Induction of *in vitro* T cell immune response against the variable region of paraprotein light chain through autologous dendritic cells

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Induktion einer *in vitro* T-Zell-Immunantwort gegen die variable Region der Paraprotein-Leichtkette durch autologe dendritische Zellen

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With these words, I declare that:
I composed the present thesis entitled **”Induction of *in vitro* T cell immune response against the variable region of paraprotein light chain through autologous dendritic cells“** independently and on my own responsibility. All used means and institutions were cited completely.

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Summary

Plasmocytoma is characterized as a tumor in which all malignant plasma cells exhibit identical rearrangement and mutation of immunoglobulin variable genes, and secrete a monoclonal immunoglobulin or its fragments (paraprotein). In the course of disease the third complementarity-determining region (CDRIII) of this immunoglobulin remains stable, i.e. there is no evidence of ongoing somatic hypermutation. Therefore, the idiotype (i.e. the unique antigenic structure) of monoclonal immunoglobulin in an individual patient could serve as a tumor-specific antigen. However, due to genetic recombination, there are several myelomas lacking production of immunogloblin heavy chain but have free monoclonal light chains. In these cases of plasmocytoma, a defined tumor-associated antigen is provided by the variable region of the corresponding light chain (V_LIgPl) in order to elicit a tumor specific immune response.

Since plasmocytoma is still an incurable disease despite some recent progress in the development of high dose chemotherapy, development of immunotherapy approaches designed to get cytotoxic T lymphocytes (CTL) may help to eradicate or control the tumor. Aim of this thesis was induction of an *in vitro* autologous T cell proliferation restricted to MHC class I molecules against V_LIgPl through autologous dendritic cells (DC) via retroviral transduction of human $CD34^+$ progenitor cells.

The cytokine requirements to differentiate $CD34^+$ progenitor cells from different sources into DC are known to be different. GM-CSF plus TNF-α ± SCF used in most protocols described not only for generation of DC but also of macrophages and neutrophils. Therefore, this work provides data that mature DC can differentiate from $CD34^+$ cells of different origins in the presence of PMA alone under serum-free conditions without generation of cellular intermediates and other lineages. This may facilitate *in vitro* studies of primed or genetically modified DC against infectious and tumor-associated antigens.

Most retroviral vectors contain a bacterial antibiotic-resistance gene such as neomycin that is strongly immunogenic. Its peptides will be presented on MHC class I molecules and activate $CD8^+$ T lymphocytes, thus skewing the elicited immune response. In order to minimize such immune action against xenogenic peptides, a novel retroviral expression-cloning vector that

directs the tumor-associated antigen of plasmocytoma fused in frame with a FLAG antibody-binding site to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor has been constructed. Thus, transfected or transduced cells can be detected using monoclonal anti-FLAG antibodies. PI-PLC releases cell surface FLAG-antigen from transduced $CD34^+$ progenitor cells indicating that the retroviral vector targets the fusion protein to the cell surface via GPI-anchor. V_LIgPl transgene expression in DC using the novel retroviral vector elicited a strong autologous T cell proliferation restricted to MHC class I molecules. This proliferative response is more prominent in PMA-derived DC compared to cytokine-derived DC indicating that PMA-derived DC are more potent in activating autologous T cell proliferation. In addition, T cell proliferation could be inhibited almost completely by CD86 antibodies.

It can be concluded from these data that autologous T cells could recognize immune epitopes within the myeloma V_LIg, which had been presented by DC on MHC class I molecules. In addition, CD86 co-stimulatory molecule plays a critical role in activation of naive T cells. This result will aid in the generation of V_LIg-based immunotherapy in multiple myeloma.

Dendritic cells, retrovirus-mediated gene transfer, V_LIg of plasmocytoma

Zusammenfassung

Das multiple Myelom ist gekennzeichnet durch eine identische Rekombination und Hypermutation der variablen Regionen im Bereich der Immunglobulingenloci in allen neoplastischen Plasmazellen. Somit produzieren die Tumorzellen ein monoklonales Immunglobulin oder seine Fragmente (Paraprotein). Daher kann der Idiotyp, die einzigartige antigene Struktur, des monoklonalen Immunglobulins bei Myelompatienten als Tumor-spezifisches Antigen dienen. Aufgrund einer genetischen Rekombination kommt es bei einigen Patienten bzw. in einigen Myelomzellen zu einem Fehlen der schweren Kette des Immunglobulins, diese Zellen jedoch produzieren freie monoklonale Leichtketten. Bei diesen Myelompatienten steht die variable Region der korrespondierenden Leichtkette (V_LIgPl) als spezifisches Antigen zur Induktion einer Tumor-spezifischen Immunantwort zur Verfügung.

Trotz einigen Fortschritts und der Entwicklung der Hochdosis-Chemotherapie bleibt das multiple Myelom eine unheilbare Erkrankung. Daher sollen Immuntherapien entwickelt werden, um zytotoxische T-Lymphozyten zu generieren, die zur Eradikation des Tumors beitragen sollen. Ziel dieser Doktorarbeit war, die Induktion einer *in vitro*-autologen T-Zell-Proliferation zu etablieren, als eine MHC Klasse I restringierte, gegen V_LIgPl gerichtete Antwort gegen autologe DC nach retroviraler Transduktion von $CD34^+$ Progenitorzellen.

Die Zytokinzusammensetzung, um $CD34^+$ Zellen zu differenzieren unterscheiden sich bekanntlicherweise in Abhängigkeit von der Herkunft der $CD34^+$ Zellen. GM-CSF und TNF-α ± SCF werden in den meisten Protokollen verwendet, dabei differenzieren aber nicht nur DC sondern auch Makrophagen und Neutrophile. In dieser Arbeit wurden DC aus $CD34^+$ Zellen verschiedenen Ursprungs durch Stimulation mit PMA in serumfreien Medien ohne die Bildung von Intermediärstadien und anderen Differenzierungslinien generiert. Dieses Ergebnis könnte *in vitro*-Studien ermöglichen unter Verwendung von Antigen-gepulsten oder genetisch modifizierten DC gegen infektiöse oder Tumor-assoziierte Antigene.

Die meisten retroviralen Vektoren enthalten ein bakterielles Antibiotika-Resistenzgen wie Neomycin-Resistenzgen, das stark immunogen wirkt. Seine Peptide könnten auf MHC Klasse I Molekülen präsentiert werden und dadurch $CD8^+$ T-Lymphozyten aktivieren, wodurch es zu einer Verschiebung der Immunantwort kommt. Um solche Immunantwort

gegen xenogene Peptide zu minimieren, ist einen neuartigen retroviralen Expressionsvektor konstruiert worden, der zu einer Expression des Tumorantigens ohne Rasterverschiebung mit einer FLAG-Antikörperbindungsstelle als Glycosylphosphatidylinositol (GPI)-verankertes Peptid führt. Transfizierte bzw. transduzierte Zellen können somit durch monoklonale anti-FLAG Antikörper nachgewiesen werden. PI-PLC führt zu einer Ablösung des FLAG-Antigens von der Oberfläche der transduzierten Progenitorzellen. V_LIgPl-Transgenexpression in DC durch den retroviralen Vektor führt zu einer starken autologen T-Zellproliferation, die MHC Klasse I restringiert ist. Die T-Zellproliferation konnte nahezu vollständig durch CD86 monoklonale Antikörper gehemmt werden. Diese proliferative Antwort ist unter Verwendung von PMA-differenzierten DC im Vergleich zu Zytokin-differenzierten DC deutlich ausgeprägter, wodurch gefolgert werden kann, daß die PMA-differenzierten im Vergleich zu den Zytokin-differenzierten DC in der Induktion einer autologen T-Zellantwort eine deutlich stärkere Potenz aufweisen.

Aus diesen Daten kann geschlossen werden, daß autologe T-Zellen immunogen Epitope innerhalb des Myelom V_LIg erkennen können, die durch DC auf MHC Klasse I Molekülen präsentiert werden. Außerdem spielt das CD86 kostimuluierende Molekül eine entscheidende Rolle in der Aktivierung naiver T-Zellen. Diese Ergebnisse könnten eine Grundlage bilden zur Etablierung einer V_LIgPl-basierten Immuntherapie beim multiplen Myelom.

Dendritische Zellen, Retrovirus-vermittelte Genübertragung, V_LIg von Plasmozytom

Contents

1 Introduction

1.1 Paraprotein

A secreted monoclonal immunoglobulin (Ig) molecule or its fragment (free heavy or light chain) accumulating in plasma is called a paraprotein. A secreted molar surplus of its light chain, escaping through the glomerular filter and appearing in urine, is called Bence Jones protein (Stevenson, 1998). Paraproteins and Bence Jones proteins are normally detected on protein electrophoresis of serum or urine, respectively, as a dense narrow band of γ-mobility (Figure 1). The presence of a paraprotein implies a monoclonal proliferation of Ig-secreting B cells or plasma cells, which can be premalignant or malignant (Samson, 2000).

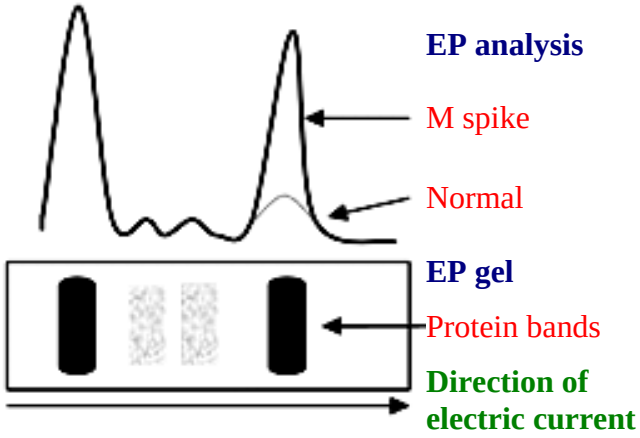


Figure 1: *Multiple myeloma serum electrophoresis shows a notable narrow "spike" of monoclonal immunoglobulins (paraproteins) of g-mobility. Electrophoresis using urine samples can also detect Bence Jones proteins. EP: electrophoresis, M: monoclonal immunoglobulin (from Multiple Myeloma Research Foundation, MMRF).*

Diseases associated with paraproteins

Diseases associated with paraproteins are classified into plasma cell malignancies, B cell lymphoid malignancies (including Waldenstrom´s macroglobulinaemia), monoclonal gammopathy of undetermined significance (MGUS) and primary amyloidosis. Plasma cell malignancies include multiple myeloma, solitary plasmocytoma of bone and extramedullary plasmocytoma. Waldenstrom´s macroglobulinaemia is a malignancy of IgM secreting

lymphoplasmacytoid cells, associated with lymphadenopathy and splenomegaly as well as marrow involvement. Other B cell tumors including chronic lymphocytic leukaemia and hairy cell leukaemia are sometimes associated with the presence of a paraprotein. MGUS is a disorder in which a paraprotein is present with no other features to suggest a diagnosis of myeloma or other B cell malignancy. Although some cases remain stable for many years, approximately 1 % cases transform to overt multiple myeloma every year. More rarely transformation to another B cell malignancy may occur. Primary amyloidosis is a condition in which a paraprotein is produced whose particular characteristics lead to deposition of fragments of immunoglobulin light chains as amyloid fibrils within tissues. Approximately 10 % of patients with myeloma will develop primary amyloid and rare cases are associated with Waldenstrom´s macroglobulinaemia and other B cell tumors (Samson, 2000).

1.2 Multiple myeloma

Multiple Myeloma (MM) is a tumor of plasma cells, occurring usually within the bone marrow but sometimes at other body sites. Neoplastic cells form discrete nodules, which erode the surrounding bone. All malignant plasma cells in plasmocytoma show identical rearrangements and mutations of immunoglobulin variable genes and secrete a monoclonal (identical) immunoglobulin (Ig) or its fragments. In the course of disease the third complementarity-determining region (CDRIII) of this immunoglobulin remain stable (Sahota *et al*., 1997; Kosmas *et al*., 1999), i.e., there is no evidence of ongoing somatic hypermutation (Ludwig *et al*., 1999). The idiotype (i.e. the unique antigenic structure) of monoclonal immunoglobulin in an individual patient can be a tumor-specific antigen (Bataille and Harousseau, 1997). However, due to genetic recombination, there are several myelomas lacking production of immunogloblin heavy chain but have free monoclonal light chains (Sakai *et al*., 1993). Therefore, in these cases of plasmocytoma, a defined tumor-associated antigen is provided by the variable region of the corresponding light chain (V_LIgPl) in order to elicit a tumor specific immune response.

In about 75 % of cases there is an obvious plasma paraprotein (in order of frequency IgG, IgA, IgD or IgE), in about 50 % a Bence Jones protein excretion exceeding 500 mg day^{-1} and in perhaps 10 % a clinically significant light-chain amyloidosis, associated with tissue

deposition of the tumor light chains or fragments thereof (Stevenson, 1998). Occasionally (< 1 %) myeloma is "non-secretory" and produces no monoclonal immunoglobulin.

Nonetheless the disease can remain asymptomatic for many years. In the symptomatic phase the most common presenting complaint is bone pain (Durie, 1996). The clinical manifestations are diverse but often include paraproteinemia, osteolytic lesions, impairment of renal function and hyporegenerative bone marrow (Dimopoulos *et al*., 1995; Clark *et al*., 1999).

1.2.1 Incidence

Multiple myeloma accounts for approximately 1 % of all cancers and 10 % of hematological malignancies (Chiriva-Internati *et al*., 2001). It is considered as a disease of older adults, presenting most commonly in individuals aged over 50 years, with a median age of 70 in women and 68 in men (Huang *et al*., 1999). The incidence of myeloma is 3-4/100,000 in the USA. This leads to approximately 13,500 new cases of myeloma in the USA each year. Myeloma is more common in blacks than whites. The incidence varies from country to country around the world from a low of 1/100,000 in China to approximately 4/100,000 in most Western industrialized countries. The male/female ratio is 3:2. The incidence rises with age. Better diagnostic techniques and the higher average age of the general population may explain the rising incidence over the last decades. However, a trend towards more frequent myeloma in patients under age 55 implies important environmental causative factors in the past 3-4 decades (Durie, 1996).

1.2.2 Pathophysiology

Multiple myeloma is now known to arise from a post germinal center B cell in the lymph node which homes to the bone marrow. Interactions with stromal cells in the marrow facilitate homing and growth of the myeloma cells. The expression of adhesion molecules such as NCAM (CD56), syndecan 1 (CD138) and PECAM 1 (CD31) on myeloma plasma cells is thought to facilitate homing to the marrow (Van Riet *et al*., 1998). In addition, neoplastic plasma cells have been found to express high levels of the IL-6 receptor (Foxwell *et al*., 1998). The stromal cells produce IL-6, which is an important growth factor for myeloma cells,

while myeloma cells produce factors such as TNF-α and IL-1β that activate osteoclasts, resulting in myeloma bone disease. These cytokines also stimulate the stromal cells to produce IL-6. Myeloma cells also produce vascular endothelial growth factor, which results in increased microvessel formation in the marrow promoting tumor growth (Samson, 2000).

Researchers have found that excess IL-6 production may result from infection with Kaposi's sarcoma-associated herpesvirus (KSHV) also called human herpesvirus-8 (HHV-8). This virus has been found in the blood of most patients with myeloma. The viral genome codes for production of a number of viral homologues of proteins involved in cell proliferation and cell death including a vIL-6 homologue. In view of the role of IL-6 in myeloma growth it seems possible that HHV-8 might also play a role in the pathogenesis of myeloma. This discovery needs to be confirmed in additional studies but, if true, represents an important advance in understanding (and possibly, treating or preventing) this cancer (Samson, 2000).

Recent studies have found that abnormalities of some oncogenes (such as c-*myc*) develop early in the course of plasma cell tumors. Changes in other oncogenes (such as N-*ras* and K-*ras*) are more often found in myeloma after bone marrow relapse and changes in tumor suppressor genes (such as p53) are associated with spread to other organs (Durie, 1996; Bataille and Harousseau, 1997). Table 1 shows chromosomal aberration in multiple myeloma (Ludwig *et al*., 1999). Deletions of tumor suppressor genes (such as p53), partial or total loss of chromosome 13 and rearrangements of band 14q32 (the immunoglobulin heavy-chain locus) and 11q13 are frequently found in multiple myeloma, and were shown to harbour prognostic significance. These cytogenetic abnormalities appear to make the myeloma more aggressive and resistant to treatment (Ludwig *et al*., 1999; Drach *et al*., 2000). Deletions of p53 and retinoblastoma (Rb)-gene lead to disruption of cell cycle control. Furthermore, loss of these tumor-suppressor genes may lead to increase autocrine secretion of IL-6 by tumor cells, since the p53 and Rb-gene products normally repress the IL-6 promoter (Santhanam *et al*., 1991). In multiple myeloma, p53 aberrations do not seem to be the initiating event (Ackermann *et al*., 1998). However, IgH translocations may be a nearly universal and possibly early event in multiple myeloma (Hallek *et al*., 1998). The chromosomal aberration was proposed as novel prognostic factor, since survival time was only 13.9 months from diagnosis for patients with a deletion of the p53 gene versus 38.7 months for patients with absence of this abnormality (Drach *et al*., 1998). Deletion of Rb (13q12) or monosomy 13 was associated with a median survival of 10 months in patients receiving standard treatment

versus 34 months for patients with other abnormalities (Seong *et al*., 1998). In addition, patients with an unfavorable karyotype had a median overall survival of 26 months versus 69^{+} months for patients with an abnormal karyotype but absence of translocation, deletion of Rb or 11q abnormalities (Ludwig *et al*., 1999).

Oncogene/tumor suppressor gene	Chromosomal aberration	Frequency (%)
retinoblastoma (Rb)	del 13q12	50 %
ras-mutations	-	34 %
p53	del 17p13	33 %
FGFR3	t (4; 14)(p16; q32)	25 %
bcl-1/cyclin D1	t (11; 14)(q13; q32)	20 %
bcl-2	t (14; 18)(q32; q21)	< 5 %
c-*myc*	t (8; 14)(q24; q32)	< 5 %

Table 1: *Molecular mechanisms of malignant transformation in multiple myeloma (Ludwig et al., 1999).*

Uncontrolled growth of plasma cells has a large range of consequences including bone marrow failure, increased plasma viscosity, suppression of normal immunoglobulin production, skeleton destruction and renal insufficiency. The characteristic sharply demarcated bone defects are due to osteoclast activation by TNF-α and IL-1β released by tumor cells. X-rays may show lytic lesions and generalized osteoporosis is also common. Vertebral collapse is frequent in myeloma patients, leading to back pain, kyphosis and loss of height and occasionally resulting in cord compression. Pathological fracture of a long bone may also occur. The bone destruction may result in hypercalcaemia, although serum alkaline phosphatase is usually normal since bone alkaline phosphatase reflects osteoblast activity and in myeloma osteoblast activity is reduced (Samson, 2000). 25-30 % of patients have some degree of renal impairment and about 5 % will present with acute renal failure. This is most commonly due to Bence-Jones protein, which damages the tubules as it passes through the kidney. The classic histological features are of fractured distal tubular casts with a surrounding chronic inflammatory infiltrate including giant cells (myeloma kidney). Much less frequently light-chain deposition may produce a form of glomerulopathy (light-chain amyloidosis). Other factors that can contribute to renal failure include hypercalcaemia, infection, dehydration, hyperuricaemia and amyloid deposition. Renal failure, which results acutely from hypercalcaemia or dehydration, is often reversible with appropriate management

but that due to Bence-Jones protein is less likely to recover (Samson, 2000). Anaemia is another common presenting future and appears to be mediated by cytokines such as IL-1β rather than being directly due to marrow replacement. In some patients renal failure may also contribute to the anaemia (Samson, 2000). About 10 % of myeloma patients develop primary amyloidosis. Kidneys usually are affected with deposition of amyloid in the glomeruli leading to generalized proteinuria and nephrotic syndrome. Peripheral neuropathy (particularly carpal tunnel syndrome), congestive cardiac failure and involvement of skin, muscle and join may also occur. Peripheral neuropathy may also occur in myeloma patients even without amyloidosis. Very high Immunoglobulin (Ig) particularly of IgA may result in hyperviscosity syndrome, with headaches, visual disturbance and loss of concentration (Samson, 2000). Increased bleeding is often accentuated by thrombocytopenia, in addition to the binding of monoclonal proteins to clotting factors and/or platelets (Durie, 1996). There is impairment of both humoral and cell-mediated immunity due to neutropenia and decreased production of polyclonal immunoglobulins leading to an increased susceptibility to infection, both bacterial and viral. Although pneumococcal pneumonia is one of the "classical" infections associated with myeloma, other bacteria such as streptococci and staphylococci are now frequently isolated. Also, Haemophilus and Herpes zoster infections are often seen (Durie, 1996).

1.2.3 Newer treatment strategies

Myeloma is still an incurable disease. Using standard chemotherapy, complete remission rates have not exceeded 5 % and the median survival has not been extended beyond 3 years (Alexanian and Dimopoulos, 1994). High-dose chemotherapy or chemo-radiotherapy with haematopoietic stem cell support would be an alternative to extend event-free and overall survival and to increase the complete response rate after therapy (Attal *et al*., 1996; Barlogie *et al*., 1997). However, relapse is still a major cause of treatment failure (Peest *et al*., 1995; Tricot *et al*., 1995). Therefore, novel alternative therapeutic strategies are much needed in these patients (Lim and Bailey-Wood, 1999; Wen *et al*., 2001). The development of immunotherapy approaches designed to get cytotoxic T lymphocytes (CTL) may help to eradicate or control residual tumor cells (Tarte *et al*., 1999). The use of professional antigen-presenting cells (APC) such as dendritic cells (DC) to generate anti-tumor response is a promising strategy (Hart, 1997; Schuler and Steinman, 1997). Vaccination of patients with DC pulsed with monoclonal Ig as tumor antigen (Ag), tumor RNA or cell lysates have already

begun in MM (Lim and Bailey-Wood, 1999; Reichardt *et al*., 1999; Hajek and Butch, 2000; Timmerman and Levy, 2000; Titzer *et al*., 2000).

1.3 Dendritic cells

Dendritic cells (DC) are found at trace levels in tissues or in circulation and are inducing many lymphocyte functions (Banchereau *et al*., 2000). During the past decade, DC have been identified as the most potent APC of the immune system (Cella *et al*., 1997; Rughetti *et al*., 2000), which play a key role in generating primary and probably secondary immune responses against specific antigens. Dendritic cells have unique migratory properties (Austyn, 1996) and the capacity to acquire, process and then present antigens in association with the appropriate accessory signals to T lymphocytes (Steinman, 1991; Hart, 1997). The role DC play in the body's natural immune response has become an important question. The increased tumor incidence in immunosuppressed patients implies that the immune system controls tumor evolution and several studies have suggested that greater numbers of DC within a tumor lead to a better prognosis. However, the existence of a malignant tumor and its inevitable progression without treatment demonstrate the ability of tumors to escape DC-mediated immune surveillance (Troy and Hart, 1997).

The superior ability of DC to present antigens to T cells has led to the development of DC-based strategies for the purpose of enhancing the immune response against tumors and infectious agents (Banchereau *et al*., 2000; Kirk and Mule, 2000). A growing number of tumor-associated antigens (TAA) have been identified that are recognized by T lymphocytes and are capable of provoking an anti-tumor immune response (Boon *et al*., 1994). Dendritic cells have now been shown to be effective in stimulating TAA-specific T lymphocyte responses *in vitro*. These results and significant DC-induced responses in animal models have encouraged the first clinical attempts (Hsu *et al*., 1996; Murphy *et al*., 1996; Nestle *et al*., 1998) to exploit DC for cellular immunotherapy against cancer. However, the application of DC in immunotherapy has been hampered by the low number of these cells and the difficulty of isolating them (Heemskerk *et al*., 1999).

Mature DC are typically characterized by expression of MHC class I, MHC class II, the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) and the dendritic cell lineage marker

CD83 (Hart, 1997). The identification of culture conditions enabling the generation of mature DC *in vitro* is useful to study their biology and to prepare large amounts of APC for immunotherapeutic purposes (Ferlazzo *et al*., 1999).

1.3.1 *In vitro* generation of DC

It is now possible to generate a large number of DC *in vitro* from either peripheral blood monocytes (Kiertscher and Roth, 1996; Zhou and Tedder, 1996) or from $CD34^+$ progenitor cells in cord blood [CB] (Santiago-Schwarz *et al*., 1992; Sato *et al*., 1998), bone marrow [BM] (Reid *et al*., 1992; Szabolcs *et al*., 1995) and peripheral blood [PB] after cytokine mobilization (Strunk *et al*., 1996; Ratta *et al*., 1998). CD34-derived dendritic cells (CD34-DC) have a preferential capacity to activate $CD8^+$ T cells (Ferlazzo *et al*., 1999). These methods used different cytokine-cocktails to stimulate *in vitro* growth and differentiation and, although time consuming, have the capacity to generate expanded DC numbers (Troy and Hart, 1997). Because cytokine receptor stimulation activates complex signalling cascades that initiate multiple responses, differentiation to other lineages is almost simultaneously induced. Thus cytokine application not only generates DC but also macrophages and neutrophils as well (Santiago-Schwarz *et al*., 1992; Szabolcs *et al*., 1995; Bender *et al*., 1996). In addition, FCS was used as culture supplement in most protocols described. There are many problems with using serum-replete medium to generate DC such as variation in the cytokine content of serum, immunogenicity of animal proteins and the risk of infectious agents. Recently, serum-free culture conditions have been described for generation of cord blood-derived dendritic cells (CB-DC) in the presence of SCF, GM-CSF, TNF-α and TGF-β1 (Bello-Fernandez *et al*., 1997). In addition, DC can be generated from mobilized peripheral blood $CD34^+$ cells in the absence of bovine products using X-Vivo 10 culture medium containing 10 % autologous serum, rhGM-CSF, rhTNF-α and rhIL-4 (Ardeshna *et al*., 2000a).

1.3.2 Antigen processing and presentation by DC

Antigen processing has been described traditionally as occurring through one of the two distinct pathways identified for endogenous and exogenous antigens. Endogenously derived antigenic peptides bind with transporter (TAP) gene products in the cytosol and are delivered

to the MHC class I compartment in the endoplasmic reticulum for incorporation into the peptide-binding groove of the MHC molecule. These MHC class I / peptide complexes are then transported to the cell surface, where they are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) (Lanzavecchia, 1996). Exogenous antigens enter the DC via endocytosis, phagocytosis or macropinocytosis (Watts, 1997) and are degraded in low pH endosomal compartments into peptide fragments that then complex with class II MHC molecules for surface presentation to $CD4^+$ T helper (Th) cells (Kleijmeer *et al*., 1995; Nijman *et al*., 1995). It has become apparent that this classic dichotomy between exogenous (MHC II restricted) and endogenous (MHC I restricted) antigen presentation does not account for all antigen processing, particularly in DC. Additional mechanisms have been identified whereby exogenously acquired antigens are processed and presented on MHC class I molecules (Watts, 1997). Thus, antigens taken up by macropinocytosis can be released as peptides to bind with TAP proteins in the cytosol for delivery to the MHC class I compartment (Norbury *et al*., 1997). Alternatively, peptides when present in high concentrations may externally exchange with peptides on surface MHC molecules, thus bypassing intracellular processing. Endogenous antigens (often autologous) can also enter the MHC class II pathway and generate significant $CD4^+$ responses (Lechler *et al*., 1996). However, for therapeutic applications the binding affinity and stimulatory activity of each such tumor derived peptide needs to be characterized.

