This was evident as tumor cells were lysed when cultured with TCL-6Dn in the presence of a transwell insert, preventing

contact between the tumor cell and T cells. As well, tumor cells were lysed when cultured in supernatant collected from stimulated TCL-6Dn. However, tumor cell lysis was higher when tumor cells were co-cultured with TCL-6Dn. The increased tumor cell lysis observed in the co-culture conditions could be due to the tumor cell being in closer proximity to the $CD4^+$ T cells secreting cytokines and not a result of cell to cell contact. Alternatively, cytokines present in the supernatant collected from stimulated TCL-6Dn may lose their efficiency after collection, resulting in lower tumor cell lysis. Meanwhile, we believe that TCL-6Dn lysed target cells via multiple mechanisms that were contact-dependent and independent.

Several cytotoxic Th1 cells that utilize the perforin/granzyme pathway to kill melanoma, lung cancer, T lymphoma and leukemia cells have been described (Thomas & Heresy, 1998a, Semino et al., 1999, Echchakir et al., 2000, Porakishvili et al., 2004). Upon recognition of HLA:peptide complexes by the TCR, perforin molecules, stored in cytoplasmic granules within the cytotoxic T cell, are released and form holes in the target cell membrane (Liu et al., 1995). Thereafter, target cell death occurs via a granzyme-mediated mechanism (Heusel et al., 1994). It is unlikely that tumor cell lysis in our study was perforin/granzyme-mediated, as this mechanism of cell killing is contact-dependent and a fast process (Liu et al., 1995). Meanwhile, our data showed that tumor cells were not lysed at 5 h, but were susceptible to lysis at 24 h, which is characteristic of Fas/FasL and cytokine-mediated cell killing (Stalder et al., 1994, Breu-Martin et al., 1995). However, to confirm TCL-6Dn did not kill via the perforin/granzyme pathway we should have examined granzyme expression in TCL-6Dn.

The primary cytotoxic mechanism used by $CD4^+$ T cells is believed to be Fas/FasL (Stalder et al., 1994). Therefore, we investigated whether TCL-6Dn killed tumor cells via Fas/FasL interactions. The interaction between FasL on the T cell and its receptor, Fas, on the target cell induces target cell death via the death domain-mediated recruitment of caspases (Reviewed in Özören & El-Deiry, 2003). All tumor cell lines used in our study expressed Fas (also known as CD95 or APO1; data not shown), which corresponds with other published studies (Thomas & Hersey, 1998a, Houston et al., 2003). In addition, Fas ligand (also known as FasL, APO1-L or CD95L) was expressed on TCL-6Dn (data not shown).

The requirement for cell to cell contact in Fas-induced killing is controversial. Ligation between Fas and FasL is required to initiate the death signal, yet a 26-kDa soluble and functional form of FasL can be released from activated T cells by metalloproteinases (Kayagaki et al., 1995, Tanaka et al., 1995). Conversely, Tanaka et al. (1998) demonstrated that the soluble form of FasL failed to initiate target cell lysis. If TCL-6Dn lysed tumor cells via the Fas/FasL pathway, mediated by soluble FasL, one would expect TCL-6Dn to lyse all Fas^+ tumor cells, regardless of p883 being present. Rather, our results showed that TCL-6Dn lysed p883-pulsed, but not all un-pulsed, Fas^+ targets. Meanwhile, we did not observe a correlation between Fas expression on the tumor cells and tumor cell lysis. In addition, TCL-6Dn failed to lyse the p883-pulsed B cell lines, which were also shown to express Fas molecules (data not shown). As a result, we do not believe TCL-6Dn lysed tumor targets via the Fas/FasL pathway.

Other studies have shown that $CD4^+$ T cells utilize cytokine-mediated mechanisms, such as IFN-γ and TNF-α, to lyse tumor cells (Mumberg et al., 1999,

Brady et al., 2000, Lindencrona et al., 2004). IFN-γ is characteristic of a Th1 response to tumors (Billiau et al., 1998) and is a multi-functional cytokine with immunomodulatory and anti-proliferative effects. The cytotoxic effect of IFN-γ may be direct (Willamson et al., 1983, Novelli et al., 1994) or indirect, as IFN-γ can up-regulate the expression of cell death receptors, such as Fas, TNF-α receptors (TNF-R) and TNF-related apoptosis-inducing ligand (TRAIL) (Ruggiero et al., 1986, Itoh et al., 1991, Sedger et al., 1999, van Geelan et al., 2003, Felli et al., 2005). However, we do not believe that IFN-γ produced by TCL-6Dn was solely responsible for tumor cell lysis because tumor cells were not lysed by IFN-γ treatment used to up-regulate HLA-DR expression.