1.3.3 Co-stimulatory molecules of DC

In addition to presenting MHC/Peptide antigen complexes, DC must (as APC) provide essential accessory signals to T lymphocytes. This requires intimate cellular contact between DC and T lymphocytes. Expression of one or both of the co-stimulator molecules B7-1 (CD80) and B7-2 (CD86) on APC are essential for effective activation of naive T lymphocytes (Guinan *et al*., 1994). These co-stimulatory molecules bind to CD28 molecule on T lymphocytes. If this fails to occur at the time of antigen recognition by the T cell receptor, a different T lymphocyte function such as anergy results (Guinan *et al*., 1994; Schwartz, 1996). CD86 is the most important ligand to induce CD28-mediated co-stimulation for $CD8^+$ T cell activation (Van Gool *et al*., 1999). On naive T cells, CD28 is the only receptor for B7 molecules. Once T cells are activated, however, they express an additional receptor called CTLA-4 (CD152). CTLA-4 closely resembles CD28 in sequence and the two

molecules are encoded by closely linked genes. CTLA-4 binds B7 molecules about 20 times more avidly than CD28 and delivers an inhibitory signal to the activated T cell. This makes the activated progeny of a naive T cell less sensitive to stimulation by the antigen-presenting cell and limits the amount of the T cell growth factor IL-2 produced. Thus, binding of CTLA-4 to B7 molecules is essential for limiting the proliferative response of activated T cells to antigen and B7 on the surface of antigen-presenting cells (Gribben *et al*., 1995, 1996; Rudd, 1996). This was confirmed by producing mice with a disrupted CTLA-4 gene. Such mice develop a fatal disorder characterized by massive proliferation of lymphocytes (Tivol *et al*., 1995). Other DC surface molecules, including CD40, probably contribute to T lymphocyte activation (McLellan *et al*., 1996) and it is possible that the DC co-stimulatory molecule repertoire, which may include other surface molecules, partially defines the type of T lymphocyte response resulting. For example, CD80 and CD86 may generate Th1- and Th2-based response, respectively (Kuchroo *et al*., 1995). Dendritic cells also produce cytokines, such as IL-12 (Heufler *et al*., 1996) and IL-7 (Mehrotra *et al*., 1995), which contribute to T cell activation and CTL differentiation. Other APC, such as B cells and macrophages, are also effective stimulators of primed T lymphocytes but the DC is special in its ability to stimulate naive T lymphocytes and hence, it is predicted to be critical in driving any initial response against TAA. Finally, a notable feature of DC relevant to their therapeutic use is that their ability to take up and process antigen declines as they become differentiated/activated into co-stimulatory cells (Troy and Hart, 1997).

1.3.4 The effect of malignancy on DC function

In a clinical study on DC within renal cell carcinoma, DC represented only a small proportion of the infiltrating leukocytes. Of these, only a small subset of DC was found to display an activated phenotype. These activated DC, when isolated, proved to be significantly stronger stimulators of T lymphocytes than either the inactivated DC or the tumor macrophages. These findings suggest that the tumor environment inhibits DC maturation and activation, providing a possible explanation for the paradox that antigen-expressing tumor cells can be recognized by T lymphocytes *in vitro* yet escape tumor surveillance and grow into lethal tumors in immunocompetent hosts (Troy and Hart, 1997).

Potential mechanisms for the defects in APC function with the result of T cell anergy observed in tumor-bearing hosts have been identified recently. Vascular endothelial growth factor, which is produced by most solid tumors, has been identified as directly responsible for inhibiting maturation of DC from precursor cells (Gabrilovich *et al*., 1996a). IL-10, also produced by many tumors (Sato *et al*., 1996), inhibits up-regulation of CD80/CD86 co-stimulatory molecules on APC (Willems *et al*., 1994), and their expression is essential for effective DC-T lymphocyte interaction. Activation of T lymphocytes by APC, in the presence of IL-10, leads to long-term anergy in the T lymphocytes (Groux *et al*., 1996). Transforming growth factor-β (TGF-β) has also been shown to indirectly inhibit the activation of T lymphocytes by DC (Lipscomb *et al*., 1993; Summers *et al*., 1999).

The effects of malignancy on DC function may not be confined to the tumor. A study in mice found that mature blood DC from tumor-bearing animals were ineffective at inducing anti-tumor CTL (Gabrilovich *et al*., 1996b), whereas DC generated from BM precursors remained fully functional. Tumor cell supernatants did not affect the function of mature DC obtained from the spleen of tumor-bearing animals, but significantly suppressed the ability to generate functional DC from the bone marrow of control mice *in vitro*. This suggests that tumor cells may release factors, which block early stages of DC maturation from precursors (Gabrilovich *et al*., 1996b). Breast cancer patients have also been suggested to have lower numbers of mature DC and these cells showed reduced ability to stimulate autologous and allogeneic mixed lymphocyte reactions (MLRs). However, the cytokine-generated DC derived from circulating precursors were again fully functional (Gabrilovich *et al*., 1997). Cytokine-generated DC from patients with renal cell carcinoma (Radmayr *et al*., 1995) and prostate cancer have also been shown to be fully functional (Tjoa *et al*., 1995). Routine DC counting is now being applied to a range of patients, including cancer patients (Troy and Hart, 1997).

1.3.5 DC as cellular immunotherapy

There are now multiple methods for providing DC with antigens to process and present (Shurin, 1996). Initial studies used extracts from tumor cell lysates with variable degree of purification. This method avoids the restriction of a define TAA but clearly increases the theoretical risk of immunizing against self-antigens (Troy and Hart, 1997). Purified proteins,

stripped tumor cells peptides (Zitvogel *et al*., 1996) and synthetic peptides (Shurin, 1996) permit a more specific immunization. Presentation of TAA by these methods may be limited by turnover of MHC complexes, but DC retain antigen for prolonged periods and migrate relatively quickly to draining lymph nodes (Barratt-Boyes *et al*., 1997). Longer lasting presentation of antigen may be obtained by DNA-based expression within the DC, but whether this is more effective remains to be established (Troy and Hart, 1997). This has been achieved by either transduction with specific cDNA (Condon *et al*., 1996) or the establishment of DC/tumor cell chimeras using cell fusion techniques (Gong *et al*., 1997). Introducing genes into DC by retroviral transduction of $CD34^+$ progenitor cells is now an established method to potentiate specific immune response against tumor antigens (Reeves *et al*., 1996).

1.4 Retroviral vectors

1.4.1 Basics of retrovirus biology

Retroviruses have been widely employed as vector systems because they can permanently express a foreign gene into mitotic cells. Moreover, they can infect virtually every type of mammalian cell, making them exceptionally versatile. The virus itself is surrounded by a phospholipid membrane, derived from the host cell from which it has arisen (Figure 2). Imbedded in the membrane are several different glycoproteins, involved in host cell recognition and facilitating virus-cell fusion events. Within this membrane envelope there is a protein capsid containing genetic material for the virus. Retroviruses contain 2 identical single-stranded RNA genomes, and are prepackaged with proteins enabling the virus to insert DNA into the host cell genome. The capsid contains reverse transcriptase, which enables the virus to convert its RNA into DNA and integrase, which helps facilitate the insertion of this viral DNA into the host's genome. The viral genome is then maintained within the cells as it undergoes normal processes, and remains in this fashion even as the virus becomes active and replicates itself (Smith, 1995).

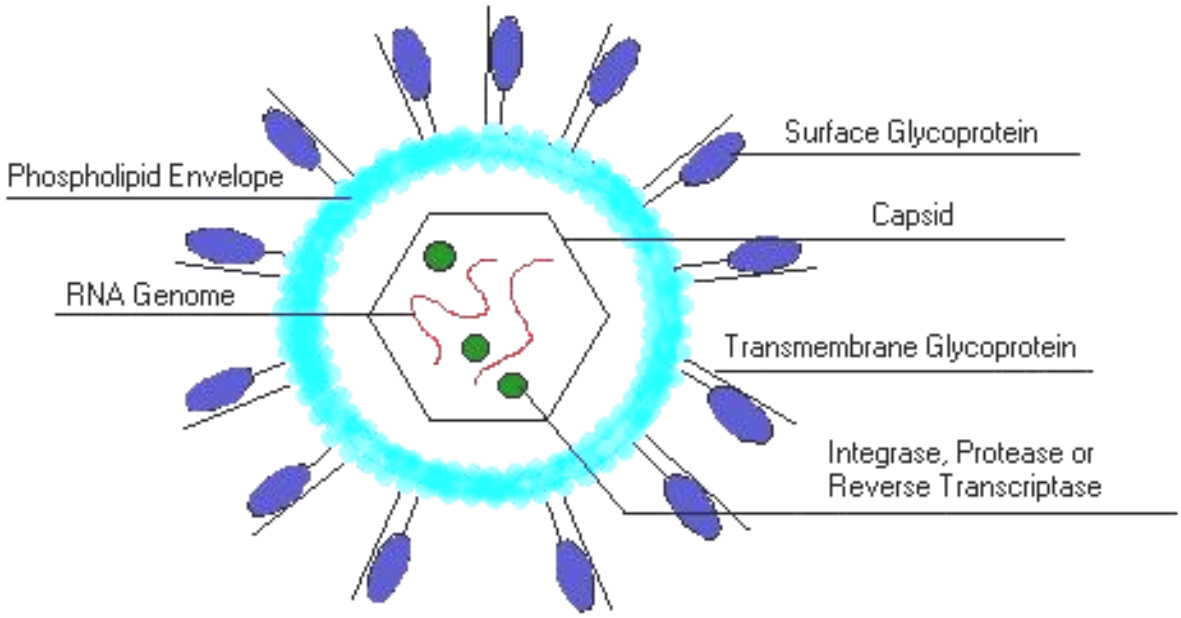


Figure 2: *Retroviral structure (Smith, 1995).*

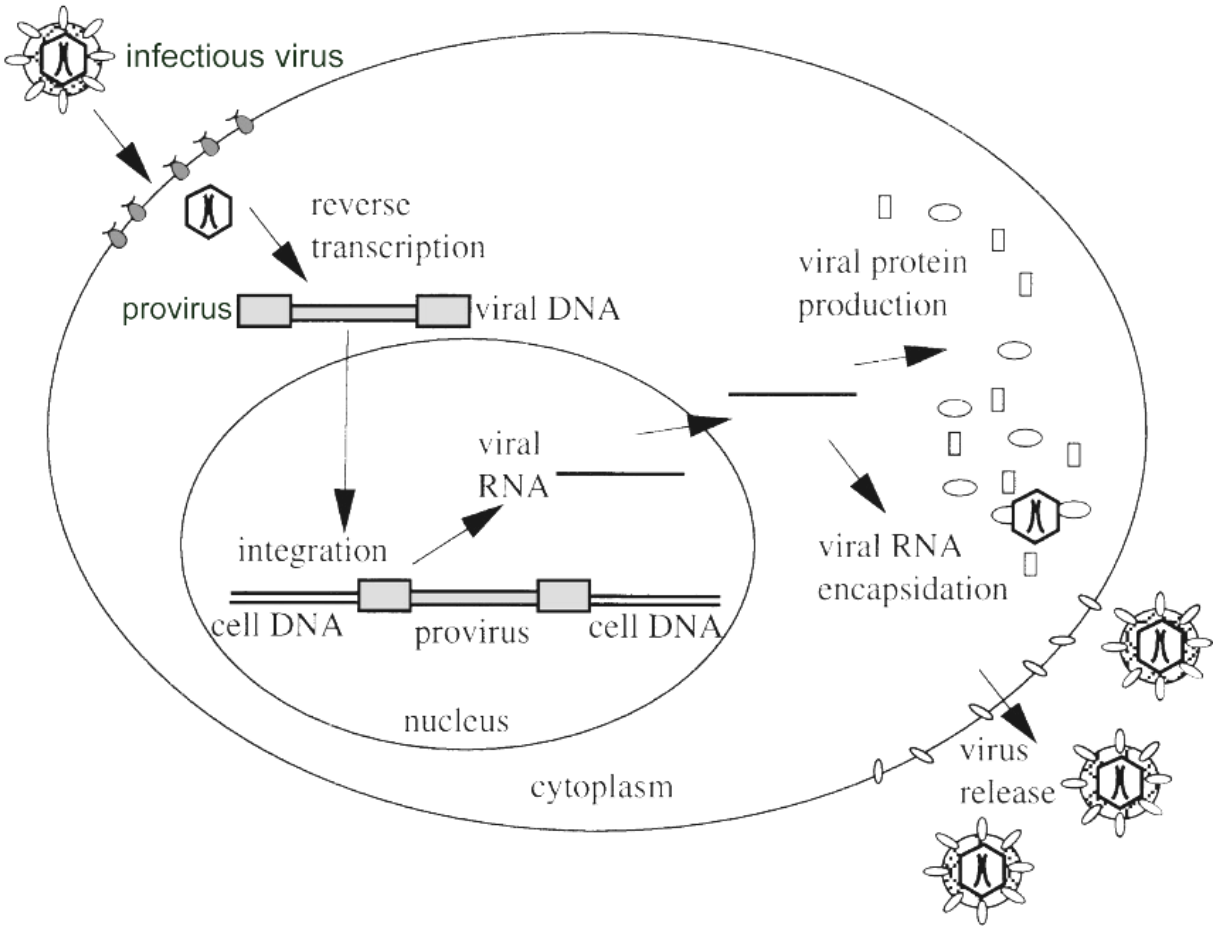


Figure 3: *Retroviral replication cycle (Buchschacher and Wong-Staal, 2000).*

1.4.1.1 Replication cycle of retroviruses

Basic features of a generic retroviral replication cycle are shown in Figure 3. Infection begins when the virus envelope glycoprotein binds to specific receptors located on the cell surface, followed by fusion of the viral envelope and cell membrane and release of the virus core into the cytoplasm. Viral RNA is uncoated, copied into a double-stranded DNA molecule and transported to the nucleus during cell division. Although they are shown in the diagram as occurring in a stepwise manner, the exact timing and mechanism of these events are unclear. Integration into chromosomal DNA enables the viral genome to be stably maintained, replicated during DNA synthesis and passed to progeny cells. This feature makes retroviral vectors useful for permanently introducing foreign genes into cells. Viral RNA is produced and translated to produce viral proteins and enzymes. Viral RNA also associates with viral proteins to form new core particles. Mature progeny virions capable of infecting new cells are formed when the cores obtain their envelopes, consisting of cellular membrane and envelope glycoprotein, when they are released from the cell (Buchschacher and Wong-Staal, 2000).

1.4.1.2 Genome structure of retroviruses

The retroviral genome varies in complexity between the different viruses in this family. A generic retrovirus, however, contains only three genes (Figure 4). *Env* encodes the glycoprotiens expressed in the viral envelope, *pol* encodes reverse transciptase and *gag* encodes the capsid proteins. Surrounding these genes are LTR's (long terminal repeats), which contains regulatory sequences, and sequences that are important as the genome inserts itself (Smith, 1995).

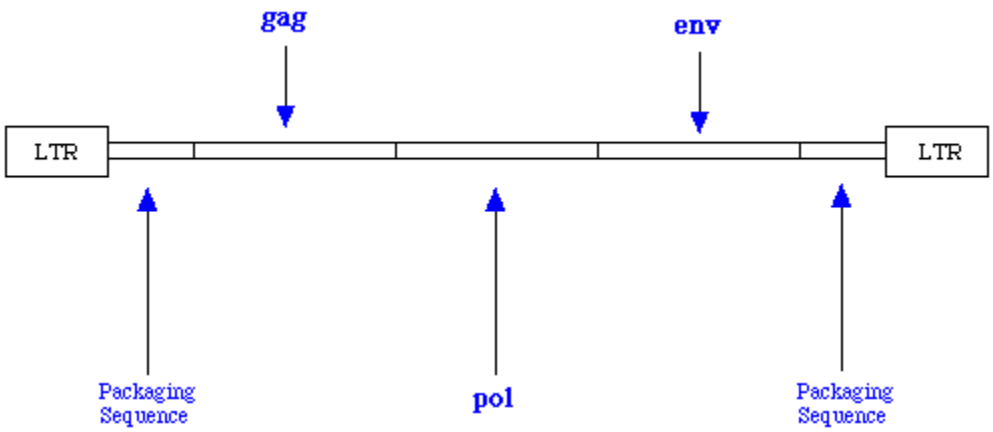


Figure 4: *General genome structure of retroviruses (Smith, 1995).*

1.4.2 Retroviral vector systems

Retroviral vectors are virus derivatives typically engineered to be replication defective. They are capable of infecting and integrating a foreign gene into a target cell but are unable to multiply and spread to other cells. Vectors are created by removing viral genes from a virus genome, leaving only the *cis*-acting sequences (regions recognized by viral proteins) necessary for a single round of replication. A diagram of the architecture of a "typical" oncoretroviral vector is shown in Figure 5. As illustrated, all viral genes are removed and replaced with either a marker gene (for example, a gene that confers drug resistance) used to monitor the vector infection of cells or another foreign gene of interest. More complex vectors capable of expressing two or more foreign genes through additional heterologous, internal viral promoters or internal ribosome entry site sequences have been designed. Figure 5 shows *cis*-acting sequences that must be present on the vector for propagation to take place. These sites are used during various stages of virus replication including reverse transcription of genomic RNA [LTR, primer binding site (pbs), polypurine tract (ppt)], integration of the viral DNA into chromosomal DNA (*att* sites), transcription of the provirus (LTR) and packaging of viral RNA into progeny virions (encapsidation site) (Buchschacher and Wong-Staal, 2000; Hu and Pathak, 2000).

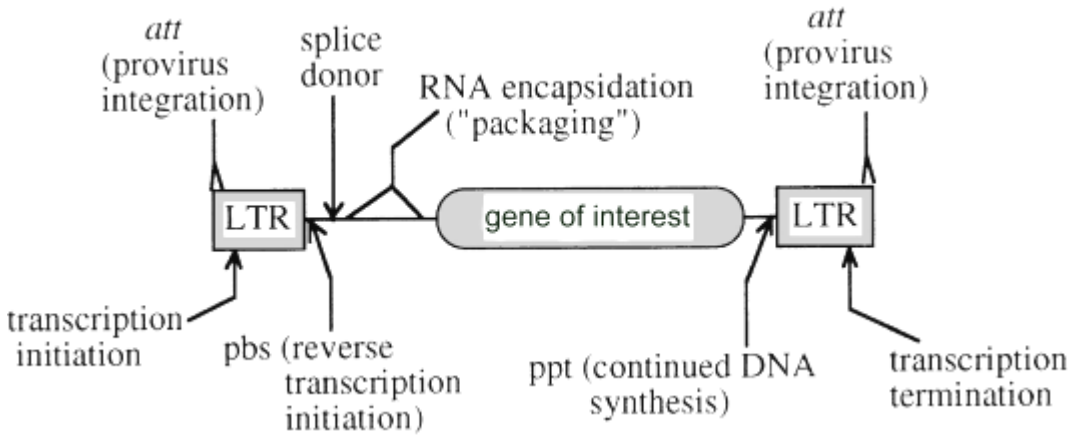


Figure 5: *cis-Acting elements of a typical retroviral vector (Buchschacher and Wong-Staal, 2000).*

Constructs containing these *cis*-acting sequences can be propagated in cultured cells and can therefore serve as vectors when *gag*, *pol* and *env* gene products are provided in *trans* (expressed from other constructs). This *trans*-complementation can be accomplished in a number of ways. Vector and wild-type virus DNA plasmid constructs can be cotransfected (introduced by chemical or electrical means) into cells in culture. When vector and wild-type

virus (also called "helper virus" because it assists in vector propagation) constructs are contained within a single cell, the gene products provided by the replication-competent virus result in the production of virus particles containing the vector RNA genome. These virions can then infect and introduce the vector genome into other cells. The fact that replicating wild-type virus is present prevents the study of a single viral replication cycle and obviously would be unacceptable for clinical use (Buchschacher and Wong-Staal, 2000; Hu and Pathak, 2000).

Techniques capable of propagating vectors without using wild-type virus have been developed. Cotransfection of a vector DNA construct and a plasmid expressing viral gene products (but not containing *cis*-acting sequences necessary for propagation) results in a single cycle of vector propagation. Because the construct(s) expressing viral proteins do not contain sequences necessary for replication, helper virus is not produced. After virions containing the vector genome are harvested from cotransfected cells and are used to infect fresh cells, the vector will not be replicating within infected cells because of lack of expressed viral proteins (Buchschacher and Wong-Staal, 2000; Hu and Pathak, 2000).

The third and probably best method for propagating retroviral vectors involves the use of packaging cell lines. There are cell lines that express viral proteins necessary for vector propagation. Such cell lines have improved greatly the efficiency by which virus containing vector genomes can be produced (Miller, 1990). Packaging cell lines are made by introducing viral genes on plasmids into cells, which are reproduced on each cell division resulting in a constant production of viral structural proteins and replication enzymes. After introduction of vector genome into these cells, vector particles are produced (Figure 6). Again, helper virus should not be produced, and the vector is propagated only over a single replication cycle.

Packaging cell lines are the preferred method to propagate a retroviral vector for two primary reasons. First, the process used to generate the virus is generally simpler and more efficient at producing a higher titer of vector virus than the cotransfection techniques. Second, the use of packaging cells reduces the possibility that wild-type virus will be regenerated inadvertently by recombination between helper and vector sequences during the transfection process; therefore, the virus is considered safer for clinical use. Nonetheless, packaging cell lines are regularly and rigorously tested for replication-competent retrovirus by using long-term culture

to amplify any regenerated wild-type virus, thus increasing the sensitivity of detection (Buchschacher and Wong-Staal, 2000; Hu and Pathak, 2000).

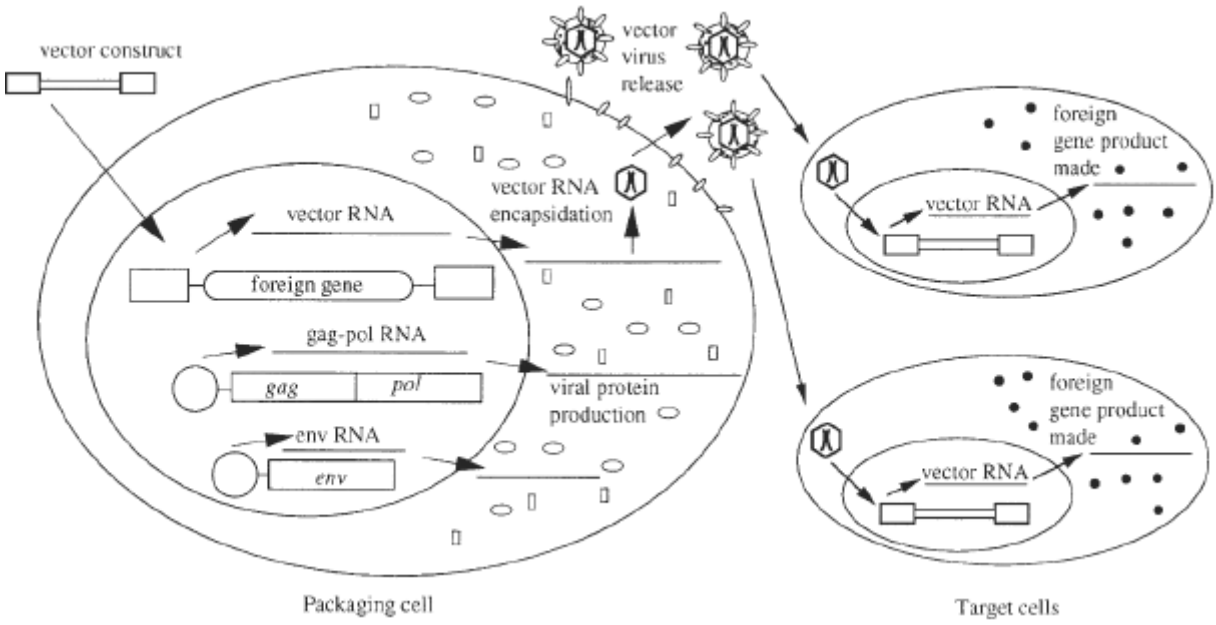


Figure 6: *Retroviral packaging cell line and viral vector production (Buchschacher and Wong-Staal, 2000).*

1.4.3 Progress with retroviral vectors

Retroviral vectors are one of the most promising systems for the transfer and the expression of therapeutic genes in human gene therapy protocols. Compared with other gene transfer systems, retroviral vectors have several advantages, including their ability to transduce a variety of cell types, to integrate efficiently into the genomic DNA of the recipient cells and to express the transduced gene at high levels. The design of different types of packaging cells has evolved to reduce the possibility of helper virus production. The host range of retroviruses has been expanded by pseudotyping the vectors with heterologous viral glycoproteins and receptor-specific ligands (Palu *et al*., 2000).

1.5 Aim of work

Since myeloma is still an incurable disease despite some recent progress in the development of high dose chemotherapy, novel alternative therapeutic strategies are much needed in these

patients. The development of immunotherapy approaches designed to get cytotoxic T lymphocytes (CTL) may help to eradicate or control residual tumor cells. The use of professional APC such as dendritic cells (DC) to generate anti-tumor response is a promising strategy.

Aim of this thesis was the induction of an *in vitro* autologous T cell proliferation restricted to MHC class I molecules against the variable region of paraprotein light chain of plasmocytoma (V_LIgPl) through autologous dendritic cells via retroviral transduction of human $CD34^+$ progenitor cells. The thesis aim was also to develop serum-free culture conditions enabling the generation of mature DC without other lineages generated to prepare large amounts of APC for *in vitro* studies.

Most retroviral vectors contain a bacterial antibiotic-resistance gene such as neomycin that is strongly immunogenic. Its peptides will be presented on MHC class I molecules and activate $CD8^+$ T lymphocytes, thus skewing the elicited immune response. In order to minimize such immune action against xenogenic peptides, aim of the thesis was also to construct a novel retroviral expression-cloning vector to direct the tumor-associated antigen of plasmocytoma fused with an artificial antibody-binding site such as FLAG to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor. Thereby, transfected or transduced cells should be detected using monoclonal anti-FLAG antibodies.

2 Materials and methods

2.1 Materials

2.1.1 Bacteria strains

DH10B (Resource Center of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics, RZPD):
F´ *mcr*A Δ(*mrr-hsd*RMS-*mrc*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K1 *rps*L *nup*G.

INVαF´ (Invitrogen BV):
F´ *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 λ^-.

TOP10F´ (Invitrogen BV):
F´ {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*msr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

XL1-Blue (Stratagene GmbH):
F´ ::Tn*10pro*A^+B^+ *lac*I^q D(*lac*Z)M15/*rec*A1 *end*A1 *gyr*A96 (Nal^r) *thi hsd*R17 (r_k^-, m_k^+) *sup*E44 *rel*A1 *lac*.

2.1.2 Cell lines

Hela (American Type Culture Collection, ATCC):
Human cervical carcinoma (Chen, 1988).

NIH/3T3 (ATCC):
Murine embryonic fibroblasts (Jainchill *et al*., 1969).

Jurkat wild type (ATCC):
Human acute T cell leukaemia, T lymphocyte (Gillis and Watson, 1980; Weiss *et al*., 1984).

GP+E86 (ATCC):
Ecotropic murine retrovirus packaging cell line (Markowitz *et al*., 1988).

PG13 (ATCC):
Pseudotyped murine retrovirus packaging cell line derived from TK-NIH/3T3 cells and based on the Gibbon ape leukemia virus, GaLV (Miller and Rosman, 1989; Miller *et al*., 1991).

ΦNX-Eco Cells (provided by Dr. Garry P. Nolan, Department of Molecular Pharmacology, Standford University School of Medicine):
High titre ecotropic human retrovirus-producing cell line derived from 293T cells (Pear *et al*., 1993; Yang *et al*., 1999).

ΦNX-Ampho Cells (provided by Dr. Garry P. Nolan):
High titre amphotropic human retrovirus-producing cell line derived from 293T cells (Pear *et al*., 1993; Yang *et al*., 1999).

2.1.3 Chemicals

The used fine chemicals were obtained from Applichem GmbH, Calbiochem-Novabiochem GmbH, ICN Biomedicals GmbH, Merck KGaA and Sigma-Aldrich Chemie GmbH. Distilled, deionized and ampura (sterile endotoxin-free) water were obtained from pharmacy of Hannover medical school (MHH).

2.1.3.1 Antibodies and fluorescence materials

Antibodies and fluorescence Materials	Clone	Isotype	Conjugate	Producer
CD1a	BL6	IgG_1 (Mouse)	PE	Immunotech GmbH
CD3	UCHT1	$IgG_{1,\kappa}$ (Mouse)	PE	DAKO
CD14	MϕP9	IgG_{2b}	FITC	Becton Dickinson GmbH
CD15	HI98	$IgM_{,\kappa}$ (Mouse)	PE	PharMingen GmbH
CD34	8G12	IgG_1 (Mouse)	FITC	Becton Dickinson GmbH
CD34	581	$IgG_{1,\kappa}$ (Mouse)	PE	Becton Dickinson GmbH
CD34	AC136	IgG_{2a} (Mouse)	FITC	Miltenyi Biotec
CD40	5C3	$IgG_{1,\kappa}$ (Mouse)	FITC	PharMingen GmbH
CD55	IA10	$IgG_{2a,\kappa}$ (Mouse)	Biotin, FITC, PE	PharMingen GmbH
CD83	HB15e	$IgG_{1,\kappa}$ (Mouse)	FITC	PharMingen GmbH
CD86 (B70/B7-2)	IT2.2	$IgG_{2b,\kappa}$ (Mouse)	Unconjugate, PE	PharMingen GmbH
Anti-FLAG M2			Biotin, FITC	Sigma-Aldrich Chemie GmbH
HLA-ABC	W6/32	IgG_{2a} (Mouse)	FITC	Serotec
HLA-ABC	B9.12.1	IgG_{2a} (Mouse)	Uconjugate, FITC	Immunotech GmbH
HLA-DR	G46-6 (L243)	$IgG_{2a,\kappa}$ (Mouse)	FITC	Becton Dickinson GmbH
HLA-DR	B8.12.2	IgG_{2b} (Mouse)	Unconjugate	Immunotech GmbH
HLA-DR	Immu-357	IgG_1 (Mouse)	PE	Immunotech GmbH
Avidin			FITC	Becton Dickinson GmbH
Streptavidin			PE	Becton Dickinson GmbH
7AAD (7-amino-actinomycin D)				PharMingen GmbH
Propidium Iodide				PharMingen GmbH
Isotype Controls	DAK-GO1	IgG_1 (Mouse)	FITC, PE	DAKO

2.1.3.2 Enzymes

The used enzymes were obtained from Roche Diagnostics GmbH, Gibco BRL, MBI Fermentas Molecular Biology GmbH, New England BioLabs, Pharmacia, Promega, Serva, Sigma-Aldrich Chemie GmbH and Stratagene GmbH.

2.1.3.3 Kits

-CD34 MultiSort Kit (Miltenyi Biotec)
-GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech)
-EndoFree Plasmid Maxi Kit (Qiagen GmbH)
-NucleoSpin® Plasmid Kit (Macherey-Nagel GmbH)
-Original TA Cloning® Kit (Invitrogen BV)
-Pan T Cell Isolation Kit (Miltenyi Biotec)
-QIAprep Spin Miniprep Kit (Qiagen GmbH)
-RNAzol™ B-Total RNA Isolation Reagent (Cinna Biotecx Labs Inc.)
-SuperFect™ Transfection Reagent (Qiagen GmbH)

2.1.3.4 Molecular weight standards

-100 bp DNA Ladder (New England BioLabs) with 1517, 1200, 1000, 900, 800, 700, 600, 500/517, 400, 300, 200, 100 bp fragments.

-1 kb DNA Ladder (Gibco BRL) with 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517/506, 396, 344, 298, 220, 201, 154, 134, 75 bp fragments.

-λ DNA-*Hind*III Fragments (Gibco BRL) with 23130, 9416, 6557, 4361, 2322, 2027, 564 bp fragments.

2.1.3.5 Oligonucleotides

The used Oligonucleotides were synthesized in MWG-Biotech. The lyophilized primers were dissolved in distilled water at a concentration of 100 µg/ml, aliquoted and stored at -20°C.

Oligonucleotide V_LIg PCR primers (Sahota *et al*., 1997)

Primer	Location	Orientation	Sequence (5' ➔ 3')
Vκ1&4	FR1	Sense	GACATCSWGATGACCCAGTCTCC
Vκ2&6	FR1	Sense	GAWRTTGTGMTGACTCAGTCTCC
Vκ3	FR1	Sense	GAAATTGTGTTGACGCAGTCTCC
Vκ5	FR1	Sense	GAAACGACACTCACGCAGTCTCC
Jκ1-4	FR4	Anti-sense	ACGTTTGATHTCCACYTTGGTCCC
Jκ5	FR4	Anti-sense	ACGTTTAATCTCCAGTCGTGTCCC
Vλ1	FR1	Sense	CAGTCTGTSBTGACKCAGCCRCCY
Vλ2	FR1	Sense	CAGTCTGCCCTGACTCAGCCTSSYT
Vλ3	FR1	Sense	TCYTMTGWGCTGACTCAGSMM
Vλ7&8	FR1	Sense	CAGRCTGTGGTGACYCAGGAGCCMTC
Vλ9	FR1	Sense	CAGCCTGTGCTGACTCAGCCACCTTC
JλC	FR4	Anti-sense	ACCKAGGACGGTSASCTKGGTSCC

Designed oligonucleotide primers for a novel retroviral vector construction

Primer	Orientation	Sequence (5' ➔ 3')
*Sac*I *Not*I Start Signal	Sense	CGAGCTCGAAGGAAAAAAGCGGCCGCATGAC CGTCGCGCGG
*Xba*I FLAG Signal	Anti-sense	TGCTCTAGAGCACTTGTCATCGTCGTCCTTGTA GTCACCCCACACGGCCGG
*Xba*I *Cla*I GPI	Sense	TGCTCTAGAGCACCCATCGATGGGCCAAATAA AGGAAGTGG
*Kpn*I *Hind*III Stop GPI	Anti-sense	CGGGGTACCCCGCCCAAGCTTGGGCTAAGTCA GCAAGC
*Xba*I 2YP999	Sense	TGCTCTAGAGCAGACATCGTGATGACC
*Cla*I 2YP999	Anti-sense	CCCATCGATGGGGTTTAATCTCCAGTCG

B = c + g + t, H = a + c + t, K = g + t, M = a + c, R = a + g, S = c + g, W = a + t, Y = c + t

2.1.3.6 Plasmids

-pBI-EGFP vector containing enhanced green fluorescent protein (EGFP) gene (Clontech GmbH)

-pBluescript SK(+) phagemid (Stratagene GmbH)

-pCR®2.1 vector (Invitrogen BV)

-pSFβN1 retroviral vector containing neomycin resistance (Neo^R) gene, kindly provided by Dr. Christopher Baum (Baum *et al*., 1995)

-pSPORT1 vector containing the first 34 amino acids of DAF (clone DKFZp434P184, RZPD)

-pT7T3D-PAC mod1 vector containing the last 37 amino acids of DAF (clone IMAGp998D23661, RZPD)

2.1.3.7 Recombinant human cytokines and growth factors

Factor	Producer
Recombinant Human Flt-3 ligand (Flt-3L)	Immunex Corp, Tebu GmbH
Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	Tebu GmbH
Recombinant Human Interleukin-3 (IL-3)	Tebu GmbH
Recombinant Human Interleukin-4 (IL-4)	Tebu GmbH
Recombinant Human Stem Cell Factor (SCF)	Amgen, Tebu GmbH
Recombinant Human Transforming Growth Factor Beta 1 (TGF-β1)	Tebu GmbH
Recombinant Human Tumor Necrosis Factor-Alpha (TNF-α)	Tebu GmbH

2.1.4 Solutions and media

2.1.4.1 Calcium phosphate precipitation method solutions for transfection of eukaryotic cells

$CaCl_2$ stock solution:

2 M $CaCl_2$ in distilled water
Filter through 0.22 µM filter.
Aliquot and store at -20°C.

Chloroquine stock solution:

50 mM Chloroquine in PBS
Filter through 0.22 µM filter.
Aliquot and store at -20°C.

Na_2HPO_4 stock solution:

5.25 g Na_2HPO_4 in 500 ml of distilled water

2 X HBS:

8.0 g NaCl
6.5 g HEPES sodium salt (Sigma-Aldrich Chemie GmbH)
10 ml Na_2HPO_4 stock solution
Adjust the pH of the solution to exactly 7.0 using NaOH or HCl.
Bring the volume up to 500 ml with distilled water.
Check pH again, the pH is very important, it must be exactly 7.0 at RT.
Filter through 0.22 µM filter.
Aliquot and store at -20°C.

2.1.4.2 Solutions and media for eukaryotic cells culture

Media:

-CellGro® DC serum-free medium (CellGenix)

-CellGro® SCGM serum-free medium (CellGenix)

-Dulbecco´s modified Eagle´s medium, DMEM (Life Technologies)

-Iscove´s modified Dulbecco´s medium, IMDM (Life Technologies)

-RPMI 1640 (Life Technologies)

-CellGro® DC serum-free medium complete (CellGro® DC-C)

CellGro® DC
2 mM L-glutamine (Biochrom KG)
100 U/ml Penicillin (Biochrom KG)
100 µg/ml Streptomycin (Biochrom KG)

-CellGro® SCGM serum-free medium complete (CellGro® SCGM-C)

CellGro® SCGM
2 mM L-glutamine
100 U/ml Penicillin
100 µg/ml Streptomycin

-DMEM complete (DMEM-C):

DMEM
10 % (v/v) FCS (PAA Laboratories GmbH)
2 mM L-glutamine
100 U/ml Penicillin
100 µg/ml Streptomycin

-DMEM for packaging cell lines GP+E86 and PG13 (DMEM-P):

DMEM
10 % (v/v) Costar FCS (Cytogen®)
2 mM L-glutamine
100 U/ml Penicillin
100 µg/ml Streptomycin

-HAT-selection medium for PG13 packaging cell line:

DMEM-P

0.2 % (v/v) 500 X HAT (Roche Diagnostics GmbH)

-HXM-selection medium (100 ml) for GP+E86 packaging cell line:

DMEM-P

150 µl Hypoxanthine, 10 mg/ml in 0.1 N NaOH (Sigma-Aldrich Chemie GmbH)

2.5 ml Xanthine, 10 mg/ml in 0.1 N NaOH (Sigma-Aldrich Chemie GmbH)

250 µl Mycophenolic acid, 10 mg/ml in 0.1 N NaOH (Sigma-Aldrich Chemie GmbH)

20-30 µl Concentrated HCl (to adjust pH)

-Phoenix packaging cell lines selection medium:

DMEM-C

1 µg/ml Diphtheria toxin (Calbiochem-Novabiochem GmbH)

300 µg/ml Hygromycin B (Roche Diagnostics GmbH)

-RPMI 1640 complete (RPMI-C)

RPMI 1640

10 % (v/v) FCS

2 mM L-glutamine

100 U/ml Penicillin

100 µg/ml Streptomycin

Saline solutions:

-Hank’s buffer saline solution, HBSS (pharmacy of MHH)

-Phosphate buffered saline solution (PBS) with or without calcium and magnesium (pharmacy of MHH)

Others:

-Ficoll-Hypaque gradient, specific gravity 1.077 g/ml (Seromed)

-Trypsin/EDTA (PAA Laboratories GmbH)

2.1.4.3 Solutions and media for bacteria culture

Luria-Bertani (LB) medium, 1L:

10 g Bacto-trypone (Difco Laboratories)
5 g Bacto-yeast extract (Difco Laboratories)
10 g NaCl
950 ml Deionized water
Adjust the pH of the solution to 7.0 with NaOH and bring the volume to 1 liter.
Autoclave on liquid cycle for 20 min.
Allow solution to cool to 55°C.
Store at RT.

LB agar plates, 1L:

Prepare LB medium as above, but add 15 g/L agar before autoclaving.
Autoclave on liquid cycle for 20 min.
Allow solution to cool to 55°C.
Add antibiotic (50 μg/ml of either ampicillin or kanamycin).
Pour into 10 cm plates.
Let harden, then invert and store at 4°C.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) stock solution (40 mg/ml):

Dissolve 400 mg X-Gal in 10 ml dimethylformamide.
Protect from light by storing in a brown bottle at -20°C.

IPTG (isopropyl-β-D-thiogalactoside) stock solution (100 mM):

Dissolve 238 mg of IPTG in 10 ml deionized water.
Filter-sterilize and store in aliquots at -20°C.

X-Gal with/without IPTG LB plates:

Warm LB plates with the appropriate antibiotic at 37°C for 10 min.

Pipet 40 µl of the X-Gal stock solution with/without 40 µl of the IPTG stock solution onto the center of each plate and spread evenly with a sterile spreader.

Allow the solution to diffuse into the plate by incubating at 37°C for 20-30 min.

Pleats are now ready to use.

SOC medium, 1L:

20 g Bacto-trypone

5 g Bacto-yeast extract

0.5 g NaCl

950 ml Deionized water

10 ml of 250 mM KCl stock solution

Adjust pH to 7.0 with 5 M NaOH and bring the volume to 980 ml with deionized water.

Autoclave on liquid cycle for 20 min.

Allow solution to cool to 55°C.

Add 10 ml of filter-sterilized 2 M glucose stock solution.

Add 10 ml of 1 M sterile $MgCl_2$ stock solution.

Store at RT or 4°C.

TSS solution:

LB medium (without pH adjustment)

30 mM $MgCl_2$

10 % (v/v) Polyethylene glycol 4000

5 % (v/v) DMSO (Sigma-Aldrich Chemie GmbH)

Autoclave on liquid cycle for 20 min.

Store at 4°C.

2.1.4.4 Standard buffer

TAE 50 X buffer (1L):

242 g Tris base

57.1 ml Glacial acetic acid

100 ml 0.56 M EDTA, pH 8.0

Adjust the pH of the solution to 7.2 bring the volume to 1 liter with distilled water.

6 X Sample buffer (6 X SB):

6 ml 50 X TAE buffer

44 ml Glycerine

0.05 % (w/v) Bromophenol blue and mix well

Ethidium bromide:

10 mg/ml in distilled H_20

2.1.5 Consumed materials

-Cannulas and Syringes (B. Braun)
-Cap lock reaction (0.5 ml, 1.5 ml and 2 ml) tubes (Eppendorf)
-Cap lock sterile disposable polypropylene RNase-free (1.5 ml and 2 ml) tubes (Eppendorf)
-Cryogenic vial, 2 ml (Nalge Nunc International)
-Filter, 0.22 μm and 0.45 μm (Millipore GmbH)
-Filter cap tissue culture (25 cm^2, 75 cm^2 and 175 cm^2) flasks (Greiner GmbH)
-Multiwell (6-, 24- and 96-well) tissue culture plates (Falcon, Greiner GmbH and Nalge Nunc International)
-Petri dishes (Greiner GmbH)
-Pipettes (Greiner GmbH)
-Pipette tips (Eppendorf)
-Sterile disposable polypropylene RNase-free tips (Eppendorf)
-Tissue culture (60 and 100 mm) dishes (Nalge Nunc International)
-Tissue culture (15 ml and 50 ml) tubes (Greiner GmbH)

2.1.6 Devices

-1214 Rackbeta liquid scintillation counter (LKB)
-2219 Multitemp II thermostatic circulator (LKB)
-Biofuge 13 (Heraeus)
-Cell culture incubators (Heraeus)
-Cell-porator® (Life Technologies)
-Centrifuge 5415 (Eppendorf)
-Centrifuge, model J2-21, rotor JA14 and JA17 (Beckmann)
-Centrifuge, model J-6B (Beckmann)
-Centrifuge, model J6-MC (Beckmann)
-Diavert fluorescence microscope (Leitz)
-Diavert light microscope (Leitz)
-Digital-pH-meter (Knick)
-Easiject™ plus electroporation (Eurogentec)
-Eclipse TE 300 fluorescence microscope (Nikon)
-FACScalibur with CellQuest software (Becton Dickinson)
-G24 environmental incubator shaker (New Brunswick Scientific Co. Inc.)
-Gel Doc 1000 (Bio-Rad Laboratories GmbH)
-GFL water bath (H. Juergens & Co.)
-Horizontal electrophoresis (Bio-Rad Laboratories GmbH and Pharmacia)
-Laminar air flow class 100 (Gelaire)
-Magnetic cell separator, MACS (Miltenyi Biotec)
-Megafuge 2.0 R (Heraeus)
-Minifuge T (Heraeus)
-PHD™ 96-well cell harvester (Cambridge Technology, Inc.)
-SpeedVac® plus SC 110A (Savant)
-Sterile GARD hood (Baker Company, Inc.)
-Thermocycler varius V45 (Landgraf)
-Thermomagnetic stirrer, MR 2002 (Heidolph Instruments)
-Thermomixer 5436 (Eppendorf)
-Thermostat 5320 (Eppendorf)
-UV-VIS Spectrophotometer, UV-1202 (Shimadzu Deutschland GmbH)
-Vortex Genie 2™ (Bender and Hobein AG)

2.2 Methods

2.2.1 Microbiological methods

2.2.1.1 Cultivation of competent cells

The bacteria were streaked onto freshly prepared agar plates containing the appropriate selective antibiotic followed by incubation overnight at 37°C. The plates were used over a period of 6 weeks when stored at 4°C.

A single colony was inoculated into 1-5 ml of LB medium containing the appropriate selective antibiotic and grown in a tube with a volume of at least 4 times the volume of culture with a constant shacking (100-250 rpm) for 12-16 h at 37°C. Growth for more than 16 h was not here performed since cells began to lyse leading to reduced plasmid yields.

The growing bacteria were stored in 30 % glycerine LB medium at -80°C.

2.2.1.2 Production of competent cells

A single colony was peaked from a freshly streaked selective plate and a starter culture of 2-5 ml LB medium containing the appropriate selective antibiotic was inoculated in a tube with a volume of at least 4 times the volume of culture. The tube was incubated for 8 h at 37°C with vigorous shaking (300 rpm). 500 µl of the starter culture was diluted into 40 ml selective LB medium in a flask with a volume of at least 4 times the volume of culture. The flask was incubated at 37°C with vigorous shaking (300 rpm) to OD_{600} = 0.4. The flask was cooled on ice and the bacteria cells were harvested by centrifugation (2000 rpm) at 4°C for 10 min. The bacteria cells were resuspended in 4 ml ice-cold TSS solution. 100-200 µl was aliquoted in sterile cap-lock eppendorf tubes (1.5 ml). The aliquots were stored at -80°C.

2.2.1.3 Transformation of competent cells with plasmid-DNA

INVαF´ and TOP10F´ competent cells were transformed in the presence or absence of 0.5 M β-mercaptoethanol, respectively following the manufacturer's instructions (Original TA Cloning® Kit, Invitrogen BV).

XL1-Blue competent cells were transformed as following:
About 50 ng plasmid-DNA or 5-10 µl ligation reaction was mixed with 50-100 µl competent cells-containing vials by stirring gently with the pipette tips and incubated on ice for 30 min. After heat shock for exactly 90 sec at 42°C without mix or shake, the vials were cooled for 3 min on ice. 250 ml of LB medium was added and the vials were incubated for 1 h at 37°C with shaking gently.

50 µl, 100 µl and 200 µl from each transformation vial were spread on separate, labeled agar plates containing the appropriate selective antibiotic and X-Gal (and IPTG in TOP10F´ competent cells). After the liquid was absorbed the plates were inverted and placed in a 37°C incubator for at least 18 h. Plates were then shifted to 4°C for 2-3 h before selecting colonies for analysis to allow proper color development.

2.2.2 Molecular biological methods

2.2.2.1 RNAzol™ B-total RNA isolation method

Homogenization:
0.1-2 × 10^7 mononuclear cells (MNC) of plasmocytoma patient (E.G) have been washed in 10 ml PBS by centrifugation for 3 min at 1600 rpm. The supernatant was removed and the cells were lysed in RNAzol™ B (0.2 ml/10^6 cells, Cinna Biotecx Labs Inc.) by repeated pipetting.

Phase separation:
Chloroform (1/10 volume of homogenate) was added and the homogenate was shaken vigorously for 15-30 sec. The sample was stored on ice for 5 min and centrifuged at 12000 x g for 15 min at 4°C. Following centrifugation, the sample forms two phases: the

lower blue phenol-chloroform phase, containing DNA and proteins and the upper colorless aqueous phase, containing RNA. The volume of the aqueous phase is about 50 % of the initial volume.

RNA precipitation:
The aqueous phase was transferred to a clean tube and equal volume of cold isopropanol was added. The sample was stored for 1 h at 4°C and centrifuged at 12000 x g for 15 min at 4°C.

RNA wash:
The supernatant was removed and the RNA pellet was washed once with 1 ml 75 % ethanol. After shaking to dislodge the pellet from the side of the tube the pellet was centrifuged for 8 min at 7500 x g at 4°C.

RNA solubilization:
The pellet was dried by air-drying or under vacuum (5-10 min). It is important not to let the RNA pellet dry completely, as this greatly decreases solubility and can lead to RNA degradation. The RNA pellet was dissolved in 50 µl autoclaved 0.1 % (v/v) diethyl pyrocarbonate treated-water (DEPC-water) by passing the solution through a pipette tip and/or incubating for 10-15 min at 55-60°C. The RNA was stored at -80°C until using.

Sterile disposable RNase-free polypropylene tubes and tips were used throughout the procedure.

2.2.2.2 Plasmid preparation

Bacterial cultures for plasmid preparation should always be grown from a single colony picked from a freshly streaked selective plate. Subculturing directly from glycerol stocks, agar stabs and liquid cultures is poor microbiological practice and may lead to loss of the plasmid. Inoculation from plates that have been stored for a long time was also considered as having lost or mutation of the plasmid. The desired clone should be streaked from a glycerol stock onto a freshly prepared agar plate containing the appropriate selective antibiotic so that single colonies can be isolated.

2.2.2.2.1 Mini-preparation of plasmid using QIAprep Spin Miniprep Kit (Qiagen GmbH) or NucleoSpin® Plasmid Kit (Macherey-Nagel GmbH)

The two methods are identical and designed for rapid and small-scale (20-40 µg) preparation of highly pure plasmid DNA (< 10 kb) from 1-8 ml of overnight *E. coli* culture. The mini-preparation of plasmid was carried out following the manufacturer's instructions. Briefly, the pelleted bacteria were resuspended in RNase A-containing buffer 1 and plasmid DNA was librated from the *E. coli* host cells by SDS/alkaline lysis (buffer 2). Buffer 3 neutralized the resulting lysate and created appropriate conditions for binding of plasmid DNA to the silica membrane in QIAprep spin or NucleoSpin plasmid column. SDS precipitate and cell debris were pelleted by the centrifugation step and the supernatant was loaded onto QIAprep spin or NucleoSpin plasmid column. If host strains with high nuclease levels were used, a washing step with buffer PB (QIAprep Spin Miniprep Kit) or AW (NucleoSpin® Plasmid Kit) was recommended. Contaminations like salts, metabolites and soluble macromolecular cellular components were removed by simple washing with ethanolic buffer. Pure plasmid DNA was finally eluted with 50 µl distilled water and stored at -20°C.

2.2.2.2.2 Maxi-preparation of plasmid using EndoFree Plasmid Maxi Kit (Qiagen GmbH)

This method was designed for purification of up to 500 µg endotoxin-free plasmid DNA. Endotoxine-free plasmid DNA improves transfection into sensitive eukaryotic cells and is essential for gene therapy research.

A single colony was peaked from a freshly streaked selective plate and a starter culture of 2-5 ml LB medium containing the appropriate selective antibiotic was inoculated in a tube with a volume of at least 4 times the volume of culture. The tube was incubated for 8 h at 37°C with vigorous shaking (300 rpm). The starter culture was diluted into 100 ml or 250 ml selective LB medium for high-copy plasmids or low-copy plasmids, respectively in a flask with a volume of at least 4 times the volume of culture. The flask was incubated at 37°C for 12-16 h with vigorous shaking (300 rpm). The bacteria cells were harvested by centrifugation (6000 x g) at 4°C for 10 min. All traces of supernatant were removed by inverting the open centrifuge tubes until all medium had been drained. The bacteria pellet was resuspended completely in 10 ml RNase A-containing buffer (P1) by vortexing. Plasmid DNA was librated

from the *E. coli* host cells by addition of 10 ml lysis buffer (P2) and mixing gently but thoroughly by inverting 4-6 times and incubation at RT for exactly 5 min. A vessel of sufficient size was used to allow complete mixing of the lysis buffer. After addition of 10 ml chilled buffer 3 and mixing immediately but gently by inverting 4-6 times, a fluffy white precipitate containing genomic DNA, proteins, cell debris and SDS became visible. The lysate was transferred into the screwed cap QIAfilter cartridge immediately to prevent later disruption of precipitate layer and incubated at RT for 10 min. A precipitate containing genomic DNA, proteins and detergents was floated and formed a layer on top of the solution. After the cap was removed, the lysate was filtered through the QIAfilter using the plunger into a 50 ml tube. Approximately 25 ml of the lysate was recovered after filtration. To obtain endotoxin-free plasmid DNA, 2.5 ml buffer ER was added to the filtered lysate. After mixing by inverting the tubes approximately 10 times and incubation on ice for 30 min, the filtered lysate was transferred to the equilibrated QIAGEN-tip 500 and moved through it by gravity. After washing twice with 30 ml buffer QC, the DNA was eluted with 15 ml Buffer QN. The eluted DNA was collected in 30 ml endotoxin-free tube and precipitated by 10.5 ml RT isopropanol and centrifuged immediately at 15000 x g for 30 min at 4°C. The supernatant was carefully decanted and DNA pellet was washed with 5 ml endotoxin-free, RT 70 % ethanol. Air-dry (5-10 min) plasmid pellet was redissolved, by rinsing the walls to recover all DNA, in a suitable volume of endotoxin-free water (ampura water). It was of big importance not to let the plasmid pellet dry completely, as this greatly decreases solubility. Endotoxin-free pure plasmid DNA was stored at -20°C. Endotoxine-free plastic pipettes and tubes were used for elution and subsequent steps.

2.2.2.3 Enzymatic modification of nucleic acids

2.2.2.3.1 Treatment of isolated total RNA with DNase 1, RNase-free (Roche Diagnostics GmbH)

To purify RNA from genomic DNA, 10 U (1 µl) DNase 1, RNase-free was added to 2 µg total RNA in a final volume of 10 µl of 1 X buffer followed by incubation for exactly 15 min at RT. To stop the reaction, 1 µl of 25 mM EDTA (Life Technologies) was added followed by incubation for 10 min at 65°C.