We believe the anti-tumor cytotoxic effect of TCL-6Dn was due to a synergistic affect of IFN-γ and TNF-α as tumor cells were cultured in various concentrations of rhTNF-α and cell lysis was undetected (data not shown). The cytotoxic effect of TNF-α occurs as TNF-α binds to its receptor, TNF-R, on the target cell surface. Upon ligand binding, downstream signals are transmitted that lead to activation of caspases and pro-apoptotic signals, resulting in target cell death (Reviewed in Baker & Reddy, 1998). IFN-γ and TNF-α have also been shown to cause cells to arrest in the G_0/G_1 phase of the cell cycle, resulting in inhibition of cell growth (Pusztai et al., 1993, Hillman et al., 1994).

Another study has shown that peptide-specific $CD4^+$ T cells kill peptide-presenting melanoma cells in-vitro using a contact-independent mechanism, which they suggested was cytokine-mediated (Brady et al., 2000). Their data also showed that melanoma cell lysis was not prevented with an anti-IFN-γ mAb, suggesting that other factors were involved. Other studies have shown that alone TNF-α and IFN-γ

cause minimal killing of tumor cell lines, while a combination of both cytokines increases killing (Willamson et al., 1983, Deem et al., 1991, Breu-Martin et al., 1995, Manos & Jones., 2001). Subsequent experiments performed in Dr. Drover's laboratory have shown that anti-IFN-γ can inhibit tumor cell lysis by p883-pulsed TCL-6Dn, while anti IFN-γ and anti TNF-α have a synergistic effect. The ability of IFN-γ to up-regulate a variety of cellular receptors, including TNF-R, suggests one mechanism responsible for the synergism between these two cytokines (Ruggiero et al., 1986, Itoh et al., 1991).

4.6. Natural Processing of the Her-2 Protein by Tumor Cell Lines

Natural processing and presentation of the endogenous Her-2 protein by tumor cells was evaluated by measuring TCL-6Dn recognition of un-pulsed HLA-DR^+ and Her-2^+ tumor cells. Other studies have shown that tumor cells can naturally process and present epitopes generated from various tumor antigens (Brady et al., 2000, Schultz et al., 2000, Kobayashi et al., 2005). Perez et al. (2002) demonstrated that Her-2^+ and class II^+ colon, pancreatic and breast cancer cells were capable of processing and presenting a Her-2-derived class II epitope. Similarly, our results suggested that some Her-2^+ and HLA class II^+ tumor cells were capable of processing and presenting the 883-899 epitope for $CD4^+$ T cell recognition.

TCL-6Dn lysed various tumor cells, in particular HT29-*0401^+ cells, that were not loaded with exogenous p883. It is possible that Her-2 protein was shed from the tumor cell surface and processed by TCL-6Dn resulting in auto-activation of the T cells. However, TCL-6Dn lysed un-pulsed DRβ1*0401^+, but not DRβ1*0401^-, Her-2^+ tumor cell lines. Thus, the p883 epitope may be naturally processed and presented by

HLA-DRβ1*0401 molecules on the tumor cells. These results correspond with data published by Perez et al. (2002) who also showed that HT29 tumor cells could process and present a Her-2-derived HLA class II epitope that differed from the p883-899 epitope by one amino acid. However, Perez et al. (2002) did not report whether they used other Her-2^+ cell lines, such as T47D.

The inability of TCL-6Dn to recognize the Her-2^+ tumor cell line T47D does not indicate this tumor cell line is unable to process endogenous tumor antigens. It is possible that HT29, but not T47D, possess the appropriate antigen processing molecules required to process and present Her-2-derived epitopes for class II presentation. Yet, T47D may process the Her-2 protein, but not generate the p883 epitope to activate TCL-6Dn. Alternatively, Her-2 expression on T47D may be modulated by pre-treatment with IFN-γ or IFN-γ generated by the activated TCL-6Dn. IFN-γ can effect the expression of several proto-oncogenes (Beatty & Paterson, 2000, Brouwers et al., 2003, Li et al., 2003, Reddy et al., 2003). In particular, IFN-γ can suppress Her-2 expression in human ovarian and prostate cancer cells (Marth et al., 1990, Marth et al., 1992, Fady et al., 1992, Kominsky et al., 2000). Therefore, IFN-γ may modulate Her-2 expression on some of the Her-2^+ tumor cell lines used in this study.

We cannot exclude the possibility that Her-2 may be shed from the tumor cell surface, processed and presented by HLA-DRβ1*0401 molecules on the $CD4^+$ T cell clone, resulting in self-activation. However, this explanation does not address why TCL-6Dn would only recognize the HLA-DRβ1*0401^+, Her-2^+ tumor cells. This explanation also implies that HT29, but not T47D, is able to shed the Her-2 protein. Recent proliferation assays conducted in Dr. Drover's laboratory showed that TCL-

6Dn failed to proliferate when cultured in supernatant collected from the Her-2^+ breast tumor cell line, SKBR3, which is thought to shed the Her-2 protein. As a result, we believe that tumor cells may be able to process the endogenous Her-2 protein.