2.2.2.3.2 First-strand cDNA synthesis (reverse transcription)

In a sterile RNase-free microcentrifuge tube, 0.5 µg (1 µl) oligo $(dT)_{15}$ primer (Promega) was added to 2 µg total RNA (treated with or without DNase 1, RNase-free) in a total volume of 12 µl DEPC-water. The tube was heated to 70°C for 5 min to linearize RNA folding within the template and immediately cooled on ice to prevent RNA folding from reforming followed by briefly centrifugation to collect the fluid at the bottom of the tube. The following components were added to the annealed primer/template in the order shown:

5 X M-MLV reaction buffer	5 µl
10 mM dNTP´s (Promega)	1.25 µl
25 U rRNasin® ribonuclease inhibitor (Promega)	0.625 µl
DEPC-water	5.125 µl
200 U M-MLV reverse transcriptase (Promega)	1 µl

The tube was mixed gently by flicking and incubated for 60 min at 42°C. After the reaction was stopped by incubation at 75°C for 10 min, the tube was cooled on ice and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed immediately thereafter.

2.2.2.3.3 Polymerase chain reaction (PCR)

A sample of the obtained cDNA (1/3 to 1/5) or 10-100 ng of plasmid DNA was used as a template in the polymerase chain reaction (PCR) using a mixture of 5' oligonucleotide FWR1 primers specific for the expressed Vκ or Vλ families together with a mixture of downstream 3' primers specific for Jκ or Jλ genes (Sahota *et al.*, 1997) or appropriate designed sense and anti-sense primers, respectively. In general, the PCR was carried out in a 50 µl volume containing:

10 X PCR buffer with 15 mM $MgCl_2$	5 µl
10 mM dNTP´s	1 µl
Primers	20 pmol each primer
DNA template	1/3 to 1/5 cDNA or 10-100 ng plasmid DNA
Sterile water	to a total volume of 49 µl
Taq polymerase (Promega)	2.5 U

Amplification consisted of an initial denaturation step of 5 minutes at 94°C, followed by 30-35 cycles of 94°C for 1 minute, 55-65°C for 1 minute and 72°C for 1 minute, with a final extension step of 5 minutes at 72°C. At least two independent PCR amplifications were performed per test. Reaction without DNA template was used as negative control. The PCR product was stored at 4°C until usage.

2.2.2.3.4 DNA digestion with restriction endonucleases

Most of the used restriction endonucleases were obtained from (New England BioLabs). Cleaving DNA substrates with restriction endonucleases was performed following the manufacturer's instructions. Generally, 10 U (1 µl) of enzyme was added to 1 µg of purified DNA in a final volume of 10 µl of the appropriate 1 X NEBuffer followed by incubation for 1 h at the recommended temperature (37°C for most restriction enzymes). The enzyme was always the last component added to the reaction (reaction components should be mixed prior to addition of enzyme). Supercoiled plasmids generally required more than I U/µg to be cleaved completely.

Cleaving a DNA substrate with two restriction endonucleases simultaneously (double digestion) was carried out for time saving. The best NEBuffer was selected to provide reaction conditions that amenable to both restriction endonucleases (to ensure almost 100 % activity). The number of units of enzyme or incubation time of the reaction was adjusted to compensate for slower rate of cleavage in case of using sub-optimal NEBuffer. In the case that no single NEBuffer was found to satisfy the buffer requirements of both enzymes, the reactions were done sequentially. Firstly, cleavage with the restriction endonucleases that required the lower salt reaction conditions was done and then the salt concentration of the reaction was adjusted (using a small volume of a concentrated salt solution) to approximate

the reaction conditions of the second restriction endonuclease. After addition the second endonuclease, the reaction was incubated to complete the second digestion. In the case that bovine serum albumin (BSA) was a buffer requirement for either enzyme, it was added to the double digest reaction because it did not inhibit any restriction enzyme. The final concentration of glycerol in any reaction should be less than 10 % to minimize the possibility of star activity. To avoid this situation, an increase in total reaction volume was necessary.

2.2.2.3.5 Ligation

To cloning the gel purified restriction DNA fragments and PCR products, ligation reaction was performed with T4 DNA ligase (Roche Diagnostics GmbH). The Original TA Cloning® Kit with linearized pCR®2.1 vector (Invitrogen BV) provides a quick, one-step cloning strategy for the direct insertion of a PCR product into plasmid vector. The linearized vector has single 3`deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. The ligation reaction was performed with 1:1 or 1:3 (vector:insert) ratio in a 10 µl volume as follows:

Gel purified PCR product or restriction DNA fragment	X µl
10 X ligation buffer	1 µl
Vector	Y µl
Sterile water	to a total volume of 9 µl
T4 DNA ligase (4.0 Weiss units)	1 µl

The ligation reaction was incubated overnight at exactly 14°C. The ligation reaction was stored at -20°C until ready for transformation.

2.2.2.3.6 Sequencing

Sequencing was performed by MWG-Biotech sequencing service using the M13 Reverse primer and M13 (-20) forward primer.

2.2.2.4 Agarose Gel electrophoresis

To visualize the isolated total RNA and separate the DNA fragments, agarose gel electrophoresis was used. 0.5 g agarose (Serva) in 50 ml 1 X TAE was boiled in a microwave. Before pouring the gel on electrophoresis plate, 2.5 µl ethidium bromide (10 mg/ml in distilled H_20) was added. Samples with (1/5 volume of sample) 6 X sample buffer (6 X SB) were loaded on the soaked plates in electrophoresis chamber filled with 1 X TAE. Electrophoresis was done at ≈ 100 V until the samples reached appropriate distance. The samples were visualized by Gel Doc 1000 (Bio-Rad) and analysed by molecular analysis software (Bio-Rad).

2.2.2.5 Gel-DNA extraction and purification

Gel-DNA extraction and purification were carried out using GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) following the manufacturer's instructions. Briefly, 300 mg (the maximum weight that can processed with this procedure) of gel slice was dissolved in 300 µl capture buffer and mixed vigorously by vortexing and incubated at 60°C (5-15 min) until the agarose was completely dissolved. The sample was passed through the GFX column to capture the DNA onto the glass fiber matrix. Matrix-bound DNA was washed with an ethanolic buffer to remove salts and other contaminants. The purified DNA was eluted from GFX column in 50 µl distilled water and stored at -20°C.

2.2.2.6 Quantification of nucleic acids

2.2.2.6.1 Photometric determination of RNA and DNA concentration

To determine the concentration and purity of the isolated total RNA and plasmid obtained from maxi-preparation method, the absorption of diluted solution was measured by UV spectrophotometer at 260 nm and 280 nm. The dilution was performed to make the absorption between 0.1 and 1.0 OD.

The used equations are:

RNA concentration (μg/μl) = absorbance at 260 nm × dilution factor × 40 × 1000

DNA concentration (μg/μl) = absorbance at 260 nm × dilution factor × 50 × 1000

Purity = absorbance at 260 nm/absorbance at 280 nm

2.2.2.6.2 Agarose gel electrophoretic determination of DNA concentration

Quantitative analysis by an agarose gel electrophoresis using λ DNA-*Hind*III Fragments as mass reference was applied to determine the concentration of gel purified DNA and plasmid obtained from mini-preparation method.

2.2.3 Cell culture

2.2.3.1 General cell culture

Generally, adherent and suspension cells were grown in a 37°C incubator containing 5 % CO_2. The saturated cultures were splited every 3 days. Cell density was maintained between 10^5-10^6 cells/ml. To split and passage adherent cells, the cells were gently rinsed with PBS and trypsinized until the cells were easily detached. Trypsinization was quenched with medium prior to subculturing in fresh medium.

2.2.3.1.1 Cell counts

Suspension and trypsinized viable cell counts were performed using a Neubauer chamber (Hawksley & Sons Limited) with at least 200 cells being counted per sample. Cell viability was assessed using trypan blue exclusion (Sigma-Aldrich Chemie GmbH).

2.2.3.1.2 Cell freezing

To freeze suspension and trypsinized cells, the cells were centrifuged at 500 x g for 5 min and the media were removed. Immediately 1 ml of cold freezing solution (90 % heat-inactivated fetal calf serum, 10 % DMSO) per 10^6 cells was added and the cells were transferred to a cold 2 ml cryogenic vial. After that the vial was placed overnight at -70°C and transferred to liquid nitrogen on the following day.

2.2.3.1.3 Cell thawing

To thaw the freezing cells, one vial was removed from liquid nitrogen and thawed rapidly at 37°C. Immediately 1 ml medium was added to the freezing vial and the cells were gently transferred to a 15 ml sterile conical screw cap tube. To allow for osmotic equilibrium, 10 ml medium was gently added and the tube was mixed by inverting. After the cells were centrifuged at 500 x g for 5 min and the supernatant was removed, the cells were resuspended in 10 ml medium (10^5-10^6 cells/ml) and cultured in filter cap 25 cm^2 tissue culture flasks at 37°C and 5 % CO_2.

2.2.3.2 Cell separation

2.2.3.2.1 Isolation of peripheral blood mononuclear cells (MNC) by density gradient centrifugation

Cord blood (CB) samples from normal full-term deliveries, human bone marrow (BM) from healthy donors and plasmocytoma patient (E.G) and G-CSF mobilized peripheral blood (PB) were used in this study. MNC were separated on Ficoll-Hypaque gradient (specific gravity 1.077 g/ml) as follows:
Diluted cells (35 ml) with PBS containing 1 % human serum albumin (pharmacy of MHH) and 0.4 % sodium citrate (pH 7.2) were carefully layered over 15 ml Ficoll in a 50 ml conical tube and centrifuged at 400 x g for 30 min at RT without brake. In case of BM and PB, cells were diluted in PBS containing 100 U/ml DNase with or without 0.02 % collagenase B (Roche Diagnostics GmbH), respectively in the presence of 2.5 mM/L $MgCl_2$ and were

shaken gently at RT for 45 min before separation on Ficoll to release cells and decrease clumps-formation. After the upper layer (diluted autologous serum) was aspirated, the undisrupted mononuclear cell layer at the interphase was transferred to a new 50 ml conical tube. The cells were washed twice with PBS, resuspended in appropriate volume of PBS and counted. All cell clumps were removed by passing cells through 30 μm nylon mesh. To lyse remaining erythrocytes, cell pellet was incubated at RT for 30 sec in sterile distilled water.

2.2.3.2.2 Isolation of monocytes

Autologous monocytes (Mo) were obtained from the same G-CSF mobilized peripheral blood by plastic adherence of MNC. After 2 h, nonadherent cells were removed by 3 X gently washing with PBS.

2.2.3.2.3 Isolation of $CD34^+$ haematopoietic progenitor cells

$CD34^+$ progenitor cells were separated from MNC by magnetic cell sorting using CD34 MultiSort Kit (Miltenyi Biotec) following the manufacturer's instructions. The CD34 MultiSort Kit allows the multi-parameter sorting of haematopoietic progenitor cells. First, $CD34^+$ cells are positively selected from peripheral blood, bone marrow or cord blood using a direct magnetic labelling system. Using MultiSort technology, the CD34 MultiSort MicroBeads have been subsequently released from the cells. Finally, the selected cell population have been magnetically labeled and resorted for other markers. After two cycles of magnetic separation, the purity of $CD34^+$ progenitor cells measured by flow cytometric analysis using noncross blocking antibody, HPCA-2 FITC (Becton Dickinson) was about 98 %.

2.2.3.2.4 Isolation of untouched $CD3^+$ T cells

Autologous T cells were purified from MNC of the same G-CSF mobilized peripheral blood by negative selection using the Pan T Cell Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions. The Pan T Cell Isolation Kit is an indirect magnetic labelling

system for the isolation of untouched T cells from human peripheral blood mononuclear cells by magnetic depletion of B cells, monocytes, NK cells, dendritic cells, early erythroid precursor cells, platelets and basophils. A cocktail of CD11b, CD16, CD19, CD36 and CD56 antibodies was used for the depletion of non-T cells. Excellent recoveries of highly pure untouched T cells were achieved by retaining the magnetically labeled non-T cells on an LS Column. T cells isolated by using the Pan T Cell Isolation Kit can be used in functional assays and signal transduction studies where a direct labelling of a T cell marker for positive selection was avoided.

2.2.3.3 Expansion of BM-$CD34^+$ progenitor cells

Ex vivo expansion of $CD34^+$ progenitor cells facilitates the identification of culture conditions useful to generate DC to study their biology and their retroviral transduction for immunotherapeutic purposes (Glimm *et al*., 1998). To test the ability to expand $CD34^+$ cells in serum-free medium, the increase in cell counts of BM-$CD34^+$ cells (5×10^4 cells/ml) cultured in complete serum-containing medium IMDM or RPMI-1640 was compared with that cultured in complete serum-free medium CellGro® SCGM or CellGro® DC containing one of the two following cytokine cocktails: Flt-3L (150 ng/ml), IL-3 (100 ng/ml), SCF (50 ng/ml) and TGF-β1 (0.5 ng/ml) or Flt-3L (300 ng/ml), IL-3 (100 ng/ml) and SCF (100 ng/ml). Cultures were incubated for 7 days at 37°C in a humidified 5 % CO_2-in-air atmosphere.

2.2.3.4 Generation of human $CD34^+$-derived dendritic cells

2.2.3.4.1 Generation of human $CD34^+$-derived dendritic cells with cytokines under serum-free conditions

CB-$CD34^+$ cells (5×10^4 cells/ml) seeded into 24-well plate (Greiner GmbH) were cultured in complete serum-free medium (CellGro® DC) containing the following cytokines that specifically reported for CB (Bello-Fernandez *et al*., 1997): Flt-3L and GM-CSF (100 ng/ml each), SCF (20 ng/ml), TGF-β1 (0.5 ng/ml) and TNF-α (10 ng/ml) for 14 days at 37°C and

5 % CO_2. The culture medium was exchanged at weekly intervals. The density of cells did not exceed 10^5 cells/ml.

Also PB-CD34$^+$ cells were cultured under the above conditions but with the following different cytokine cocktails specifically reported for PB (Garbe *et al.*, 1998): Flt-3L (150 ng/ml), IL-3 (100 ng/ml), SCF (50 ng/ml) and TGF-β1 (0.5 ng/ml) for 1st week, Flt-3L (150 ng/ml), GM-CSF (100 ng/ml), IL-4 and SCF (50 ng/ml each) and TGF-β1 (0.5 ng/ml) for 2nd week and Flt-3L (150 ng/ml), GM-CSF (100 ng/ml), IL-4 and SCF (50 ng/ml each), TGF-β1 (0.5 ng/ml) and TNF-α (2 ng/ml) for 3rd and 4th weeks. At day 26, 20 ng/ml TNF-α was added to induce maturation.

2.2.3.4.2 Generation of human CD34$^+$-derived dendritic cells with phorbol ester (PMA) under serum-free conditions

CB- or PB-CD34$^+$ cells at a concentration of 5×10^4 cells/ml were cultured in 24-well plate containing complete serum-free medium (CellGro® DC). Cultures were stimulated with 15 ng/ml PMA (Sigma-Aldrich Chemie GmbH) and incubated for 7 days at 37°C and 5 % CO_2. PB-CD34$^+$ cells were also stimulated with PMA plus 10 ng/ml TNF-α or plus 100 ng/ml ionomycin (Calbiochem-Novabiochem GmbH). Expanded BM-CD34$^+$ cells in complete CellGro® SCGM containing Flt-3L (300 ng/ml), IL-3 (100 ng/ml) and SCF (100 ng/ml) were stimulated too with PMA.

2.2.3.5 Transfection

2.2.3.5.1 Transfection of cells via electroporation

Cells ($0.1\text{-}2 \times 10^7$) were centrifuged at 700 x g washed once in cell culture medium without serum or glutamine and resuspended in a final volume of 400-1000 µl medium containing retroviral vector (10-40 µg). Electroporations were carried out in 0.4 cm electrode gab cuvettes at different voltage and capacity using Cell-porator® (Life Technologies) or Easiject™ plus electroporation (Eurogentec) at room temperature or 4°C. Cells were grown in cell culture medium supplemented with 10 % FCS, 2 mM glutamine, 100 U/ml penicillin, and

100 µg/ml streptomycin at 37°C and 5 % CO_2. After 48-72 h cells were analyzed for transfection by flow cytometer.

2.2.3.5.2 Transfection of cells via SuperFect^{TM} Transfection Reagent (Qiagen GmbH)

SuperFect^{TM} Transfection Reagent represents a new class of activated-dendrimer transfection reagent designed for outstanding transfection results (Tang *et al*., 1996). It possesses a defined spherical architecture, with branches radiating from a central core and terminating at charged amino groups. It assembles DNA into compact structures, optimizing the entry of DNA into the cells. SuperFect-DNA complexes possess a net positive charge that allows them to bind to negatively charged receptors (e.g. sialylated glycoproteins) on the surface of eukaryotic cells. Once inside the cell, SuperFect reagent buffers the lysosome after it has fused with the endosome, leading to pH inhibition of lysosomal nucleases. This ensures stability of SuperFect-DNA complexes and the transport of intact DNA to the nucleus.

Different cell numbers ($0.1\text{-}2.5 \times 10^6$) were seeded in 100-mm tissue culture dishes the day before transfection in order to determine the optimal transfection conditions. 10 µg retroviral vector diluted in 300 µl cell growth medium containing no serum, proteins or antibiotics was combined with 60 µl of Superfect^{TM} Transfection Reagent at room temperature for 10 min followed by the addition of 3 ml cell growth medium containing all additives including serum and antibiotics. The medium of the overnight cultures was removed and replaced with a mixture of Superfect-DNA. The complex incubation time on cells was 2.5 h. Analysis was performed 48 h post-transfection.

2.2.3.5.3 Transfection of adherent cells via calcium phosphate precipitation method (Pear *et al*., 1993):

A total of 2×10^6 adherent cells were cultured in 3 ml medium in 60-mm tissue culture dishes (Nalge Nunc International) the night prior to transfection. About 5 min prior to transfection, chloroquine (a final concentration of 25 µM) was added to each dish to inhibit lysosomal DNases by neutralizing vesicle pH. To a 15 ml tube the following components were added in the order shown:

5-10 µg (≈ 5-10 µl) DNA (DNA was added in a drop to a side of tube)

≈ 429-434 µl sterile distilled water (DNA was washed to bottom of tube with water)

61 µl 2 M $CaCl_2$ (the solution was mixed thoroughly with finger tapping)

500 µl total volume

500 µl 2 X HBS (The solution was vigorously bubbled with automatic pipettor for 15 sec)

HBS/DNA solution was added immediately dropwise to the above medium. After dishes were rocked gently forward and backward a few times to distribute DNA/$CaPO_4$ particles, the cells were incubated at 37°C and 5 % CO_2 for 24 h. Subsequently, the medium was changed to 3 ml fresh 10 % FCS medium. After 24-48 h cells were analyzed for transfection by flow cytometer.

2.2.3.6 Working with retroviruses

2.2.3.6.1 Growing retroviral packaging cells

Ecotropic and pseudotyped murine retrovirus packaging cells (GP+E86 and PG13, respectively) were cultured in DMEM-P medium at 37°C in 5 % CO_2. At least one week before transfection or infection, GP+E86 and PG13 cells were cultured in HXM- or HAT-selection medium, respectively.

Human ecotropic and amphotropic packaging cells (ΦNX-Eco and ΦNX-Ampho) were cultured in DMEM-C medium at 37°C in 5 % CO_2. At least one week before transfection or infection, the cells were cultured in DMEM-C medium that contained diphtheria toxin (1 µg/ml) for selection of the ecotropic and amphotropic envelope gene and hygromycin B (300 µg/ml) for selection of the gag-pol genes.

The work with transfected ecotropic and non-transfected packaging cells were occurred in S1 (safety level 1) labor but that with transfected or infected amphotropic and pseudotyped packaging cells were occurred in S2 (safety level 2) labor.

2.2.3.6.2 Virus production

The ecotropic and amphotropic packaging cells were transfected with the retroviral vector-DNA via electroporation, calcium phosphate precipitation method or SuperFect™ Transfection Reagent. Mock (no retroviral vector-DNA) transfected cells were used as negative control. To monitor the transfection efficacy, the cells were transfected with retroviral vectors containing EGFP. Cultures were maintained at 37°C and 5 % CO_2 for 24–48 h, then at 32°C and 5 % CO_2 for 16–24 h. Viral supernatants were harvested and filtered using a 0.45 μm filter (Millipore GmbH), then stored at -80°C. ΦNX-Eco virus stocks with titers > 1×10^6 colony-forming units (CFU) per ml, as determined by titration on NIH/3T3, were used for transduction of murine pseudotyped packaging cells, PG13.

2.2.3.6.3 Retroviral transduction of murine pseudotyped packaging cells, PG13

A total of 2×10^6 PG13 cells were cultured in 12 ml medium in 100-mm tissue culture dishes. Following overnight incubation, the packaging cells were overlaid with 10 ml thawed retrovirus-containing supernatants (> 1×10^6 CFU/ml) or no retrovirus-containing supernatant (mock) obtained from ΦNX-Eco packaging cells supplemented with protamine sulphate (5 μg/ml, Sigma-Aldrich Chemie GmbH) and incubated at 37°C in 5 % CO_2 for 16-24 h. At 72 h post infection, viral supernatants were harvested in complete serum-free medium CellGro® SCGM supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and filtered using a 0.45 μm filter, then stored at -80°C. General recommendation for harvesting the retrovirus-containing supernatants was at 32°C. Here, pseudotype retrovirus-containing supernatants were harvested at 37°C because PG13 rapidly looses viral titer when grown at the lower temperature (Reeves *et al*., 2000). Virus stocks with titers > 5×10^5 CFU/ml, as determined by titration on Hela, were used for transduction of human $CD34^+$ progenitor cells.

2.2.3.6.4 Retroviral transduction of $CD34^+$ haematopoietic progenitor cells

Different methods were used for retroviral transduction of $CD34^+$ haematopoietic progenitor cells in serum-free medium to determine the optimal transduction conditions. Firstly, $CD34^+$ progenitor cells (5×10^4) were stimulated for 48 h with Flt-3L (300 ng/ml), IL-3 (100 ng/ml) and SCF (100 ng/ml) in complete serum-free medium CellGro® SCGM. Subsequently, cells were infected with 1 ml mock or undiluted amphotropic or pseudotyped viral supernatants (> 5×10^5 CFU/ml, made in serum-free medium) in the presence of protamine sulphate (5 µg/ml) and cytokines with or without centrifugation at 1000 x g for 2 h at room temperature. After that cells were incubated at 37°C in 5 % CO_2. On the next day, supernatants were removed and cells were cultured in fresh medium supplemented with cytokines. Three rounds of consecutive transductions were performed on days 3, 5 and 7, respectively. The efficiency of the transduction was monitored one week after the last infection.

Cells were also transduced by adhesion on the recombinant human fibronectin fragments CH-296, RetroNectin (Takara) as follows (Hanenberg *et al.*, 1996):
Non-tissue culture treated (24-well) plates (Greiner) were coated with 420 µl 96 µg/ml RetroNectin at RT for 2 h. The CH-296 solution was removed and the plates were blocked with 420 µl PBS/2 % Human serum for 30 min at RT. After plates were washed with HBSS/2.5 % (v/v) 1M HEPS (Applichem GmbH), 1 ml cells/undiluted amphotropic or pseudotyped viral supernatant with cytokines were added and incubated overnight at 37°C and 5 % CO_2. On the next day cells were harvested, washed and cultured in fresh medium containing cytokines at 37°C and 5 % CO_2 in a new 24-well plate. The transduction efficacy was monitored after 3 to 5 days by flow cytometric analysis or fluorescence microscope.

2.2.3.6.5 Viral titer determination

The titer of virus stocks obtained from ecotropic or amophotropic and pseudotyped packaging cells were determined by titration on NIH/3T3 or Hela, respectively. Flow cytometric analysis of EGFP expression was performed to determine the titer of the viral supernatant. In brief, 2×10^5 cells/well were plated in 6-well tissue culture plates the night before infecting the cells with the retrovirus. The next day, cells from 3 wells were trypsinized and counted to

determine the average number of cells per well at the time of exposure to retrovirus. Serial dilutions (1:10) of the viral supernatant in a final volume of 1 ml of medium were prepared and added to each well in the presence of protamine sulphate (5 μg/ml). Cells were centrifuged at 1000 x g for 5 min at room temperature and then incubated at 37°C in 5 % CO_2. The following day, 2.0 ml of medium were added, and cells were incubated for 2 more days. Three days after viral infection, the transduced cells were trypsinized and resuspended in 2 ml of medium for flow cytometric analysis assay. Live cells were gated based on scatter/side scatter profile and analyzed to determine the percentage of positive cells. The titer [colony-forming units (CFU) per ml] was calculated as:
(% positive cells × cell number at initial viral exposure)/viral volume (ml) applied when transduction was not saturated, < 80 % (Galipeau *et al*., 1999)**.**

2.2.3.7 Flow cytometric analysis

Adherent and suspension cells (10^5) were harvested, washed twice and resuspended in staining buffer (PBS plus 0.2 % BSA) in 96-well plates. Phenotypic analysis of cells was performed by flow cytometer (FACScalibur with CellQuest software, Becton Dickinson) using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-marked mAbs in appropriate concentrations following the manufacturer's instructions. Nonspecific binding was blocked by the addition of FcR blocking reagent (Human IgG, Miltenyi Biotec). Appropriate conjugated isotype-matched Abs were used as controls. To exclude dead cells, 7-amino-actinomycin D (7-AAD, PharMingen) was added to each sample. Ten thousand cells from each sample were analyzed on flow cytometer. Data presentation was performed using WinMDI version 2.8 program.

2.2.3.8 FLAG expression and PI-PLC release

Cells transfected or transduced with pSFβ_S(FLAG)$_\omega$DAF or pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF retroviral vectors were washed twice and resuspended in PBS with or without 1 U/ml *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C, PI-PLC (ICN Biomedicals GmbH). After incubation for 60 min at 37°C, cells were analyzed for FLAG expression by

flow cytometer using Anti-FLAG M2 (Sigma-Aldrich Chemie GmbH). Mock-transduced cells were used as negative control.

2.2.4 Immunological methods

2.2.4.1 Processing and presentation of SuperAg and soluble whole Ag by PMA-derived DC

To test the ability of PMA-generated DC to process and present SuperAg and soluble whole Ag, γ-irradiated (3000 rad ^{137}Cs) Mo or DC from day 7 PMA-treated PB-$CD34^+$ cell cultures were plated in triplicate wells of 96-well flat-bottom plates (Nalge Nunc International) at 2×10^4 cells/well. Purified autologous T cells (1×10^5 cells/ml) in complete RPMI-1640 medium were added to the Mo- or DC-containing wells without or with 3 μg/ml staphylococcal enterotoxin B, SEB (Sigma-Aldrich Chemie GmbH) or 10 μg/ml preservative-free tetanus toxoid, TT (Calbiochem-Novabiochem GmbH). Also autologous T cells were plated in triplicate wells of 96-well flat-bottom plates with media alone (no stimulus) or with media containing SEB or TT. Cultures were incubated for 7 days at 37°C in a humidified 5 % CO_2-in-air atmosphere. T cell proliferation was assessed after 0.5 μCi/well [^{3}H]-thymidine (Amersham Pharmacia Biotech) addition for the final 18 h of culture. Cells were harvested using a 96-well cell harvester, and [^{3}H]-thymidine incorporation was measured using a Beta scintillation counter.

2.2.4.2 Autologous T cell proliferation assay

γ-irradiated (3000 rad ^{137}Cs) mock-transduced or retroviral-transduced, PMA-derived or cytokine-derived, DC were plated in triplicate wells of 96-well flat-bottom plates at different concentration. Purified autologous T cells (1×10^5 cells/ml) in complete RPMI-1640 medium were added to the DC-containing wells. Also, transduced DC or autologous T cells were plated in triplicate wells of 96-well flat-bottom plates with media alone as a control for assay. Cultures were incubated for 7 days at 37°C in a humidified 5 % CO_2-in-air atmosphere. T cell proliferation was assessed after 0.5 μCi/well [^{3}H]-thymidine addition for the final 18 h of culture. Cells were harvested using a 96-well cell harvester, and [^{3}H]-thymidine incorporation was measured using a Beta scintillation counter. Proliferative index (PI) value was calculated

as the ratio between the mean CPM of T cells in the presence and in the absence of transduced DC in the cultures.

2.2.4.3 MHC restriction of T cell response

To study MHC restriction of T cell response, antibodies against MHC class I (HLA-ABC, clone B9.12.1, Immunotech GmbH), class II (HLA-DR, clone B8.12.2, Immunotech GmbH), or an isotypic control mouse IgG at 20 μg/ml were added to the cultures at the initiation of the autologous proliferation assay.

2.2.4.4 Inhibitory effect of CD86 antibodies on T cell response

To study the role of CD86 (B7-2) co-stimulatory molecule in T cell activation, antibodies against CD86 (clone IT2.2, PharMingen GmbH) or an isotypic control mouse IgG at 20 μg/ml were added to the cultures at the initiation of the autologous proliferation assay.

2.2.5 Statistical analysis

Data are presented as mean values ± SD. For comparison of two groups, unpaired t tests and Mann-Whitney test (U-test) were used. For comparison of three or more groups, Ordinary one-way ANOVA and Kruskal-Wallis test were used. *P* values of < 0.05, < 0.01 and < 0.001 were considered statistically significant, highly significant and very highly significant, respectively.

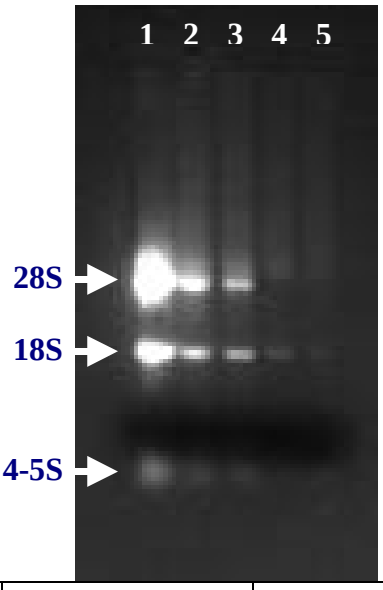
3 Results

3.1 V_L gene usage by tumor cells

In order to amplify tumor specific transcripts of the variable region of paraprotein light chain (V_L), reverse transcriptase-polymerase chain reaction (RT-PCR) of total RNA isolated from mononuclear cells (MNC) of heparinized bone marrow aspirate of a plasmocytoma patient (E.G) was performed.

Figure 7 shows that 2 to 40 μg of total RNA were isolated from $0.1\text{-}2 \times 10^7$ MNC fraction of the plasmocytoma patient. The final preparation of RNA was free of DNA and protein contamination and had a 260/280 ratio higher than 1.8. RT-PCR was carried out using a mixture of 5' oligonucleotide FWR1 primers specific for the expressed Vκ or Vλ families together with a mixture of downstream 3' primers specific for Jκ or Jλ genes (Sahota *et al*., 1997). The size of the amplified plasmocytoma variable region of immunoglobulin light chain (V_LIgPl) was about 350 pb (Figure 8).

The V_LIgPl used by the tumor cells was identified as repeated identical V_L-J_L sequences obtained after cloning of PCR products (Figure 9). Non-identical sequences were presumably from normal plasma cells in the aspirate (Hawkins *et al*., 1994; Sahota *et al*., 1997). Sequencing alignment was made to current EMBL/GenBank and V-BASE sequence directories (Cook and Tomlinson, 1995) using MacVector 4.0 sequence analysis software (International Biotechnologies Inc). Sequence analysis of V_LIg of the plasmocytoma patient revealed a V segment of the $V_{\kappa IV}$ subgroup (Figure 10).



Lanes	Number of MNC	Total RNA	Purity %
1	2×10^7	40 µg	1.8
2	1×10^7	13 µg	1.84
3	5×10^6	8 µg	1.9
4	2×10^6	3.5 µg	1.87
5	1×10^6	2 µg	1.82

Figure 7: *Total RNA isolation from different cell numbers of MNC of heparinized bone marrow aspirate of a plasmocytoma patient (E.G).*

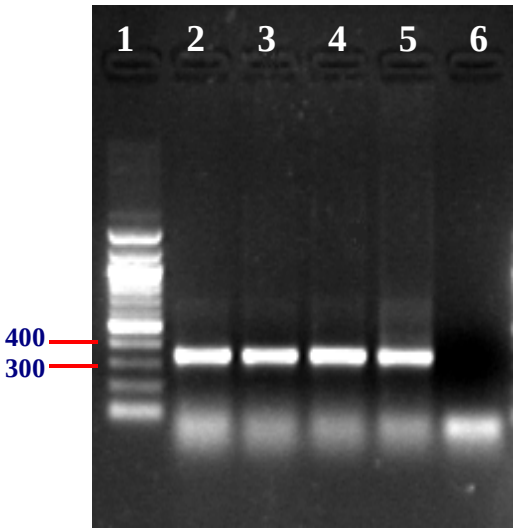


Figure 8: *V_LIgPl RT-PCR. Lane 1, 100 bp DNA Ladder; lane 2-5, four independent PCR amplifications and lane 6, negative control.*

Alignment for V segment

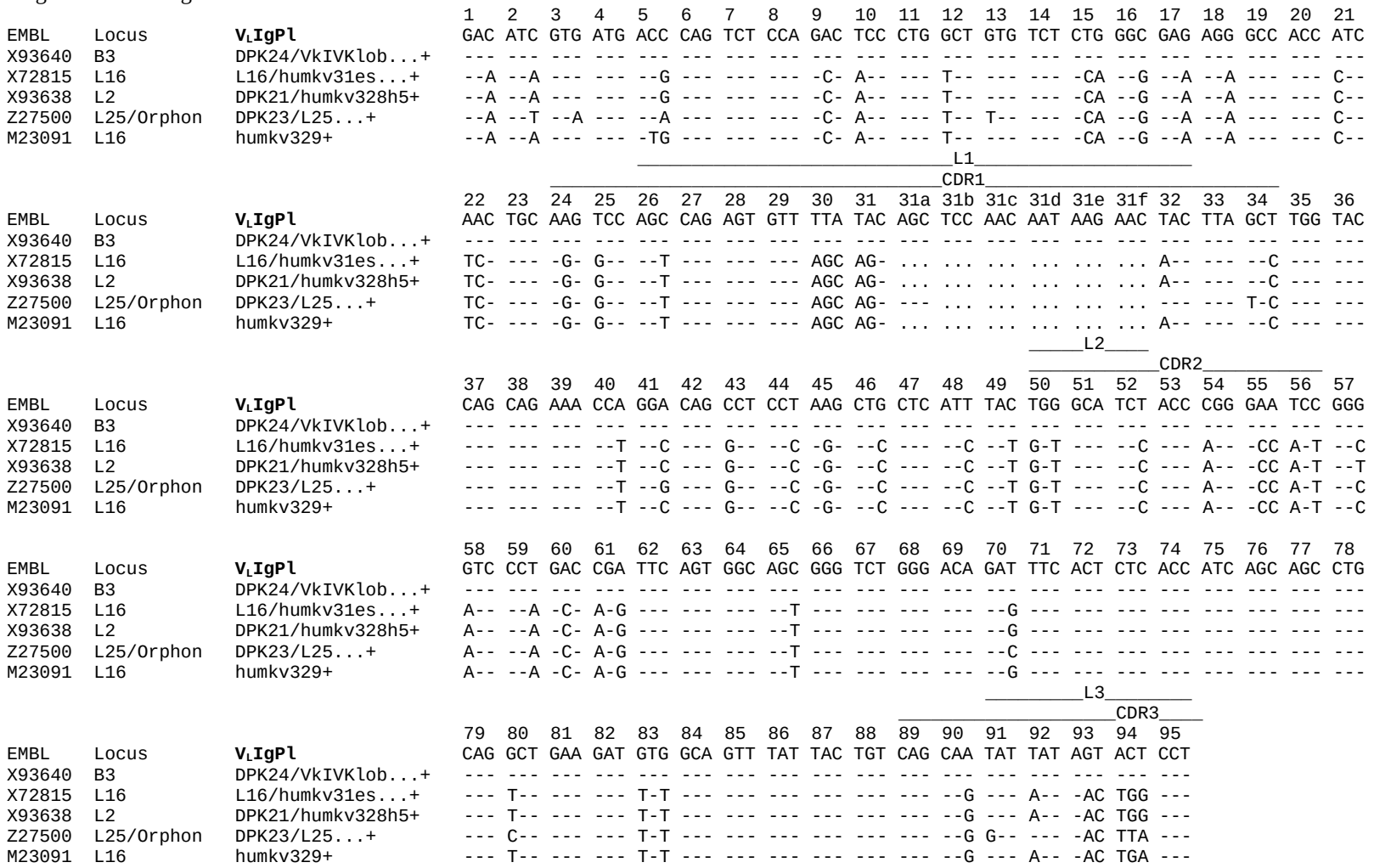
```
           1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20  21
2YP999     GAC ATC GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT GTG TCT CTG GGC GAG AGG GCC ACC ATC
7YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
8YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
9YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1YP999     --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
4YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10YP999    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
5YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                          _____________________________L1_____________________
                     _____________________________________CDR1_____________________________
           22  23  24  25  26  27  28  29  30  31  31a 31b 31c 31d 31e 31f 32  33  34  35  36
2YP999     AAC TGC AAG TCC AGC CAG AGT GTT TTA TAC AGC TCC AAC AAT AAG AAC TAC TTA GCT TGG TAC
7YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
8YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
9YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
4YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- -G- --- --- --- --- --- ---
10YP999    --- --- --- --- --- --- --- --- --- -K- --- --- --- --- --- --- --- --- --- --- ---
5YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                           _____L2____
                                                     ______________CDR2_____________
           37  38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57
2YP999     CAG CAG AAA CCA GGA CAG CCT CCT AAG CTG CTC ATT TAC TGG GCA TCT ACC CGG GAA TCC GGG
7YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
8YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
9YP999     --- --- --- --- --- --- --- -Y- --- --- --- --- --- --- --- --- --- --- --- --- ---
1YP999     --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- --- ---
4YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10YP999    --- --- --- --- --R --- --- -S- --- --- M-- --- --- --- --- --W --- --- --- --- ---
5YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

           58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76  77  78
2YP999     GTC CCT GAC CGA TTC AGT GGC AGC GGG TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGC CTG
7YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
8YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
9YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
4YP999     --- --- --- --- --- --- --- --- -K- --- --- --- --- --- --- --- --- --- --- --- ---
10YP999    --- --- --- --- --- --- --- --- --- -M- --- --- --- --- --- --- --- --- --- --- ---
5YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                                                        _________L3________
                                                  ______________________CDR3____
           79  80  81  82  83  84  85  86  87  88  89  90  91  92  93  94  95
2YP999     CAG GCT GAA GAT GTG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGT ACT CCT
7YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
8YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
9YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
1YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
4YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
10YP999    --- S-- --- --- --- --- --- --- -M- --- --- --- --- --- --- --- ---
5YP999     --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --C
```

Alignment for J segment

```
2YP999     TGTCAGCAATATTATAGTACTCCTCGGGGGCCACCTTCGGCCAAGGGACACGACTGGAGATTAAACGT
7YP999     ------------------------------------------------------------------
8YP999     ------------------------------------------------------------------
9YP999     ------------------------------------------------------------------
1YP999     ----------------------------------.-------------------------------
4YP999     ------------------------------------------------------------------
10YP999    ------------------------------------------------------------------
5YP999     ----------------T------CACTTTCGGCGGAGG-A----A-TGG-AAT-AAAC-T........
```

Figure 9: *Repeated identical V_L-J_L sequences of a plasmocytoma patient. Dashes indicate sequence identity, and points indicate loss of nucleotides. K = g + t, M = a + c, R = a + g, S = c + g, W = a + t, Y = c + t.*

Alignment for V segment



The sequence uses a V segment of the V_{KIV} subgroup.

Figure 10: *Sequence analysis of V_LIg gene expressed in a plasmocytoma patient (V_LIgPl). Dashes indicate sequence identity, and points indicate loss of nucleotides. Analysis performed by DNAPLOT Version 2.0.1 using V BASE Version 1.0.*

3.2 Retroviral expression-cloning vectors

The most widely used retroviral vectors contain an antibiotic-resistance gene such as neomycin (Neo^R) that is strongly immunogenic. Its peptides will be presented on MHC class I molecules and activate $CD8^+$ T lymphocytes, thus skewing the elicited immune response. To avoid this, a novel retroviral expression-cloning vector that directs the tumor-associated antigen of plasmocytoma marked with a FLAG antibody-binding site to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor has been constructed. Thus, transfected or transduced cells can be detected using monoclonal anti-FLAG antibodies.

The GPI-anchored construct has been chosen because of its stable transgene surface expression without a major dependence on the transcription rate, which is limiting for constructs using nerve growth factor receptor (NGFR) or epidermal growth factor (EGF) fusion peptides (Austin *et al.*, 2000). For targeting the transgene to a GPI-linked protein, a GPI-linked protein that is endogenously expressed in DC such as decay-accelerating factor (DAF) has been used in order to avoid an imbalance of presentation of foreign peptides apart from V_LIgPl.

3.2.1 Retroviral vectors construction

3.2.1.1 pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF

The novel retroviral vector pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF has been constructed as follows:

A) Construction of plasmid Bluescript SK(+) containing the FLAG sequence in frame between the two hydrophobic ends of DAF:
The ***Sac*I *Not*I Start-codon NH_2(34a.a)DAF FLAG *Xba*I** in frame has been constructed from pSPORT1 contained 5' end of DAF and transformed in DH10B (clone DKFZp434P184) by PCR using *Sac*I *Not*I Start Signal sense primer and *Xba*I FLAG Signal anti-sense primer and was inserted into pBluescript SK(+) by *Not*I and *Xba*I digestion. In addition, the ***Xba*I *Cla*I DAF(37a.a)COOH Stop-codon *Hind*III *Kpn*I** in frame has been constructed from pT7T3D-PAC mod1 contained 3' end of DAF and transformed in DH10B (clone IMAGp998D23661) by PCR using *Xba*I *Cla*I GPI sense primer and *Kpn*I *Hind*III Stop GPI

anti-sense primer and was inserted into the above pBluescript SK(+) containing ***Not*I Start-codon NH_2(34a.a)DAF FLAG *Xba*I** in frame by *Xba*I and *Kpn*I digestion (Figure 11***A***).

B) Construction of plasmid Bluescript SK(+) containing the FLAG-V_LIgPl in frame between the two hydrophobic ends of DAF:
The ***Xba*I V_LIgPl *Cla*I** in frame has been constructed by PCR using *Xba*I 2YP999 sense primer and *Cla*I 2YP999 anti-sense primer and was inserted into the above pBluescript SK(+) containing ***Not*I Start-codon NH_2(34a.a)DAF FLAG *Xba*I *Cla*I DAF(37a.a)COOH Stop-codon *Hind*III *Kpn*I** in frame by *Xba*I and *Cla*I digestion (Figure 11***B***).

C) Construction of the retroviral vector that directs the FLAG-V_LIgPl fusion protein to the plasma membrane via GPI-anchor:
The novel retroviral vector pSFβ$_S$(FLAG-V_LIgPl)$_\omega$DAF has been constructed by replacing the Neo^R (neomycin resistance) fragment of pSFβN1, kindly provided by Dr. Christopher Baum (Baum *et al.*, 1995) with ***Not*I Start-codon NH_2(34a.a)DAF FLAG *Xba*I V_LIgPl *Cla*I DAF(37a.a)COOH Stop-codon *Hind*III** by *Not*I and *Hind*III digestion (Figure 11***C***).

3.2.1.2 pSFβ$_S$(FLAG)$_\omega$DAF

The vector pSFβ$_S$(FLAG)$_\omega$DAF without the V_LIgPl has been constructed by replacing the Neo^R (neomycin resistance) fragment of pSFβN1 with ***Not*I Start-codon NH_2(34a.a)DAF FLAG *Xba*I *Cla*I DAF(37a.a)COOH Stop-codon *Hind*III** by *Not*I and *Hind*III digestion.

3.2.1.3 pSFβEGFP

The Neo^R of pSFβN1 was replaced by enhanced green fluorescent protein gene (EGFP), isolated as a 763-bp fragment from pBI-EGFP plasmid by *Not*I and *Hind*III digestion, to generate pSFβEGFP used as control for transfection and transduction efficacy.

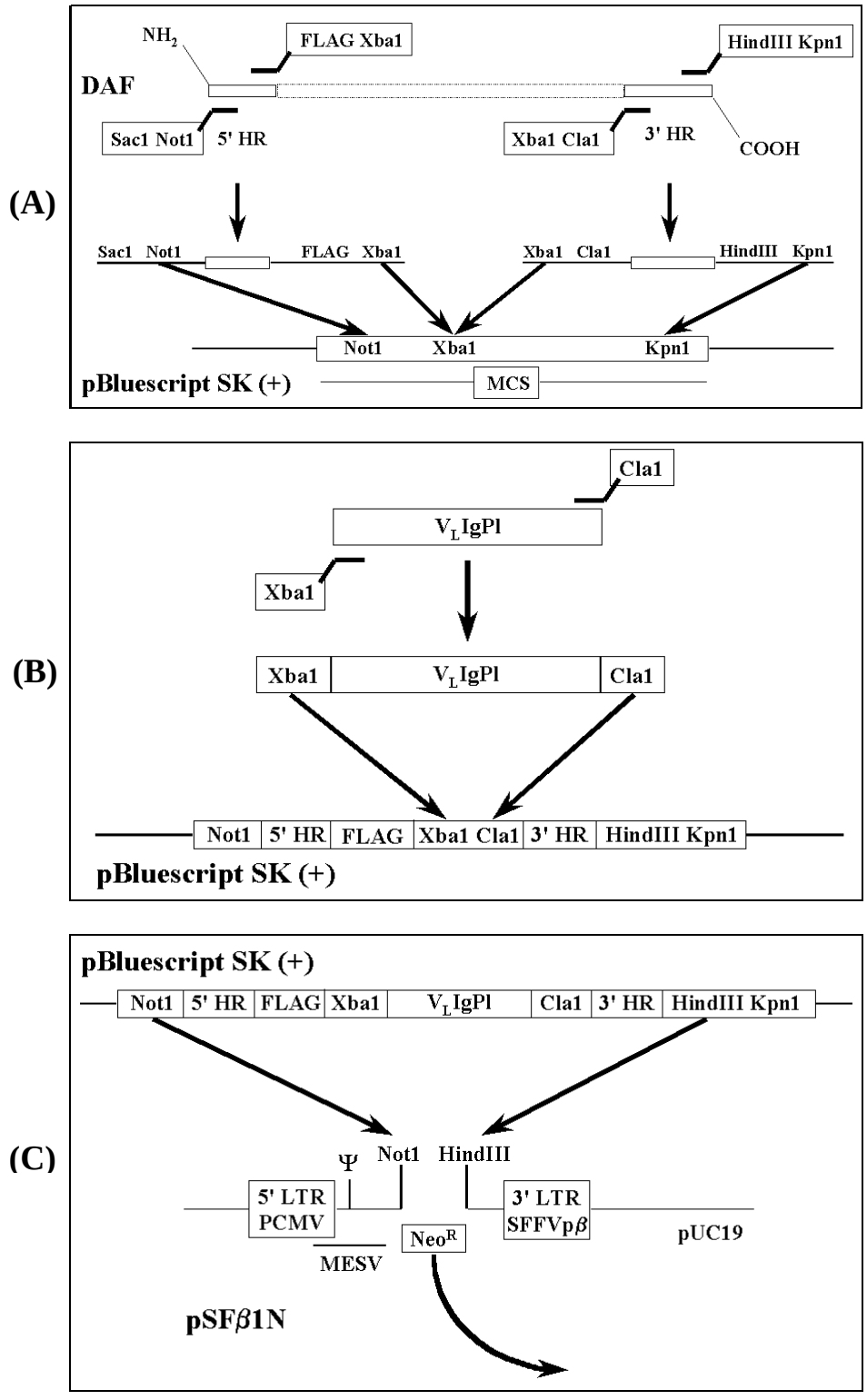


Figure 11: *Steps of the pSFb_S(FLAG-V_LIgPl)$_w$DAF retroviral vector construction:* ***(A)*** *Construction of plasmid Bluescript SK(+) containing the FLAG in frame between the two hydrophobic ends of DAF,* ***(B)*** *Construction of plasmid Bluescript SK(+) containing the FLAG-V_LIgPl in frame between the two hydrophobic ends of DAF and* ***(C)*** *Construction of the retroviral vector that directs the FLAG-V_LIgPl fusion protein to the plasma membrane via GPI-anchor. 3' HR, 3' hydrophobic region of DAF; 5' HR, 5' hydrophobic region of DAF; LTR, long terminal repeat; MESV, murine embryonic stem cell virus; PCMV, PCC4-cell-passaged mutant virus; SFFVp, polycythemic strain of the spleen focus-forming virus and y, indicates the presence of retroviral packaging sequence.*

The objective of this work was to induce efficient transfer and expression of V_LIgPl gene in DC. Therefore, the SFFVp/MESV hybrid retroviral vector backbone has been chosen, which was designed to overcome transcriptional inefficiency and silencing associated with retroviral gene transfer into myeloid progenitors and haematopoietic stem cells (Baum *et al*., 1995).

Figure 12 demonstrates the schematic diagram of the retroviral vectors pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF, pSFβ_S(FLAG)$_\omega$DAF and pSFβEGFP. The novel retroviral vector, pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF, contains FLAG-V_LIgPl fusion protein in frame between the two ends of DAF. The first 34 and the last 37 amino acids of DAF are hydrophobic domains cleaved upon attachment to the GPI-anchor molecule (Moran *et al*., 1991). In addition, the cleavage attachment site (ω) is located within the carboxyterminus leading to GPI-linked transgene expression provided by this vector (Figure 13). The GPI-anchor contains phosphatidylinositol, carbohydrate and ethanolamine and is though to be preassembeld in the endoplasmic reticulum (Masterson *et al*., 1989; Udenfriend and Kodukula, 1995; Kinoshita and Inoue, 2000). Anchor addition apparently takes place in the endoplasmic reticulum (Bangs *et al*., 1985, 1986; Ferguson *et al*., 1986; Udenfriend and Kodukula, 1995; Kinoshita and Inoue, 2000), after which the protein is transported to the cell surface via the Golgi apparatus (Caras, 1991). Xba1 and Cla1 restriction sites are not present in any type of human V genes. Thus, any type of the V_LIg after Xba1 and Cla1 restriction sites addition can be directly cloned into this novel retroviral expression-cloning vector in order to obtain the segment of interest in frame with the recognition sequences of DAF. As a result, the vector pSFβ_S(FLAG)$_\omega$DAF without the V_LIgPl directs the FLAG to the plasma membrane and can be used as a control for immune response.

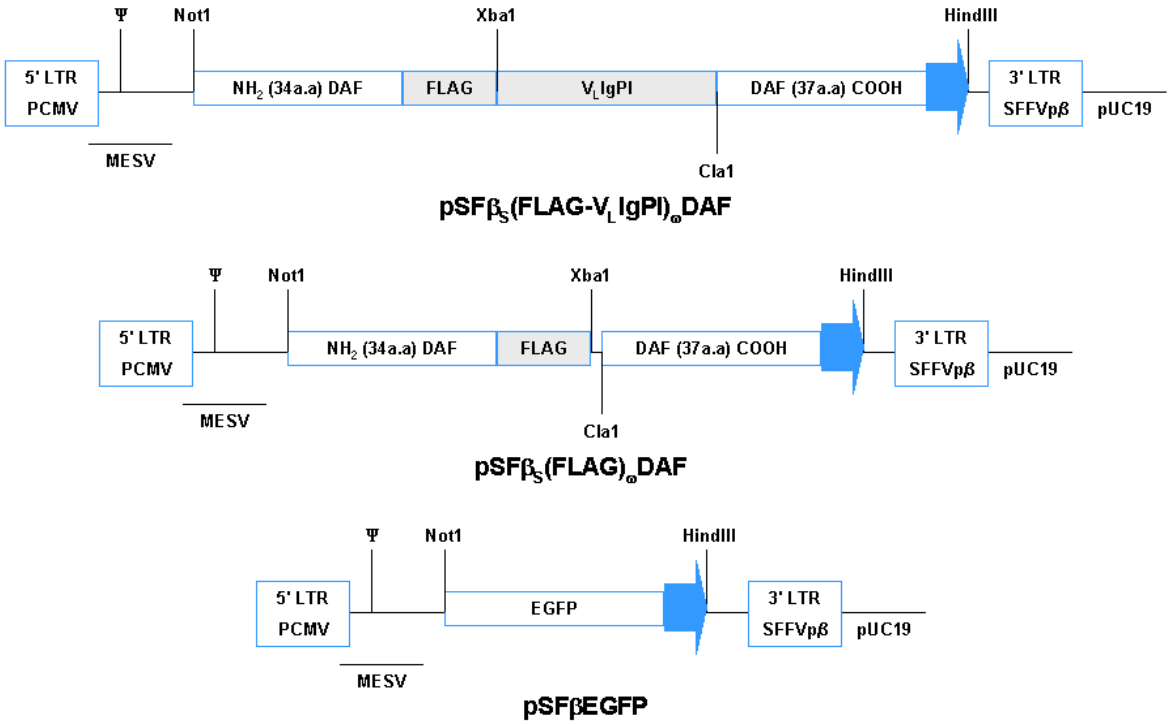


Figure 12: *Schematic diagram of the retroviral vectors pSFb$_S$(FLAG-V$_L$IgPl)$_w$DAF, pSFb$_S$(FLAG)$_w$DAF and pSFbEGFP. LTR, long terminal repeat; MESV, murine embryonic stem cell virus; PCMV, PCC4-cell-passaged mutant virus; SFFVp, polycythemic strain of the spleen focus-forming virus and y , indicates the presence of retroviral packaging sequence.*

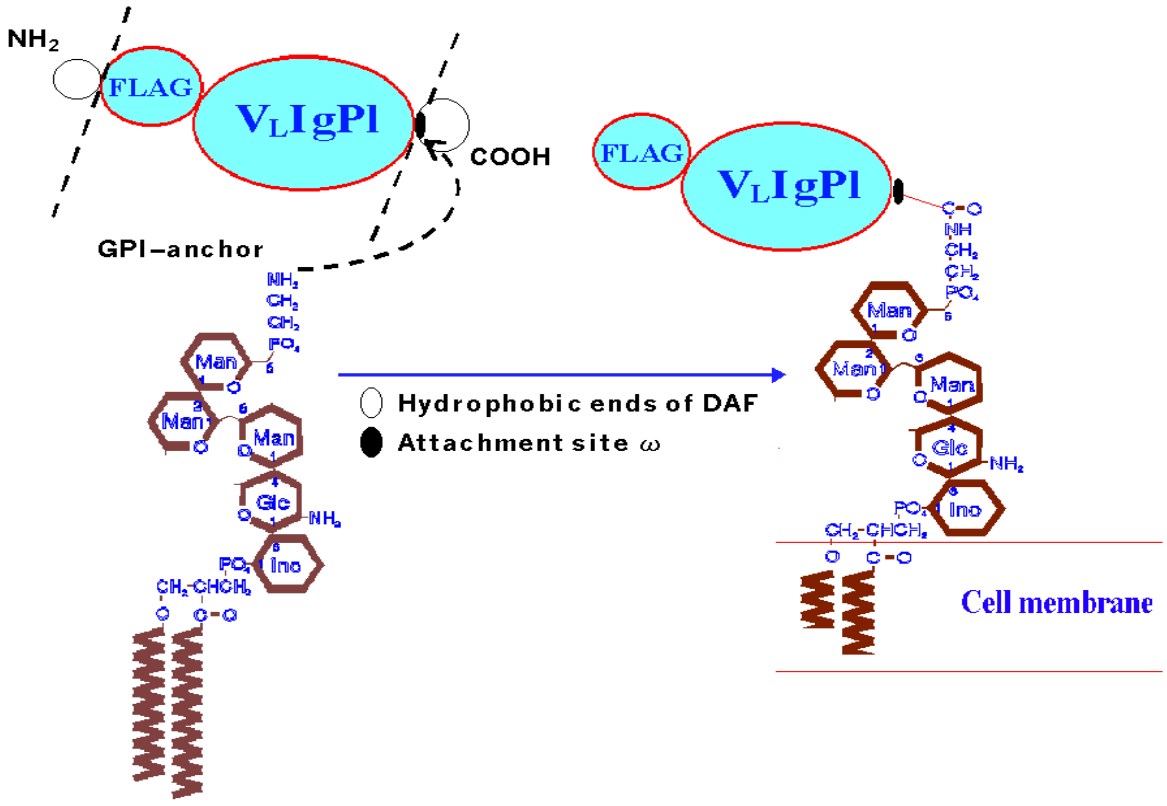


Figure 13: *Schematic diagram of the GPI attachment to the FLAG-V$_L$IgPl fusion protein.*

3.2.2 Testing the novel retroviral vector through transient expression of FLAG in Jurkat cells

Jurkat wild type cells were transfected with pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF retroviral vector via electroporation to make sure that it targets the FLAG-V_LIgPl fusion protein to the cell surface. Different electroporation protocols were carried out to determine the optimal transfection conditions. The best result (13.2 %) obtained at higher cells number (2×10^7) in a final volume of 500 µl medium containing 40 µg retroviral vector at 350 V, 1050 µF and RT (Table 2).