4.7. Potential Implications of Findings

TCL-6Dn responded differently to p883 presented by various APCs. Whether TCL-6Dn recognized differences in the antigen-presenting cell and adjusted their cytokine production accordingly is unknown. In this study, we showed that TCL-6D produced IFN-γ when p883-pulsed tumor cell lines and B cell lines were used as APCs. However, we did not address whether TCL-6Dn produced additional cytokines in response to Ag-pulsed tumor cells. It would be interesting to discern whether TCL-6Dn generates a different cytokine profile when it recognizes Ag-pulsed tumor cells than in response to Ag-loaded DCs. We believe that TCL-6Dn may have produced IL-2 in response to Ag-loaded DCs, but not Ag-loaded tumor cells. This experiment could explain why TCL-6Dn proliferated in response to Ag-loaded DCs, but not other APCs.

There are examples of $CD4^+$ T cells that lyse HLA class II^+ tumor cells, yet most tumor cells are constitutively HLA class II negative. However, $CD4^+$ T cells that secrete cytokines upon activation can potentially up-regulate class II expression on tumor cells or initiate tumor cell lysis by cytokine-mediated mechanisms. This is why we set out to examine the exact mechanism used by TCL-6Dn to lyse the Ag-loaded tumor cells. Whether TCL-6Dn used a cytokine- or Fas-mediated mechanism could be determined with blocking mAbs specific for Fas, IFN-γ and TNF-α. Although data from Dr. Drover's laboratory (data not shown) determined that

blocking the interaction between IFN-γ and TNF-α and their appropriate receptors decreased killing of the Ag-loaded tumor cells, tumor cell lysis was not completely abrogated. This could be due to insufficient blocking of the IFN-γ and TNF-α or TCL-6Dn may utilize additional mechanisms to lyse the antigen-loaded tumor cells.

Apart from our *in-vitro* findings, the presence of Her-2-specific cytotoxic $CD4^+$ T cells among the infiltrating T cells in Her-2 positive carcinoma *in-vivo* remains undetermined. Potentially, this could be investigated using HLA-class II tetramers carrying the Her-2 883-899 peptide in the context of relevant HLA-DR molecules, such as HLA-DRβ1*0401, in $DR4^+$ cancer patients. Therefore, binding of the tetramer could identify p883-specific infiltrating $CD4^+$ T cells from Her-2^+ and HLA-DRβ1*0401^+ carcinomas. However, even if tumor cells are capable of antigen presentation *in vivo*, the precise location of Ag-presentation may remain unclear. Tumor antigen presentation may occur at the tumor site as approximately 52% of tumors contain moderate to large numbers of infiltrating T cells (Oldford et al., 2004). Alternatively, antigen presentation could occur at the regional lymph node as tumor cells migrate to the lymph node (Leong et al., 2006, Al-Batran et al., 2005, Yang et al., 1997). It is unlikely that naïve $CD4^+$ T cells with an appropriate TCR would be exposed to Ag at the tumor site or that tumor cells lacking co-stimulatory molecules could activate naïve $CD4^+$ T cells. However, tumor antigens that are shed may be picked up and processed by intratumoral professional APCs. This could induce HLA class II expression on tumor cells through the release of cytokines and enable the tumor cell to present tumor-antigen derived peptides. Alternatively, tumor cells can metastasize via the lymphatic circulation, draining into the lymph whereby Ag-presentation by the tumor cell could occur.

Her-2 epitope 883 to 899 may be a naturally processed epitope expressed on the surface of some tumor cells. This is consistent with results published by Perez et al (2002) who showed natural processing and presentation of a similar Her-2 epitope by tumor cell lines. Although Kobayashi et al. (2000) showed the Her-2 p883-899 epitope is naturally processed by professional APCs, this does not imply the whole Her-2 protein is processed via the endocytic pathway. Other studies have shown this occurrence with MAGE-A3 tumor antigen as epitopes 281- 295 and 247-258 were presented by MAGE-$A3^+$ tumor cells to $CD4^+$ T cells, whereas epitopes 114-127 and 121-134 were not expressed on the surface of MAGE-$A3^+$ tumor cells (Chaux et al., 1999, Manici et al., 1999). Therefore, it would be interesting to determine if the whole Her-2 protein is processed by identifying additional Her-2-derived antigenic peptides that are presented on the surface of Her-2^+ tumor cells.