Cell number	DNA amount	Final volume	Voltage	Capacity	Temperature	FLAG expression
1×10^6	10 µg	1000 µl	250 V	800 µF	4°C	0.93 %
1×10^6	10 µg	1000 µl	250 V	800 µF	RT	1.36 %
5×10^6	20 µg	800 µl	250 V	800 µF	4°C	4.99 %
5×10^6	20 µg	800 µl	250 V	800 µF	RT	5.5 %
1×10^7	30 µg	800 µl	250 V	800 µF	4°C	6.8 %
1×10^7	30 µg	800 µl	250 V	800 µF	RT	7 %
2×10^7	40 µg	800 µl	350 V	1050 µF	4°C	10.3 %
2×10^7	40 µg	500 µl	350 V	1050 µF	RT	13.2 %

Table 2: *FLAG expression in Jurkat wild type cells transfected with pSFb_S(FLAG-V_LIgPl)$_w$DAF retroviral vector via different electroporation protocols. RT: room temperature.*

In order to verify GPI-anchoring of the transgene, phosphatidylinositol-specific phospholipase C (PI-PLC) was used that specifically releases proteins bound to the membrane via GPI-anchor (Moran *et al*., 1991). As shown in Figure 14, FLAG signals from transfected cells could be released by PI-PLC indicating that the retroviral vector targets the fusion protein to the cell surface via GPI-anchor.

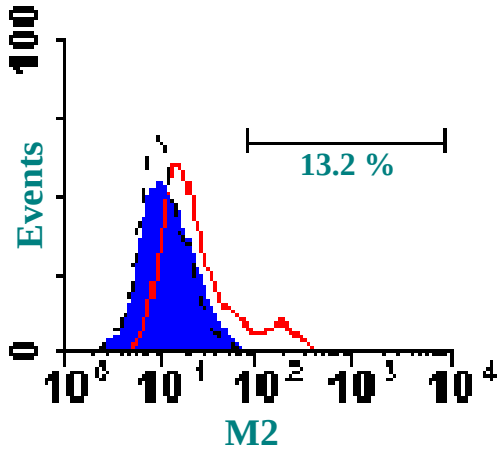


Figure 14: *PI-PLC dependent release of cell surface FLAG of transfected Jurkat wild type cells with pSFb$_S$(FLAG-V$_L$IgPl)$_w$DAF indicating that this retroviral vector targets the fusion protein to the cell surface via GPI-anchor. Transfected cells treated with or without PI-PLC are indicated by dotted and solid lines, respectively. Shaded areas represent the non-transfected cells. M2: Anti-FLAG antibody-binding.*

3.3 Serum-free culture conditions for *in vitro* generation of dendritic cells

The identification of culture conditions enabling the generation of DC *in vitro* are useful to study their biology and to prepare large amounts of APC for immunotherapeutic purposes (Ferlazzo *et al*., 1999). It is now known that CD34-derived dendritic cells (CD34-DC) have a preferential capacity to activate $CD8^+$ T cells (Ferlazzo *et al*., 1999) and CD86 is the most important ligand to induce CD28-mediated co-stimulation for $CD8^+$ T cell activation (Van Gool *et al*., 1999). DC can be generated *in vitro* from $CD34^+$ progenitors in cord blood [CB] (Santiago-Schwarz *et al*., 1992; Sato *et al*., 1998), bone marrow [BM] (Reid *et al*., 1992; Szabolcs *et al*., 1995) and peripheral blood [PB] after cytokine mobilization (Strunk *et al*., 1996; Ratta *et al*., 1998). However, the cytokine requirements to differentiate CB- and PB-$CD34^+$ progenitor cells into DC are known to be different (Garbe *et al*., 1998). GM-CSF plus TNF-α ± SCF used in most protocols described not only for generation of DC but also of macrophages and neutrophils (Santiago-Schwarz *et al*., 1992; Szabolcs *et al*., 1995; Bender *et al*., 1996). In addition, FCS used as culture supplement in most of the protocols described contains additional foreign antigens leading to potential skewing of the resulting immune response.

Phorbol ester (PMA) is a stable analogue of 2,3-diacylglycerol that activates the classical (α, β1, β2 and γ) and new (δ, ε, η, θ and μ) isoforms of protein kinase C [PKC] (Nishizuka,

1995). PMA-mediated signaling induced the expression of the RelB transcription factor, suggesting a pathway by which genetic events involved in DC differentiation are initiated (Davis *et al.*, 1998). Recently, it was reported that PMA-induced PKC activation specifically leads to differentiation of bone marrow $CD34^+$ progenitor cells to mature DC in serum-containing medium and no other lineages are generated (Davis *et al.*, 1998; St. Louis *et al.*, 1999). Here the ability to differentiate $CD34^+$ progenitor cells from different origins into DC under serum-free conditions in the presence of PMA was studied.

3.3.1 *Ex vivo* expansion of $CD34^+$ progenitors under serum-free conditions

Ex vivo expansion of $CD34^+$ progenitor cells facilitates the identification of culture conditions useful to generate DC to study their biology and retroviral transduction for immunotherapeutic purposes (Glimm *et al.*, 1998). To test the ability to expand $CD34^+$ progenitor cells under serum-free conditions, the increase in cell counts of BM-$CD34^+$ cells cultured in complete serum-containing medium IMDM or RPMI-1640 was compared with that cultured in complete serum-free medium CellGro® SCGM or CellGro® DC containing either Flt-3L, 150 ng/ml; IL-3, 100 ng/ml; SCF, 50 ng/ml and TGF-β1, 0.5 ng/ml or Flt-3L, 300 ng/ml; IL-3, 100 ng/ml and SCF, 100 ng/ml (Table 3).

Experiment	Expansion (fold)							
	Complete serum-containing media				Complete serum-free media			
	IMDM		RPMI-1640		CellGro® SCGM		CellGro® DC	
	1st cytokine cocktail	2nd cytokine cocktail	1st cytokine cocktail	2nd cytokine cocktail	1st cytokine cocktail	2nd cytokine cocktail	1st cytokine cocktail	2nd cytokine cocktail
1	3.16	4.35	3.00	4.36	2.90	4.70	2.40	3.30
2	3.55	4.80	3.70	4.50	3.90	5.50	3.10	3.39
3	4.19	5.11	3.77	5.21	4.21	4.94	3.60	3.90
Mean ± SD	3.63 ± 0.52	4.75 ± 0.38	3.49 ± 0.43	4.69 ± 0.46	3.67 ± 0.68	5.05 ± 0.41	3.03 ± 0.60	3.53 ± 0.32

Table 3: *Expansion of BM-$CD34^+$ cells in serum-free media compared with serum-containing media and with different cytokine cocktails. BM-$CD34^+$ cells (5 ´ 10^4 cells/ml) cultured in complete serum-containing medium IMDM or RPMI-1640 or complete serum-free medium CellGroÒ SCGM or CellGroÒ DC containing either 1st cytokine cocktail (Flt-3L, 150 ng/ml; IL-3, 100 ng/ml; SCF, 50 ng/ml and TGF-b1, 0.5 ng/ml) or 2nd cytokine cocktail (Flt-3L, 300 ng/ml; IL-3, 100 ng/ml and SCF, 100 ng/ml). Cultures were incubated for 7 days at 37°C and 5 % CO_2.*

Figure 15***A*** shows that there is no statistical difference (P = 0.344, n = 12) in expansion between serum-containing media and serum-free media. The highest (4.36 ± 0.91, n = 6) and lowest (3.28 ± 0.51, n = 6) expansions were in CellGro® SCGM and CellGro® DC, respectively (Figure 15***B***). The expansion using cytokine cocktail 2 is significantly better (P = 0.0005, n = 12) than that using cytokine cocktail 1 (Figure 15***C***). These findings demonstrate that the best conditions for expansion of $CD34^+$ cells are provided in serum-free medium (CellGro® SCGM) containing Flt-3L (300 ng/ml), IL-3 (100 ng/ml) and SCF (100 ng/ml). In the case that 50 % of the cytokine-containing medium was exchanged after 3 days and the density of cells did not exceed 10^5 cells/ml, the increase of cells was 18.5-fold. Therefore, the above cytokine cocktail facilitates retroviral transduction.

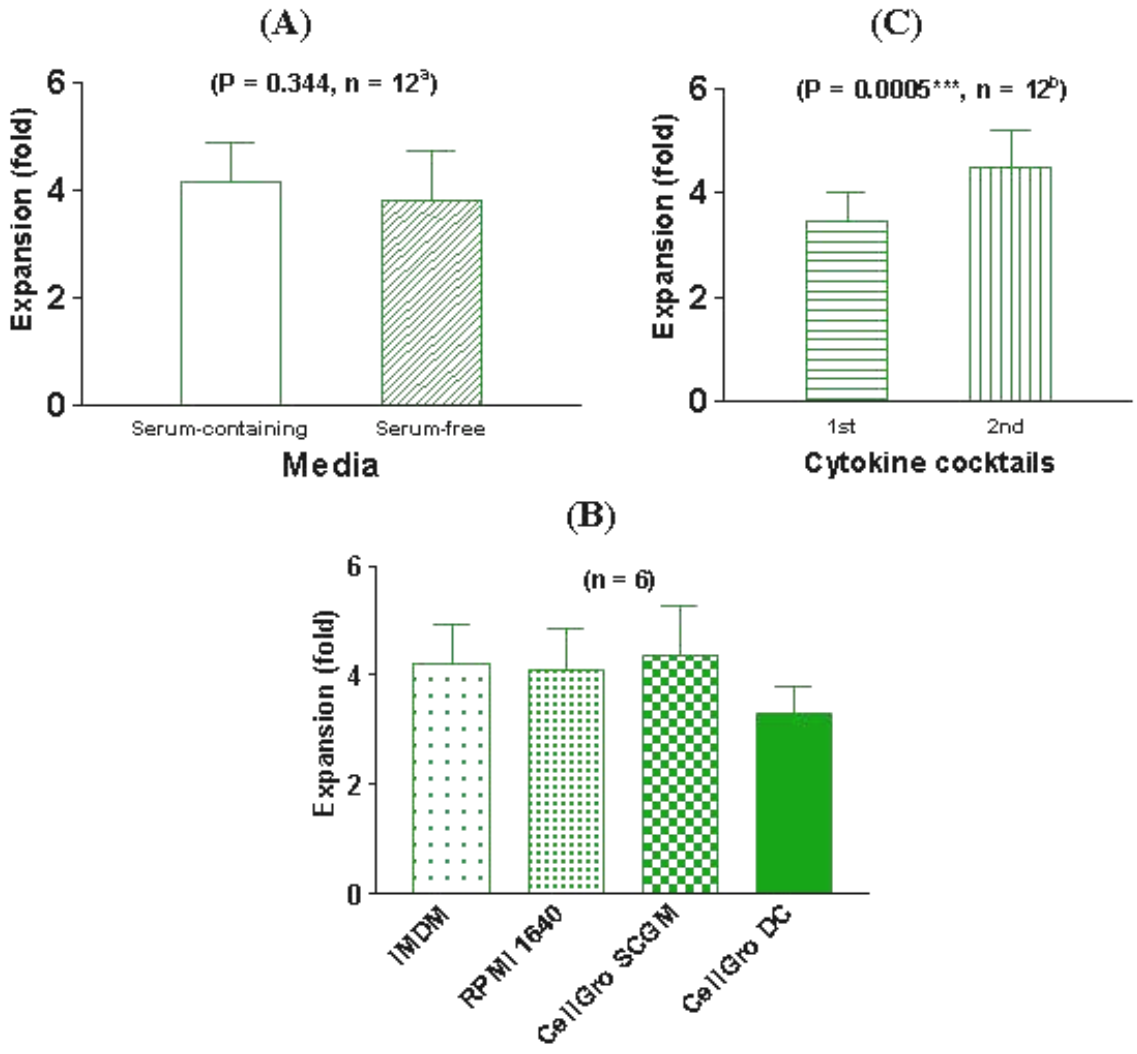


Figure 15*: Effect of* ***(A)*** *serum* ***(B)*** *different media and* ***(C)*** *different cytokine cocktails on in vitro expansion of $CD34^+$ progenitor cells.*
[a] *Unpaired t-tests*
[b] *Ordinary one-way ANOVA*
*** *Very highly significant*

3.3.2 PMA-derived dendritic cells

3.3.2.1 Phorbol ester induces differentiation of $CD34^+$ progenitors from different origins to DC under serum-free conditions

For DC generation out of $CD34^+$ cells by cytokine cocktails, large amounts of cytokines are necessary. In addition, cells generated by such cytokine cocktails are not only confined to the DC monocytic lineage. In order to analyze DC function in Ag presentation of V_LIgPl peptides, a more homogeneous DC fraction is needed. Therefore, PMA was used, which has the capability to trigger DC differentiation out of BM derived $CD34^+$ cells without contamination with other cell lineages. Here, the ability of PMA to differentiate CB- and PB-$CD34^+$ progenitor cells to DC using serum-free conditions has been examined.

By day 7, about 40-60 % of CB- and PB-$CD34^+$ progenitor cells cultured in serum-free medium (CellGro® DC) with 15 ng/ml PMA became large and loosely adherent and a subset developed long dendrites and hair-like cytoplasmic projections (Figure 16), a morphology reported to be characteristic of DC (Hart, 1997). Morphologic changes were present within one day of stimulation and completed within three days. They were stable over at least 2 weeks in culture.

Expression of the co-stimulatory molecule CD86 (B7-2), and the DC lineage marker CD83 typically characterize mature DC. Phenotypic analysis at day 7 after PMA-induction of CB- and PB-$CD34^+$ progenitor cells (adherent and non-adherent) demonstrated that all cells were positive for both CD83 and CD86 and negative for CD1a (Figure 17). In addition these cells were $CD1a^-$ throughout all experiments. These data are in accordance with the finding that PMA induces DC differentiation without causing cell proliferation and the generation of cellular intermediates (Davis *et al.*, 1998).

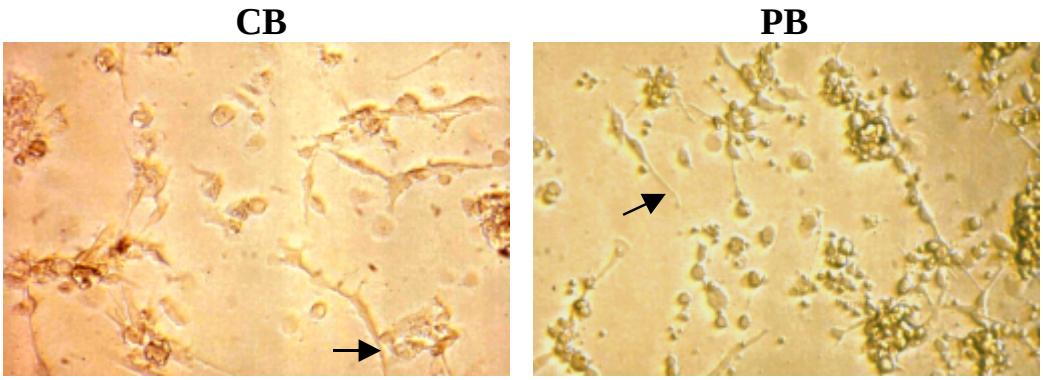


Figure 16: *Typical morphology of cord blood (CB)- and cytokine mobilized peripheral blood (PB)-CD34$^+$ cells cultured in serum-free medium (CellGro$^{\hat{O}}$ DC) upon stimulation with PMA alone for 7 days (arrows indicate elongated dendrites).*

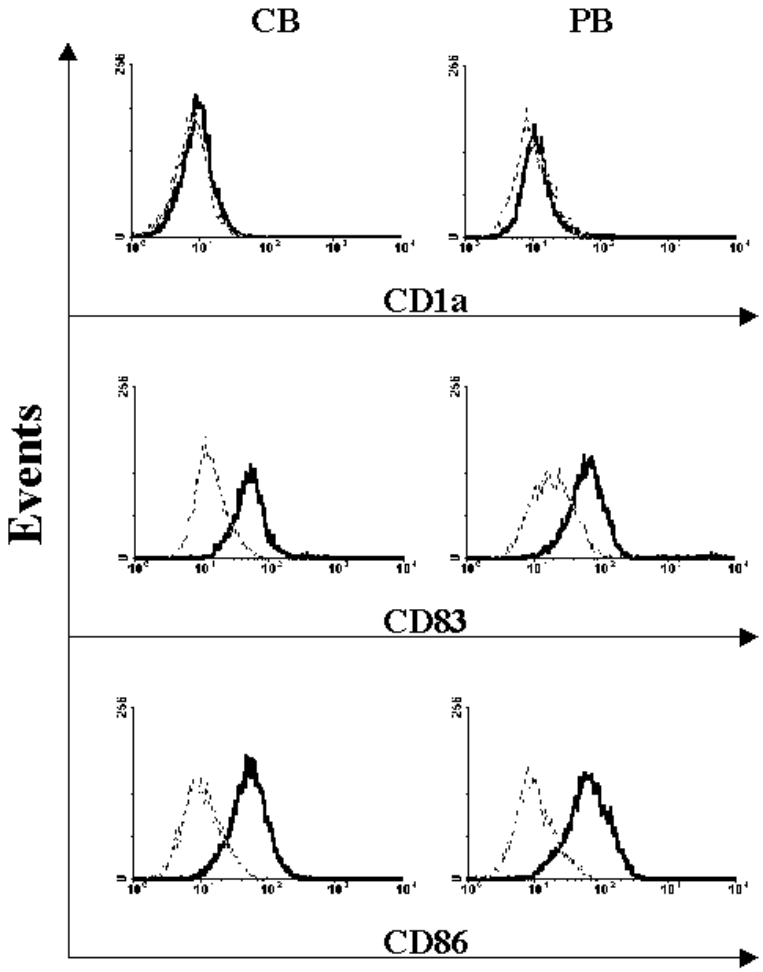


Figure 17: *Surface Ag phenotype characterization of day 7 PMA-treated cord blood (CB)- and cytokine mobilized peripheral blood (PB)-CD34$^+$ cell cultures in serum-free medium. Total cells in cultures (adherent and non-adherent) were analyzed by flow cytometric analysis. Isotype-matched controls are indicated by dotted lines. Results are representative of three different experiments.*

3.3.2.2 Phorbol ester induces differentiation of *ex vivo* expanded $CD34^+$ progenitors to DC in serum-free medium

Murine oncoretroviral vectors require cell division for integration into the nucleus. Therefore, a round of expansion of $CD34^+$ cells is required before their differentiation into DC in the presence of PMA. The efficiency of DC differentiation by PMA after expansion of $CD34^+$ cells needs to be investigated.

To test if morphological and phenotypic changes induced by PMA were affected by pretreatment of BM-$CD34^+$ cells with a cytokine cocktail that induced a large expansion under serum-free conditions, the cells were expanded for 7 days in CellGro® SCGM containing Flt-3L (300 ng/ml), IL-3 (100 ng/ml) and SCF (100 ng/ml) and then differentiated in CellGro® DC containing 15 ng/ml PMA until day 14.

Figure 18 shows that the DC differentiation induced by PMA under serum-free conditions was not affected morphologically by pretreatment of BM-$CD34^+$ cells with the above cytokine cocktail that induced $CD34^+$ cell expansion connected with myeloid, but not with DC differentiation, as described previously (Davis *et al*., 1998). In addition, the cells were positive for CD83 and CD86 and negative for CD1a and CD14 (Figure 19). Table 4 demonstrates that at day 14, 2.94 ± 0.42-fold increase of the cell number was obtained. Because the used cytokines facilitate murine oncoretroviral transduction (Glimm *et al*., 1998), the method described in this study may be used preferentially for introducing genes into DC by retroviral vectors.

3.3.2.3 $CD86^+$ yield of PMA-derived DC compared with that of cytokine-derived DC under serum-free conditions

To study the efficacy of PMA to generate mature DC in serum-free medium, PMA-derived DC were compared with cytokine-derived DC using the same serum-free culture conditions. The maturation of cultured human blood DC was delineated by surface analysis of the co-stimulatory molecule B7-2 (CD86), because it is the most important ligand to induce CD28-mediated co-stimulation for $CD8^+$ T cell activation (Van Gool *et al*., 1999).

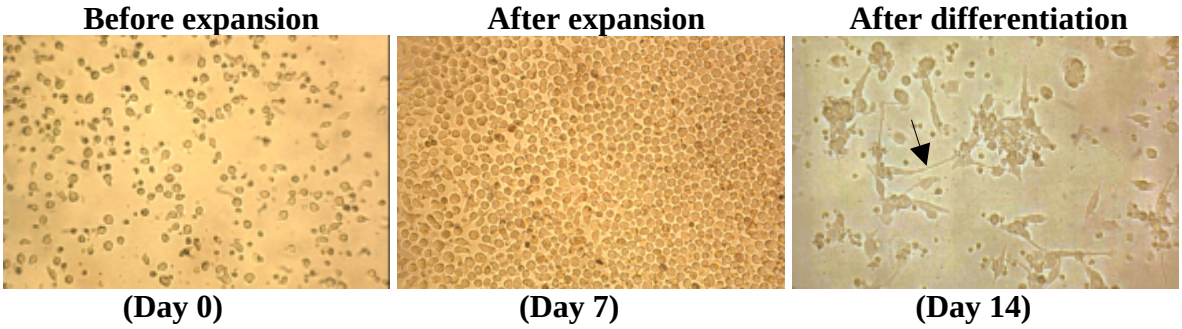


Figure 18: *Morphology of PMA-derived DC from ex vivo expanded BM-CD34$^+$ progenitor cells (arrow indicates elongated dendrite).*

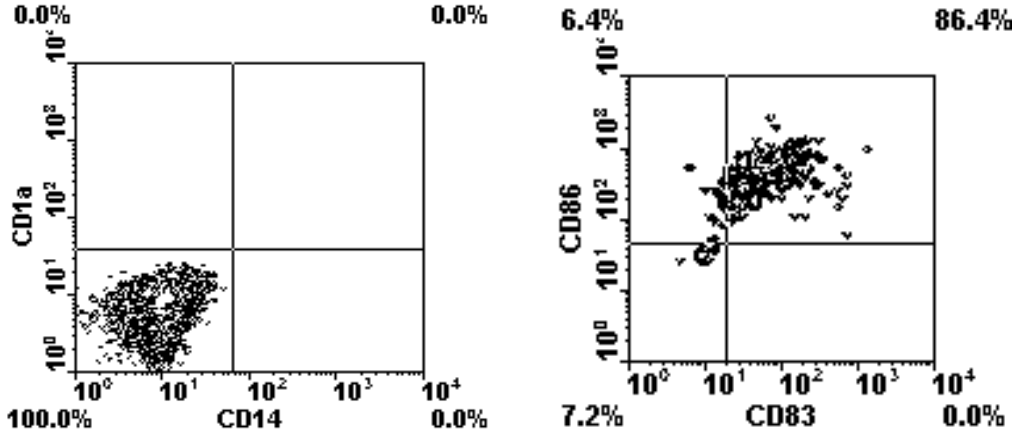


Figure 19: *Surface Ag phenotype characterization of day 7 PMA-derived DC from ex vivo expanded BM-CD34$^+$ progenitor cells.*

Experiment	Cell number	
	After expansion (Day 7)	After differentiation (Day 14)
1	2.35×10^5 (4.70)	1.48×10^5 (2.96)
2	2.80×10^5 (5.60)	1.80×10^5 (3.60)
3	2.24×10^5 (4.48)	1.28×10^5 (2.56)
4	2.05×10^5 (4.10)	1.29×10^5 (2.57)
5	2.59×10^5 (5.18)	1.50×10^5 (3.00)
Mean ± SD	(4.81 ± 0.59)	(2.94 ± 0.42)

Table 4: *Viable cell counts of BM-CD34$^+$ cells after expansion and differentiation for one week with PMA under serum-free conditions. The data enclosed in brackets represent the fold increase in cell number.*

$CD86^+$ yield was calculated as the ratio of the output number of $CD86^+$DC relative to the input number of $CD34^+$ progenitor cells with the following equation:

$CD86^+$ yield = Number of viable $CD86^+$DC at the end of experiment × 100 / the input number of $CD34^+$ progenitor cells (5×10^4)

In case of a shift, as found in PMA-induced DC, all cells have been considered to be positive. In contrast, $CD86^+$ and $CD86^-$ cells in cytokine-induced DC have been separable because not all cells shifted towards a positive CD86-expression. In this case only positive cells have been considered as induced DC (Figure 20).

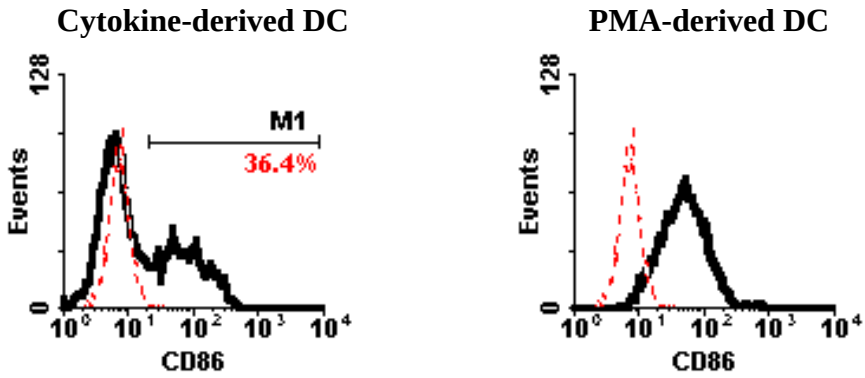


Figure 20: *CD86 expression in cytokine- and PMA-derived DC. Isotype-matched controls are indicated by dotted lines.*

It is well known that the cytokine requirements to differentiate CB- and PB-$CD34^+$ progenitor cells into DC are different (Garbe *et al.*, 1998). Therefore, $CD34^+$ progenitors from CB and PB in parallel experiments were used for this experiment.

$CD86^+$ yield was 28.0 % ± 7.0 and 44.6 % ± 7.5 for CB- and PB-derived dendritic cells upon stimulation with PMA for one week in serum-free medium (CellGro® DC), respectively (Figure 21). In contrast, the yield was 15.3 % ± 5.6 and 28.1 % ± 7.5 for CB- and PB-derived DC stimulated with different cytokine cocktails specifically for CB and PB differentiation, respectively, and for indicated periods of time in the same serum-free medium as mentioned in material and methods. These data suggest that under serum-free conditions $CD86^+$ yield was increased by PMA compared to cytokine cocktails.

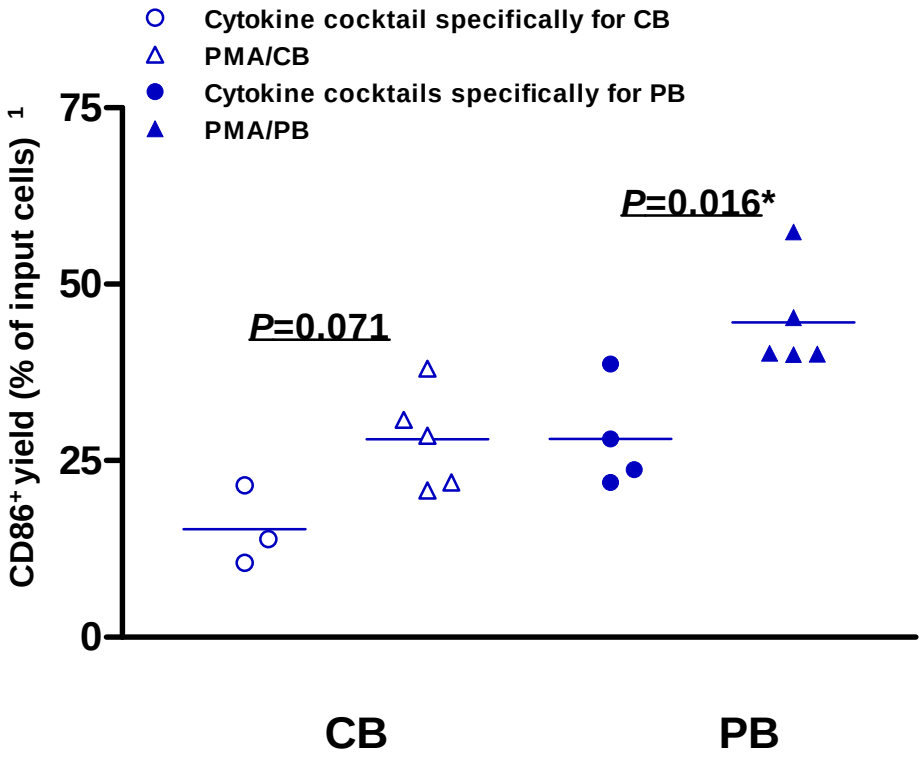


Figure 21: *$CD86^+$ yield of CB- and PB-derived dendritic cells stimulated with PMA for one week or with different cytokine cocktails specifically for CB and PB in serum-free medium (CellGro^{Ò} DC) and for indicated periods of time as mentioned in material and methods. CB, cord blood; PB, cytokine mobilized peripheral blood.*
[1] Number of viable $CD86^+$DC at the end of experiment ´ 100 / 5 ´ 10^4
****** Significant, P < 0.05, Mann-Whitney test (U-test)*

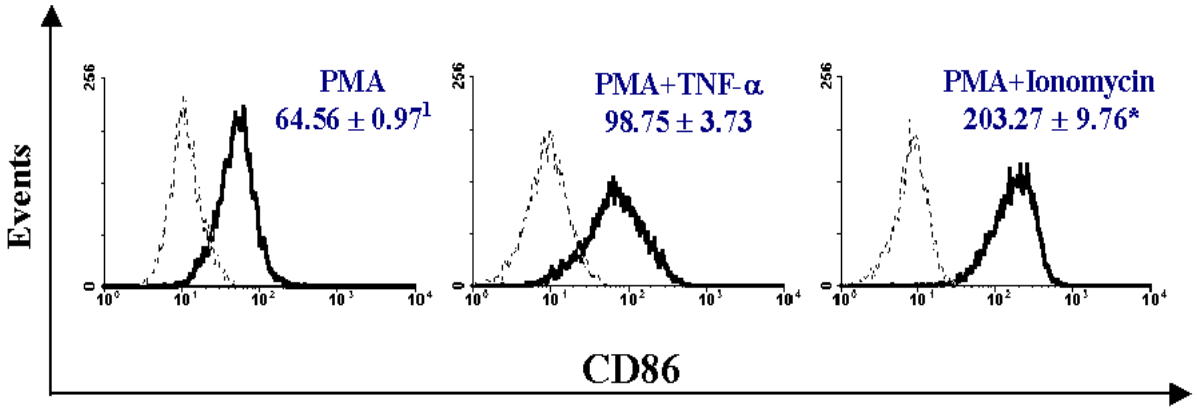


Figure 22: *$CD86^+$ expression of PB-derived dendritic cells after one week stimulation with serum-free medium (CellGro^{Ò} DC) containing PMA alone or plus TNF-α or ionomycin. Isotype-matched controls are indicated by dotted lines. Results are representative of three different experiments.*
[1] Mean fluorescence
****** Significant, P < 0.05, Kruskal-Wallis test*

3.3.2.4 Effect of addition TNF-α or ionomycin to the serum-free medium containing PMA on the output number of viable cells and $CD86^+$ expression of the $CD34^+$-derived dendritic cells

PB-$CD34^+$ cells were also stimulated with PMA plus 10 ng/ml TNF-α or plus 100 ng/ml ionomycin in complete serum-free medium (CellGro® DC) for 7 days to study their effect on PMA-derived DC.

Figure 22 demonstrates that up-regulation of CD86 was most pronounced with addition of the calcium ionophore ionomycin. However, the output number of viable cells after differentiation was decreased by PMA plus TNF-α ($P > 0.05$) or ionomycin ($P < 0.05$) compared with that in PMA alone (Figure 23). This observation is in accordance with the finding that the percentage of apoptotic cells of stimulated KG1 myeloid cell line with PMA plus TNF-α or ionomycin was increased compared with that stimulated with PMA alone (St. Louis *et al*., 1999).

3.3.2.5 PMA-generated DC from $CD34^+$ progenitors are functional APCs

The functional hallmark of DC is their ability to activate T cells (Caux *et al*., 1990; Rosenzwajg *et al*., 1996; Zhou and Tedder, 1996). While T cell proliferation was induced by autologous PMA-generated DC in the presence of SEB or TT, PMA-generated DC can't induce the proliferation of autologous T cells in the absence of Ag (Figure 24). This lack of proliferation, in the absence of Ag, makes it unlikely that T cell proliferation is due to phorbol ester carryover. In addition, SEB and TT did not induce T cell activation in the absence of PMA-derived DC. SEB-mediated T cell activation is not MHC restricted and does not require antigen (Ag) processing but TT-mediated T cell proliferation is MHC restricted and require both Ag processing and presentation. As presented in Figure 24, only minimal proliferation can be demonstrated for T cells with added autologous monocytes in the presence of SEB or TT. In contrast, after adding autologous $CD34^+$-derived DC, a marked proliferation could be induced.

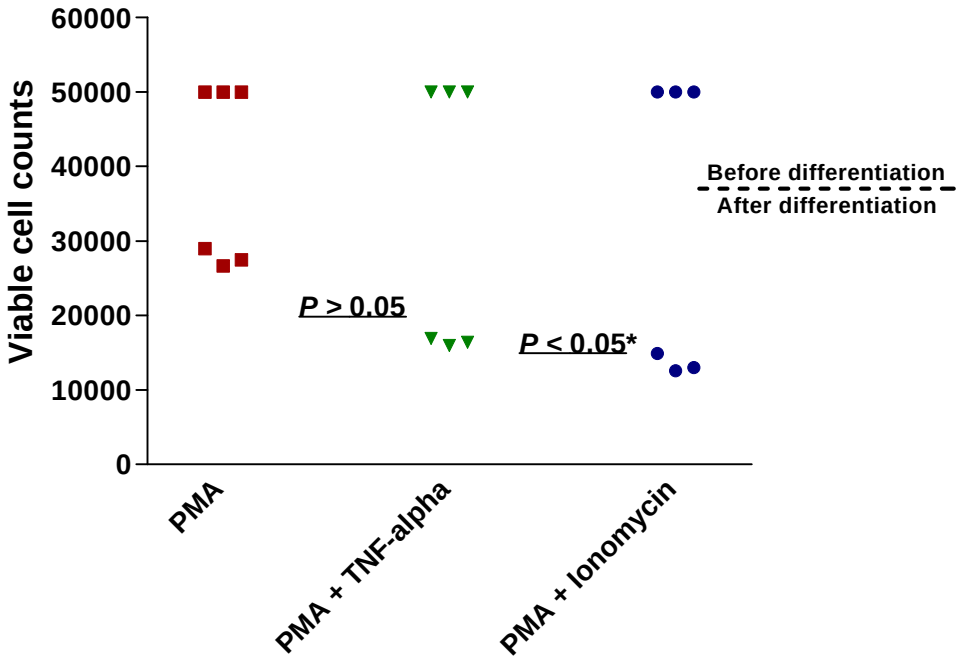


Figure 23: *Viable cell counts of PB-derived dendritic cells after one week stimulation with serum-free medium (CellGro^{Ò} DC) containing PMA alone or plus TNF-a or ionomycin.*
******* *Significant, P < 0.05, Kruskal-Wallis test*

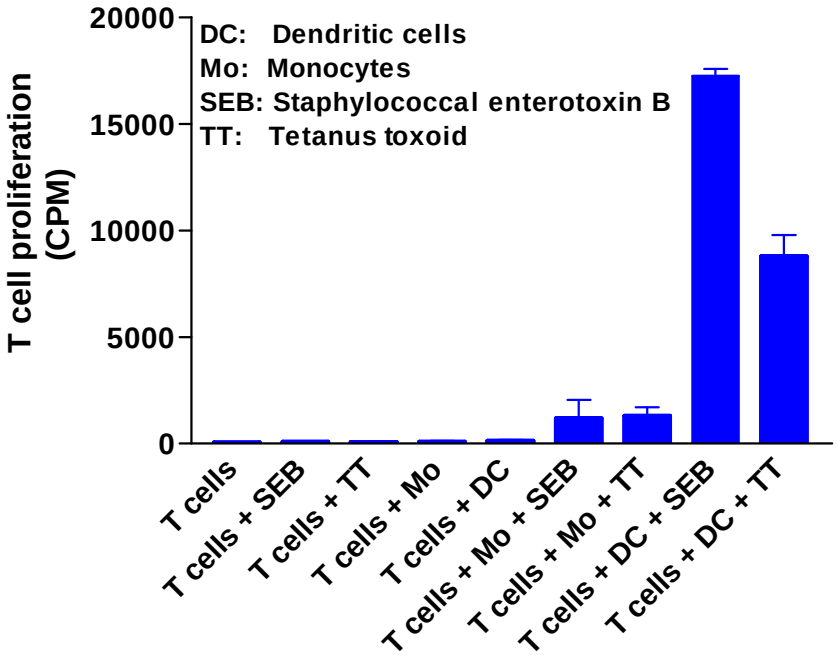


Figure 24: *Autologous T cell proliferation induced by PMA-generated DC, from PB-CD34^{+} cells, pulsed with staphylococcal enterotoxin B (SEB) or preservative-free tetanus toxoid (TT). Monocytes or day 7 g-irradiated DC (2 ´ 10^{4} cells) were added to purified autologous T cells (1 ´ 10^{5} cells) in the absence or presence of 3 mg/ml SEB or 10 mg/ml TT. As negative controls, autologous T cells were plated in triplicate wells of 96-well flat-bottom plates with media alone (no stimulus) or with media containing SEB or TT. Data are presented as the mean CPM ± SD of triplicate cultures.*

3.4 Retroviral transduction

3.4.1 High titer virus production

The efficacy of GP+E86 ecotropic producer cell line to produce high-titer murine helper-free retroviruses (that are capable of delivering genes to dividing murine cells) appears to be limited by the low transfectability of these packaging cells. Figure 25 shows that the percentage of transfected cells expressing EGFP not exceeded 3 % after transfection with pSFβEGFP retroviral vector using electroporation or SuperFect™ Transfection Reagent.

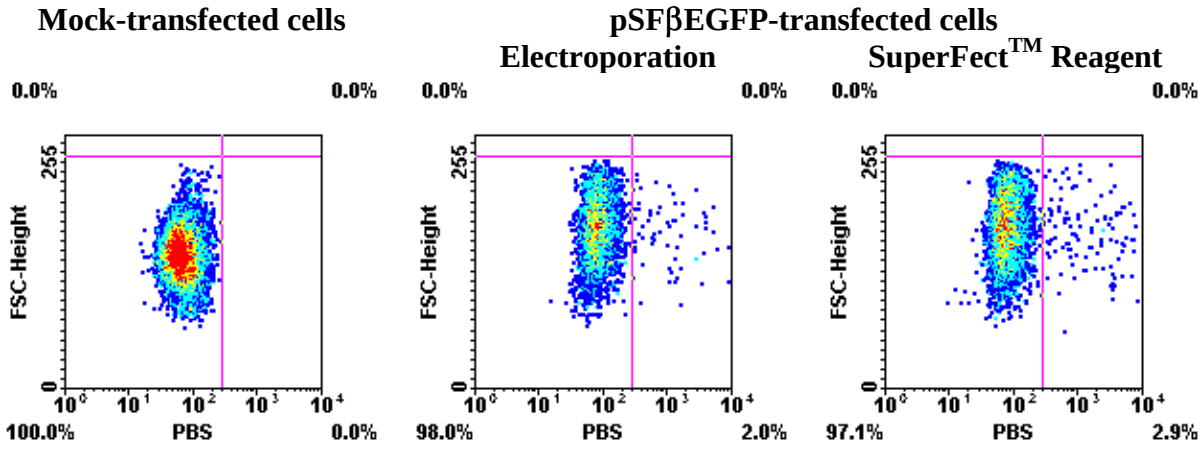


Figure 25: *Flow cytometric analysis of ecotropic GP+E86 packaging cells transfected with pSFbEGFP retroviral vector containing EGFP via electroporation or SuperFect™ Transfection Reagent. Mock (no retroviral vector-DNA) transfected cells were used as negative control.*

Recently, the laboratory of Nolan (Dr. Garry P. Nolan, Department of Molecular Pharmacology, Standford University School of Medicine) created a new producer cell line [Phoenix Eco (ΦNX-Eco) and Phoenix Ampho (ΦNX-Ampho)] derived from the highly transfectable 293T cell line to generate high-titer helper-free retroviruses by transient transfection (Pear *et al*., 1993; Yang *et al*., 1999). The new producer cell line Phoenix was highly transfectable with SuperFect reagent than calcium phosphate precipitation method (Figure 26). The highest transfection efficiency (74.4 %) and a virus titer (4×10^6 CFU/ml) was obtained with the cells that were 2×10^6 at seeding, 24 h before transfection, using SuperFect reagent (Figure 27).

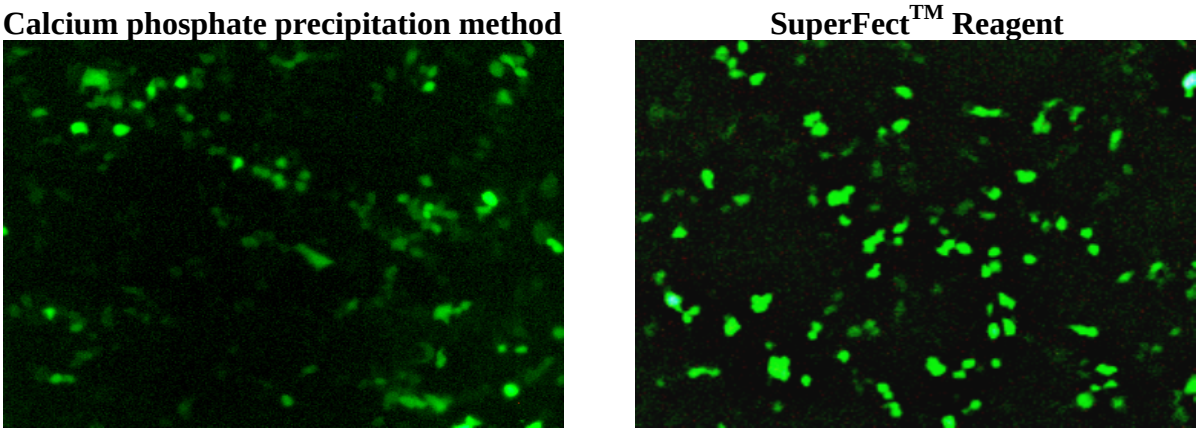


Figure 26: *Fluorescence microscopic image of transient transfected Phoenix cells with pSFbEGFP retroviral vector via calcium phosphate precipitation method or SuperFect™ Transfection Reagent.*

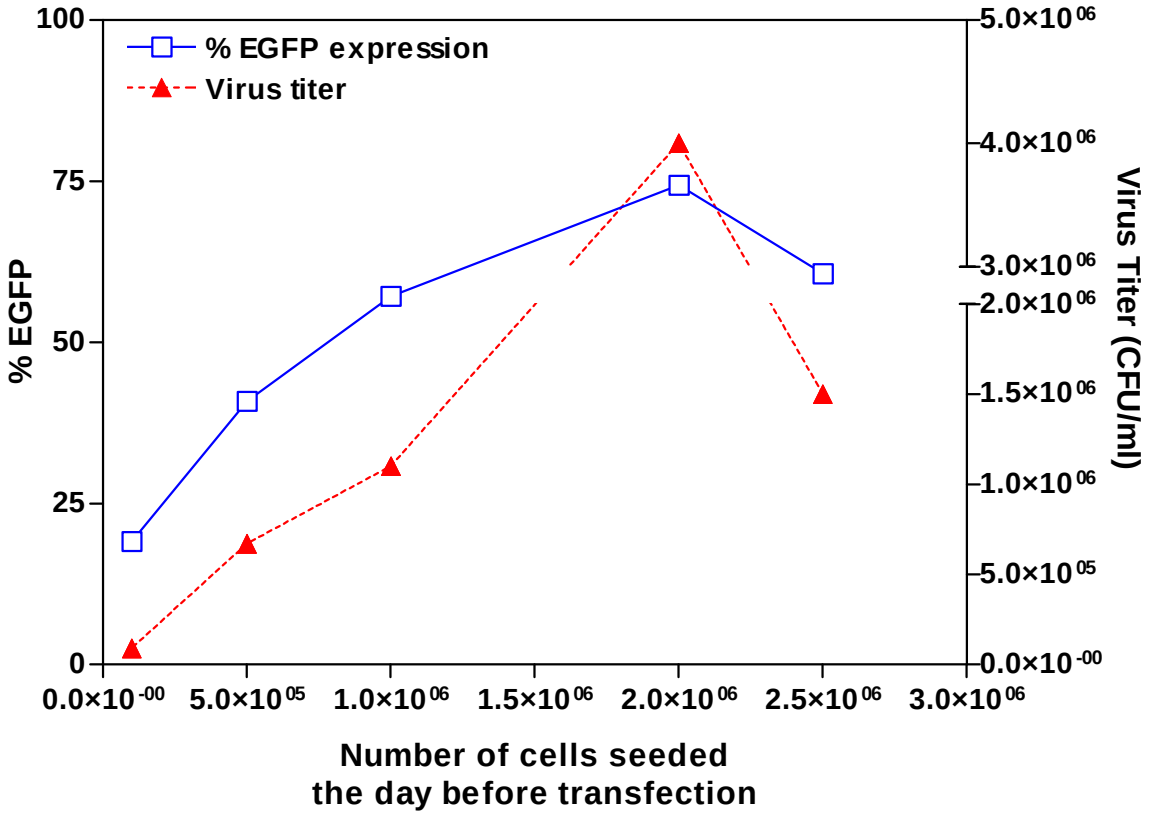


Figure 27: *Transient transfection efficiency as measured by EGFP expression and virus titer production of Phoenix cells transfected at different confluent percentages with pSFbEGFP retroviral vector via SuperFect™ Transfection Reagent. Different cell numbers (0.1-2.5 ´ 10^6) were seeded in 100-mm tissue culture dishes the day before transfection. Transfections were occurred with 10 mg retroviral vector/60 ml SuperFect reagent. The complex incubation time on cells was 2.5 h. Analysis was performed 48 h post-transfection.*

In this study, transient transfection of human ecotropic packaging cells, ΦNX-Eco using a SuperFect reagent was utilized to produce high-titer murine recombinant retroviral vectors (> 1×10^6 CFU/ml) that used for transduction of murine pseudotyped packaging cells PG13 to generate high-titer (> 5×10^5 CFU/ml) retroviruses pseudotyped with gibbon ape leukemia *env* protein (to increase the transduction efficiency to dividing human progenitor cells). The pseudotype retrovirus-containing supernatants were harvested under serum-free conditions (CellGro® SCGM), to avoid skewing the resulting immune response by foreign antigens present in FCS, at 37°C as described previously (Glimm *et al*., 1998) because PG13 rapidly looses viral titer when grown at the lower temperature (Reeves *et al*., 2000).

3.4.2 $CD34^+$ progenitor cells transduction

The expansion of primitive progenitor cells in culture should facilitate retroviral transduction because retroviral vectors require target cell division for efficient integration into cellular genome (Glimm *et al*., 1998). The data in Table 3 and Figure 15 indicated that $CD34^+$ progenitor cells were expanded highly in CellGro® SCGM containing Flt-3L, 300 ng/ml; IL-3, 100 ng/ml and SCF, 100 ng/ml. Here, different methods were used for retroviral transduction of $CD34^+$ haematopoietic progenitor cells in serum-free medium to determine the optimal transduction conditions. The best result were obtained with the following conditions: $CD34^+$ progenitor cells (5×10^4) were stimulated firstly for 48 h with the above cytokine cocktail in complete serum-free medium CellGro® SCGM. Subsequently, cells were infected with 1 ml pseudotyped viral supernatants (> 5×10^5 CFU/ml, made in serum-free medium) in the presence of protamine sulphate (5 μg/ml) and cytokines with centrifugation at 1000 x g for 2 h at room temperature. Cells were then incubated at 37°C in 5 % CO_2. On the next day, supernatants were removed and cells were cultured in fresh medium supplemented with cytokines. Three rounds of consecutive transductions were performed on days 3, 5 and 7, respectively. The efficiency of the transduction was monitored one week after the last infection. Figure 28 exhibits the surface antigen phenotype characterization of $CD34^+$ progenitor cells after expansion and transduction under the above conditions. The cells were negative for CD1a, CD15 and CD14. In addition, only 3.9 % ± 0.3 % (n = 3) were $CD86^+$. On the other hand, the level of CD34 expression was 78.9 % ± 4.7 % (n = 3). Cell viability during expansion and transduction under serum-free conditions was greater than 90 %.

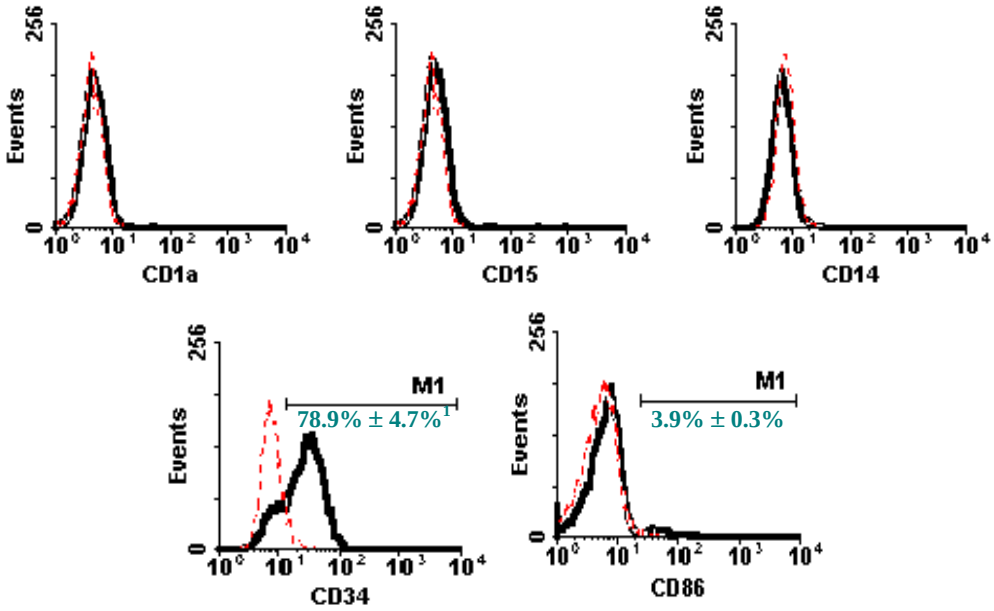


Figure 28: *Surface Ag phenotype characterization of CD34$^+$ progenitor cells after expansion and transduction in serum-free medium CellGro^{Ò} SCGM containing Flt-3L (300 ng/ml), IL-3 (100 ng/ml) and SCF (100 ng/ml). Isotype-matched controls are indicated by dotted lines.*
[1]Mean ± SD of three different experiments

Cell surface FLAG expression of pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF-transduced CD34$^+$ progenitor cells was 45.9 % ± 4.0 % (n = 3) one week after the last infection (Figure 29). FLAG signals from pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF-transduced CD34$^+$ progenitor cells could be released by PI-PLC indicating that the retroviral vector targets the fusion protein to the cell surface via GPI-anchor (Figure 29).