While HLA class II molecules generally present peptides from exogenous proteins, cytosolic antigen-derived peptides have been eluted from class II molecules (Reviewed in Zhou & Blum, 2004). Where antigen processing and loading of peptides derived from cytosolic proteins occurs is unclear. Studies have shown that cytosolic proteins, such as GAD and influenza viral proteins require proteasomal and endosomal compartments of the Class I antigen processing for the presentation of class II epitopes (Lich et al, 2000, Tewari et al., 2005, Dörfel et al., 2005). Other studies have shown that cytosolic proteins, such as the EBV nuclear proteins and the NeoR antigen, are processed by acidic proteases in the lysosomal compartments of the class II processing pathway (Nimmerjahn et al., 2003, Paludan et al., 2005). Whether the Her-2 protein is processed via the class I or class II antigen processing pathway and loaded onto HLA class II molecules remains undetermined.

Since Her-2 is expressed at low levels in normal cells, high-affinity $CD4^+$ T cells specific for Her-2-derived epitopes may be rendered tolerant or deleted (Reviewed in Sprent, 2001). The presence of Her-2 on normal cells could trigger an autoimmune response if high affinity $CD4^+$ T cells reacted with APCs presenting low levels of Her-2-derived peptides. However, it is possible that epitopes expressed on normal cells are below the threshold level for T cell recognition, while over-expression in tumor cells can initiate a Her-2 specific T cell response. Whether antigen-presenting tumor cells would generate low-, intermediate- or high-affinity $CD4^+$ T cells *in vivo* is undetermined. Low affinity T cells may be inefficient in providing tumor protection *in vivo*, whereas high affinity T cells could trigger an autoimmune response (Zeh et al., 1999). However, if tumor antigens are processed in non-endocytic compartments, tumor cells could present different tumor peptides than professional APCs. Therefore, tumor cells could stimulate a different repertoire of $CD4^+$ T cells that may not initiate an auto-immune response.

In conclusion, we have demonstrated that Her-2 p883-specific $CD4^+$ T cells recognize some Her-2^+ and HLA class II^+ tumor cell lines, indicating this epitope may be expressed on the surface of tumor cells. Since various Her-2 epitopes recognized by $CD8^+$ T cells have been discovered (Fisk et al., 1995, Rongcun et al., 1999, Baxevanis et al., 2002, Sotiropoulou et al., 2003), incorporation of the Her-2 p883 epitope with class I-restricted epitopes in vaccines could result in a more efficient and longer lasting anti-tumor immune response. This $CD4^+$ T cell clone may also be of importance for immunotherapeutic strategies that utilize antigen-specific T cells, as TCL-6Dn was activated to proliferate and generate cytokines

when Ag-loaded DCs were used as APCs, but TCL-6Dn was cytotoxic toward Ag-loaded tumor cells.

CHAPTER 5. REFERENCES

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CHAPTER 6. APPENDIX

APPENDIX 1

Table 6.1. The Amount of ^{3}H-thymidine incorporated by 1 x 10^4 cells of non-induced and IFN-γ-induced tumor cell lines (shown in Figure 3.18) following 18 h labelling with 5 μCi/ml of tritiated-thymidine (6.7 Ci/mmol).

Cell Line	cpm values obtained for 1x10^4 cells of each cell line	
	Non-induced	IFN-γ-induced
T47D	6253	2643
T47D-*0401	5301	5499

Table 6.2. The Amount of ^{3}H-thymidine incorporated by 1 x 10^4 cells of non-induced and IFN-γ-induced tumor cell lines (shown in Figure 3.19) following 18 h labelling with 5 μCi/ml of tritiated-thymidine (6.7 Ci/mmol).

Cell Line	cpm values obtained for 1x10^4 cells of each cell line	
	Non-induced	IFN-γ-induced
T47D	9418	7094
T47D-*0401	5494	4294
HT29	2490	4536
HT29-*0401	6954	2432
MDA MB 435	5528	1069
MDA MB 435-*0401	5041	895

Table 6.3. The Amount of ^{3}H-thymidine incorporated by 1 x 10^4 cells of non-induced and IFN-γ-induced tumor cell lines (shown in Figure 3.20) following 18 h labelling with 5 μCi/ml of tritiated-thymidine (6.7 Ci/mmol).

Cell Line	cpm values obtained for 1x10^4 cells of each cell line	
	Non-induced	IFN-γ-induced
HT29	2490	4536
HT29-*0401	6954	2432

Table 6.4. The spontaneous and maximum release of ^{51}Cr from the ^{51}Cr-labelled tumor cell lines shown in Figure 3.17.

Tumor Cell Line	Un-pulsed		p883-pulsed	
	Spontaneous Release	Maximum Release	Spontaneous Release	Maximum Release
T47D	606.1	4859.9	694.1	4684.2
T47D*0401	1281.3	6194.6	1164.9	6165.0
HT29	708.6	6807.0	651.1	6589.2
HT29*0401	455.6	1925.5	462.6	1858.8