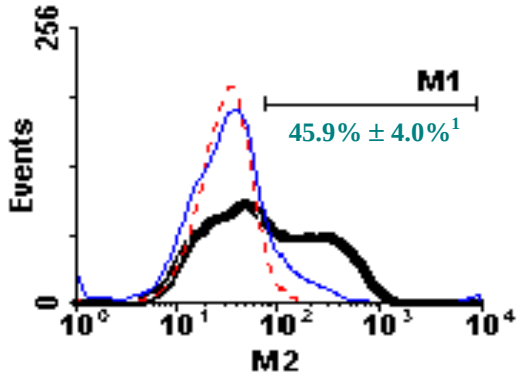


Figure 29: *FLAG expression and PI-PLC release of pSFb$_S$(FLAG-V_LIgPl)$_w$DAF-transduced CD34$^+$ progenitor cells. Thin and thick lines indicate transduced cells treated with or without PI-PLC, respectively. Dotted line represents the mock-transduced cells. M2: Anti-FLAG antibody-binding.*
[1]Mean ± SD of three different experiments

3.4.3 V_LIgPl gene expression in dendritic cells

Figure 30 shows typical morphology of cytokine- and PMA-derived DC obtained from transduced $CD34^+$ progenitor cells under-serum free conditions. Cell aggregates and numerous cytoplasmic protrusions (veils) are a characteristic feature of cytokine- and PMA-derived DC, morphology reported to be characteristic of mature DC (Hart, 1997). These veils usually extend from all parts of the cells. Occasionally, cells were observed with polarized veils (Thurnher *et al*., 1997). In addition, a subset of PMA-derived DC developed long dendrites.

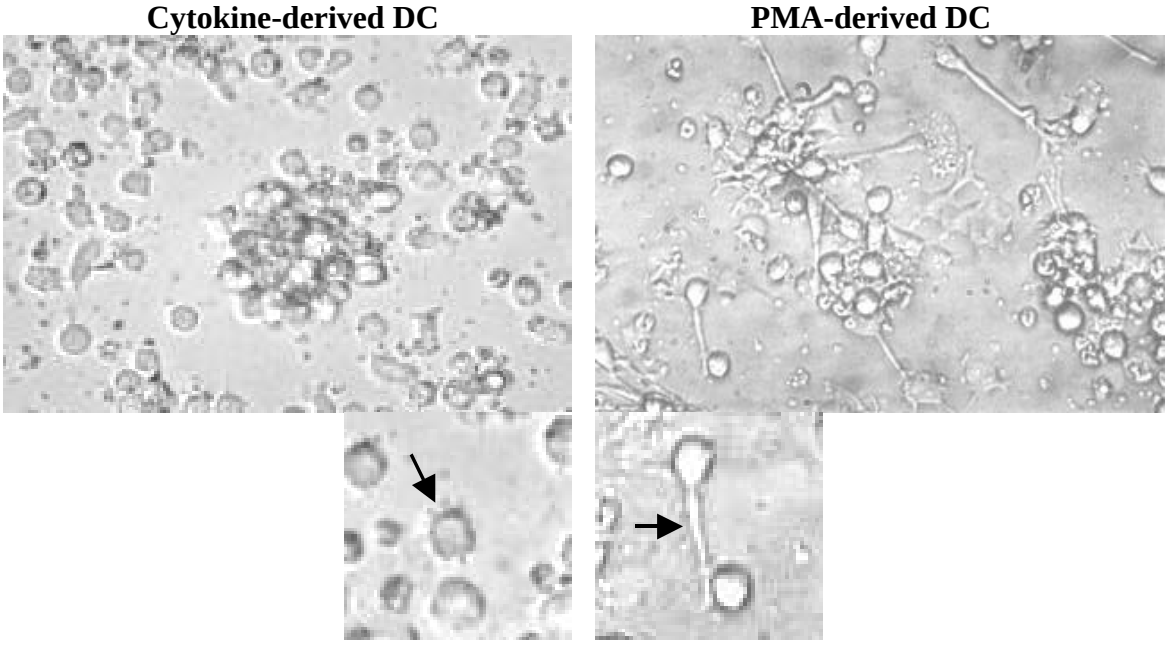


Figure 30: *Morphology of the cytokine- and PMA-derived DC obtained from $pSFb_S(FLAG\text{-}V_LIgPl)_wDAF$-transduced $CD34^+$ cells under serum-free conditions. Arrows indicate hair-like cytoplasmic projections and elongated dendrite.*

Table 5 shows that all PMA-derived DC were negative for CD1a and positive for CD40, CD83, CD86 and MHC-class I and II. On the other hand, in cytokine-derived DC only 52.2 % ± 5.3 %, 41.8 % ± 3.1 %, 26.3 % ± 2.4 % and 14.4 % ± 5.2 % (n = 3) were $CD86^+$, $CD1a^+$, $CD83^+$ and $CD40^+$, respectively.

Surface antigen	Cytokine-derived DC	PMA-derived DC
CD1a	(41.8 % ± 3.1 %[1], ++)	-
CD40	(14.4 % ± 5.2 %, +)	+
CD83	(26.3 % ± 2.4 %, +)	++
CD86	(52.2 % ± 5.3 %, ++)	+++
MHC class I	++++	++++
MHC class II	++++	++++

Table 5: *Surface Ag phenotype characterization of cytokine- and PMA-derived DC obtained from transduced CD34$^+$ cells cultured in serum-free medium CellGro^{Ò} DC for indicated periods of time as mentioned in material and methods. Each + indicates an increase of 0.5 logs in mean fluorescent intensity versus isotype matched control and - and ( ) indicate all cells are negative or subpopulation only is positive, respectively.*
[1]Mean ± SD of three different experiments

In addition, co-expression of V_LIgPl gene and CD86, the most important ligand to induce CD28-mediated co-stimulation for CD8$^+$ T cell activation, increased in PMA-derived DC compared with cytokine-derived DC; 53.8 % ± 2.8 % (n = 3) versus 32.1 % ± 2.1 % (n = 3), respectively (Figure 31). These data indicated that PMA-derived DC are more homogeneous and mature than cytokine-derived DC.

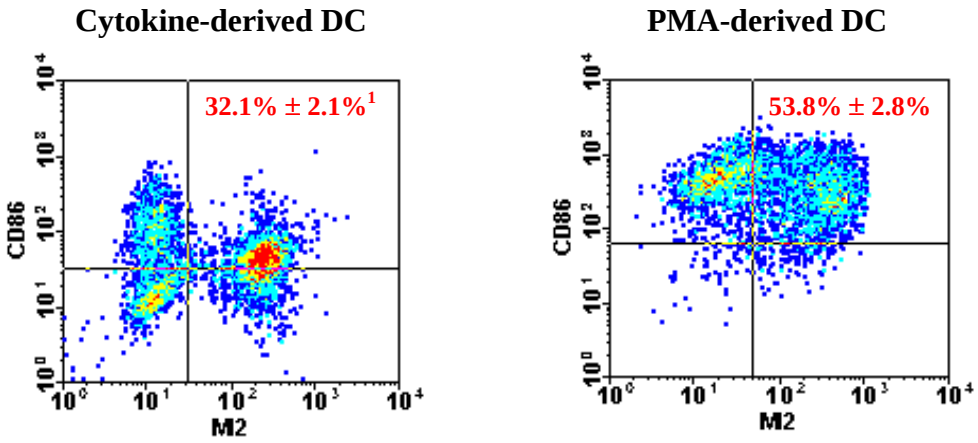


Figure 31: *Co-expression of V_LIgPl gene and CD86 of the cytokine- and PMA-derived DC obtained from pSFb$_S$(FLAG-V_LIgPl)$_w$DAF-transduced CD34$^+$ cells under serum-free conditions. M2: Anti-FLAG antibody-binding.*
[1]Mean ± SD of three different experiments

3.5 An *in vitro* autologous T cell response against V_LIgPl

3.5.1 V_LIgPl-expressing dendritic cells induce an *in vitro* autologous T cell proliferation

Figure 32 demonstrates the successful activation and proliferation of autologous T cells by pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF-transduced DC. This proliferative response is more prominent in PMA-derived DC (PI = 328.54) compared to cytokine-derived DC (PI = 143.29) indicating that PMA-derived DC are more potent in activating autologous T cell proliferation. The proliferative response could be augmented by increase of the percentage of pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF-transduced DC (Figure 33).

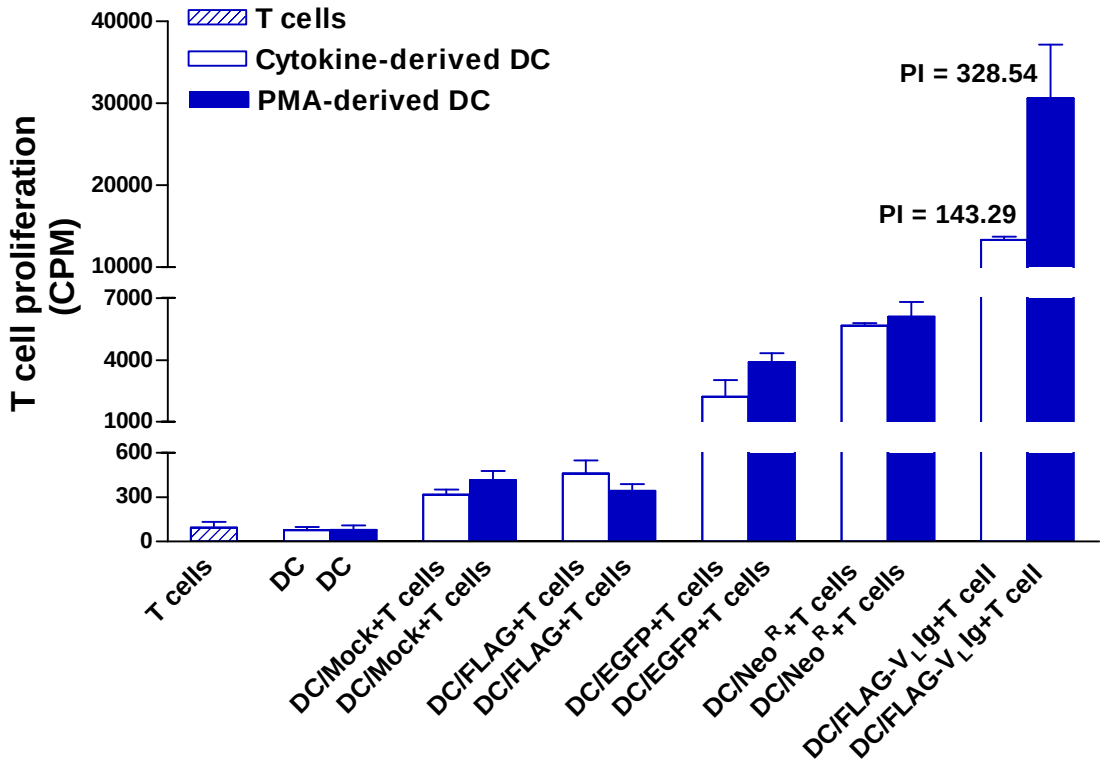


Figure 32: *Autologous T cell proliferative response induced by cytokine-derived DC compared with PMA-derived DC obtained from transduced CD34$^+$ cells. Mock-transduced DC were used as negative control. In addition, transduced DC or autologous T cells were plated in triplicate wells of 96-well flat-bottom plates with media alone. Data are presented as the mean CPM ± SD of triplicate cultures. Proliferative index (PI) value is the ratio between the mean CPM of T cells in the presence and in the absence of transduced DC in the cultures. DC/Mock, mock-transduced DC; DC/FLAG, pSFb$_S$(FLAG)$_w$DAF-transduced DC; DC/EGFP, pSFbEGFP-transduced DC; DC/Neo^R, pSFb1N-transduced DC; DC/FLAG-V_LIg, pSFb$_S$(FLAG-V_LIgPl)$_w$DAF-transduced DC.*

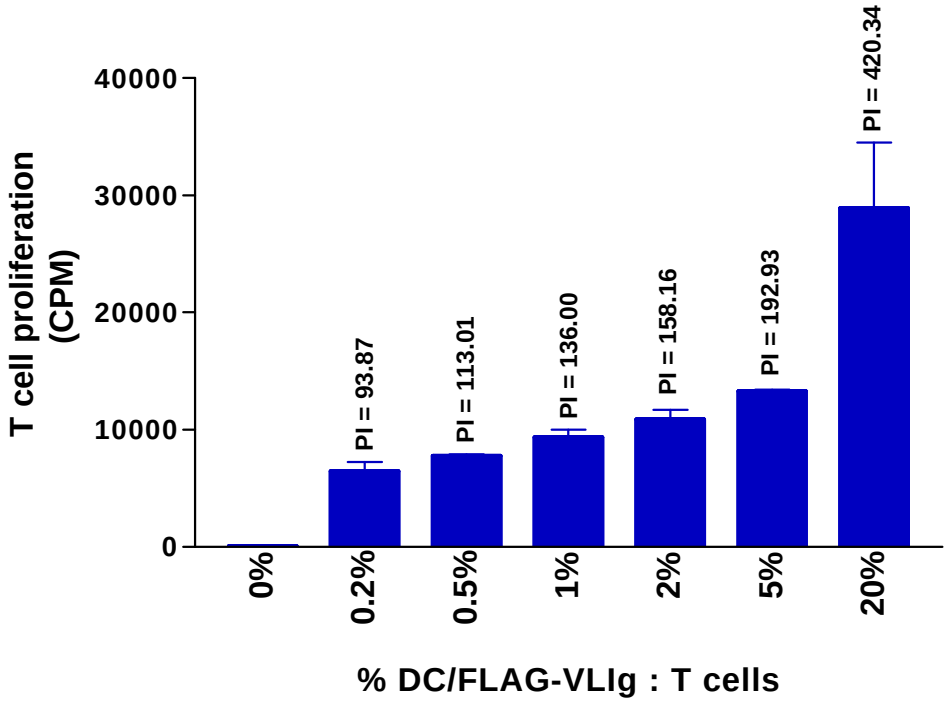


Figure 33: *Autologous T cell proliferative response induced by different concentration of PMA-derived DC obtained from pSFb$_S$(FLAG-V_LIgPl)$_w$DAF-transduced CD34$^+$ cells. Data are presented as the mean CPM ± SD of triplicate cultures. Proliferative index (PI) value is the ratio between the mean CPM of T cells in the presence and in the absence of transduced DC in the cultures.*

The absence of autologous T cell proliferation in the presence of mock- or pSFβ$_S$(FLAG)$_\omega$DAF-transduced DC indicates that the proliferation induced by pSFβ$_S$(FLAG-V_LIgPl)$_\omega$DAF-transduced DC is specific for V_LIg of plasmocytoma (Figure 32). On the other hand, moderate proliferation in the presence of autologous DC transduced with pSFβN1 (containing Neo^R) or pSFβEGFP (containing EGFP) has been observed (Figure 32). This data supports the idea that neomycin and EGFP by themselves are immunogenic.

3.5.2 MHC-restriction of T cell response

To determine MHC restriction of the V_LIgPl-induced proliferation, the inhibitory effect of anti-HLA antibodies on their autologous T cell proliferation was studied. Figure 34 demonstrates that autologous T cell proliferation was almost completely inhibited by monoclonal antibody against MHC class I (HLA-ABC). There was only minor inhibition by antibodies against MHC class II (HLA-DR). In addition, no significant inhibition was found with control mouse mAbs. These data indicated that the elicited proliferative response is not

directed against the constructed whole GPI-linked fusion protein present at the surface of transduced DC. Proliferation is rather stimulated by fragments of the transduced peptide presented on MHC class I molecules.

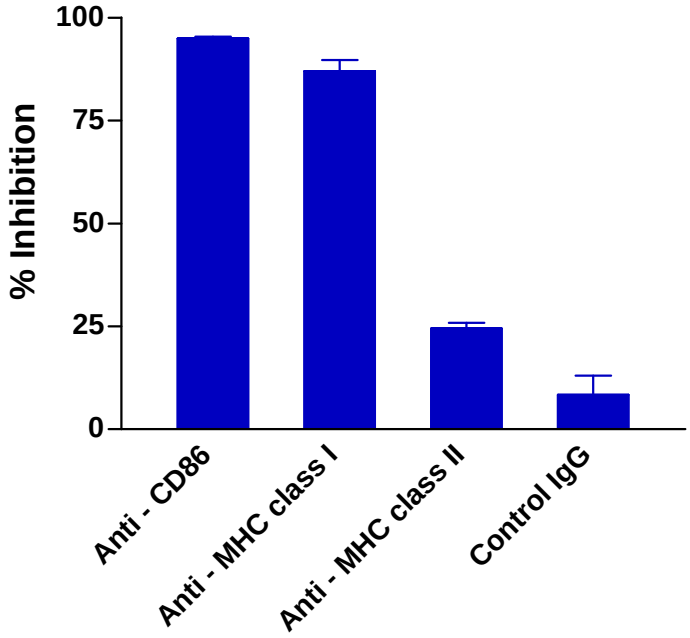


Figure 34: *Inhibition (%) of autologous T cell proliferation, induced by pSFb$_S$(FLAG-V$_L$IgPl)$_w$DAF-transduced DC, by antibodies against MHC class I (HLA-ABC), class II (HLA-DR), CD86 (B7-2) or an isotypic control mouse IgG. Data are presented as the mean CPM ± SD of triplicate cultures.*

3.5.3 Inhibitory effect of CD86 antibodies on T cell response

The expression of co-stimulatory molecules on APC plays a major role in the activation of T cells. Therefore, the inhibitory effect of CD86 antibodies on autologous T cell proliferation induced by V_LIgPl-transduced DC was examined. As shown in Figure 34, autologous T cell proliferation was almost completely blocked by CD86 antibodies indicating the critical role of this co-stimulatory molecule in activation of naive T cells.

4 Discussion

Multiple myeloma (MM) is a malignant disease commonly affecting elderly individuals and characterized by accumulation of mature plasma cells in the bone marrow. Although it is possible to induce transient remission of disease by chemotherapy, the disease is essentially incurable. Therefore, novel approaches are much needed (Lim and Bailey-Wood, 1999; Wen *et al*., 2001).

The pathophysiology of paraproteinemia could be elucidated by an increasing knowledge on normal B cell development. Early in normal B cell development the variable (V), diversity (D) and joining (J) segments of the immunoglobulin (Ig) heavy and light chain genes are rearranged to produce a functional V(D)J sequence coding for the antibody-combining site of the Ig molecule. This sequence is the same in all cells belonging to a particular B cell clone and can be used as a marker of the clone (Ludwig *et al*., 1999; Samson, 2000). The recombinations take place within the bone marrow without antigenic stimulation. Virgin B cells migrate then to the lymph nodes. In the primary antibody response, they give rise to lymphoblasts that in turn differentiate into short-lived plasma cells secreting IgM. In secondary antibody response the lymphoblasts migrate into germinal centers of the lymph nodes and, as a result of exposure to antigen, mutations take place in V(D)J sequence which improve antigen affinity (somatic hypermutation). During germinal center reaction, B cells with high affinity to antigens presented by follicular dendritic cells escape apoptosis to give rise to memory B cells, which can produce IgM secreting lymphoplasmacytoid cells or can undergo isotype class switching (Ig heavy chain constant region exchange) to produce cells secreting antibodies of different isotypes (IgG, IgA, IgE or IgD). These cells do not proliferate within lymph nodes but home to bone marrow and develop there into long-lived plasma cells. Isotype switching does not affect antibody specificity, but alters effector functions that an antibody can engage (Ludwig *et al*., 1999; Samson, 2000).

The malignant clone for MM is derived from a cell, somatically hypermutated. The V genes show no clonal diversity and the V sequence remains stable during the course of disease. Thus, it is generally believed that the initial neoplastic event in MM occurs in a single post germinal-center B cell because at this stage in B cell development somatic hypermutation has ceased (Ludwig *et al*., 1999). Therefore, the idiotypic protein (Id) secreted by neoplastic

plasma cells represents an ideal tumor-specific antigen for immune targeting (Sahota *et al*., 1997; Kosmas *et al*., 1999). This antigen is supposed to exhibit a low immunogenicity (Lim and Bailey-Wood, 1999). Most studies addressing the capacity to elicit an anti-idiotypic immune response have analyzed the target antigen as a paraprotein taken up by DC (Lim and Bailey-Wood, 1999; Reichardt *et al*., 1999; Hajek and Butch, 2000; Timmerman and Levy, 2000; Titzer *et al*., 2000) and presented on almost MHC class II molecules. However, the response *in vivo* seems to be modest and transient (Wen *et al*., 2001). Idiotypic gene transfer into dendritic cells represents an alternative for induction of an anti-idiotypic immune response. Because internally produced peptides are presented differently (on almost MHC class I molecules), this procedure may elicit a more efficient cytotoxic immune response.

Due to genetic recombination, in several myelomas neoplastic cells don't produce immunogloblin heavy chain leading to the appearance of free monoclonal light chains (Sakai *et al*., 1993). Therefore, in most cases of plasmocytoma, the variable region of the corresponding light chain (V_LIgPl) can be used as a defined tumor-associated antigen. This thesis provides evidence that a considerable syngeneic T cell proliferation restricted to MHC class I molecules can be elicited *in vitro* against V_LIgPl through autologous dendritic cells via a retroviral transduction of human $CD34^+$ progenitor cells under serum-free conditions. The V_LIgPl gene used by the tumor cells in this study was a V segment of the V_{kIV} subgroup.

4.1 A novel retroviral expression-cloning vector

Retroviral vectors are able to introduce new genes with stable expression and at high efficiency into the genome of many types of mammalian cells, including primitive haematopoietic cells (Miller, 1992).

The objective of this work was to induce efficient transfer and expression of V_LIgPl gene in DC. Therefore, the SFFVp/MESV hybrid retroviral vector backbone has been chosen, designed to overcome transcriptional inefficiency and silencing associated with retroviral gene transfer into myeloid progenitors and haematopoietic stem cells (Baum *et al*., 1995). Due to the mechanism of reverse transcription, the integrated provirus contains the leader sequence of the murine embryonic stem cell virus (MESV) and a modified LTR of the spleen focus-forming virus (SFFVp) in its 5'end. This LTR has been re-engineered to avoid

recombination of the provirus and minimize the risk of producing helper viruses. The U3 region of SFFVp showed higher enhancer activity than that of Moloney murine leukemia virus (M-MLV). The presence of both retroviral *cis* elements allows efficient and long-term gene expression in the myeloid compartment (Baum *et al*., 1995).

The Adoption of marker genes that can be rapidly monitored and used for efficient selection of transduced cells will facilitate the improvement of gene marking protocols. To date the neomycin phosphotransferase (Neo) gene has been the most widely used marker in clinical studies (Rill *et al*., 1992; Brenner *et al*., 1993; Dunbar *et al*., 1995; Cornetta *et al*., 1996; Heslop *et al*., 1996). However, the introduction of Neo is hampered by non-specific toxicity of neomycin and by potential immunoclearence of transduced cells (Brenner, 1996). Furthermore, selection is time-consuming. Cytochemical markers, such as β-galactosidase (Strair *et al*., 1990; Bagnis *et al*., 1995), also have important limitations (Giaretta *et al*., 2000). In fact, The presence of endogenous activity in some cell types, the need to use fluorogenic substrates and the potential for passive transfer of the enzymatic activity to untransduced neighboring cells make this marker quite unreliable (Giaretta *et al*., 2000). Therefore, in this study, a novel retroviral expression-cloning vector that directs the tumor-associated antigen of plasmocytoma marked with a FLAG antibody-binding site to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor has been constructed. The FLAG epitope consists of eight-amino acid peptide (DYKDDDDK) recognized by the commercially available monoclonal antibodies (M1, M2 and M5), raised against this peptide (Knappik and Pluckthun, 1994). Using FLAG expression at the cell surface transduced cells can be immunoselected. It is not likely to obscure other epitopes, domains, or alter function, secretion, or transport of the fusion protein because of the small size of the FLAG peptide tag. The three anti-FLAG monoclonal antibodies (M1, M2 and M5) differ in their binding abilities based on the location of the FLAG sequence in the fusion protein. The M1 antibody requires the FLAG to be at the very N-terminus of the fusion protein for high affinity binding and recognizes the sequence as small as DYKD or DYKDE (Knappik and Pluckthun, 1994). The M5 antibody binds with high affinity if the FLAG sequence is at the very N-terminus of the fusion protein and preceded by a methionine. Antibody M2 is the most versatile in its applications because it recognizes a FLAG-tagged fusion protein independently of the intramolecular location of the tag. M1 requires calcium ions for binding, whereas M2 and M5 are not calcium-dependent (Brizzard *et al*., 1994).

The GPI-anchored construct has been chosen because of its stable transgene surface expression without a major dependence on transcription rate. Low transcription rate might be a limiting factor of classical membrane proteins or fusion proteins such as nerve growth factor receptor [NGFR] or epidermal growth factor [EGF] (Austin *et al*., 2000). For targeting the transgene to a GPI-linked protein, the GPI anchoring determining sequences within decay-accelerating factor (DAF) normally expressed in dendritic cells themselves have been used thus avoiding additional unphysiological peptides expressed in DC. DAF is a GPI-linked membrane glycoprotein that regulates complement by binding activated complement fragments, C3b and C4b, thereby inhibiting amplification of the complement cascade on host cell membranes (Nicholson-Weller *et al*., 1982; Medof *et al*., 1984; Kinoshita *et al*., 1986). The GPI-anchor consists of an ethanolamine phosphate, three mannose residues and a nonacetylated glucosamine linked to phosphatidylinositol (Masterson *et al*., 1989; Schubert *et al*., 1994; Udenfriend and Kodukula, 1995; Kinoshita and Inoue, 2000). GPI-anchor preassembly and addition to the protein apparently takes place in the endoplasmic reticulum (Bangs *et al*., 1985, 1986; Ferguson *et al*., 1986; Schubert *et al*., 1993; Udenfriend and Kodukula, 1995; Kinoshita and Inoue, 2000). Thereafter, the protein is transported to the cell surface via the Golgi apparatus (Caras, 1991). Two elements are required for anchor addition, COOH-terminal hydrophobic domain (presumably removed during anchor attachment) and a second element, the cleavage/attachment site, located 10-12 residues NH_2-terminal to the signal (Caras *et al*., 1989; Udenfriend and Kodukula, 1995). In addition, lacking of NH_2-terminal signal peptide of DAF (resides –34 to –1), that is necessary for translocation across the membrane of the endoplasmic reticulum, failed to produce GPI-anchored protein suggesting that membrane translocation is necessary for anchor addition (Caras, 1991). Therefore, nascent proteins destined to be processed to a GPI-anchor are supposed to contain two hydrophobic signal peptides, one at NH_2 terminus and another at the COOH terminus (Udenfriend and Kodukula, 1995; Tiede *et al*., 1999). Both are cleaved sequentially. After action of the NH_2-terminal signal peptidase, the transamidase reaction will yield the mature GPI-linked protein (Amthauer *et al*., 1993; Udenfriend and Kodukula, 1995; Ramalingam *et al*., 1996; Hiroi *et al*., 2000). The transamidation reaction involves formation of an activated carbonyl intermediate (enzyme-substrate complex) with the ethanolamine moiety of the preassembed GPI unit serving as a nucleophil (Maxwell *et al*., 1995; Ramalingam *et al*., 1996; Ohishi *et al*., 2001). To address these requirements, a fusion protein containing FLAG-V_LIgPl in frame between the two ends of DAF has been constructed. The first 34 and the last 37 amino acids of DAF are hydrophobic domains cleaved upon attachment to the GPI-anchor

molecule (Moran *et al*., 1991). In addition, the cleavage attachment site (ω) is located within the carboxyterminus leading to GPI-linked transgene expression provided by this vector.

The pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF retroviral vector successfully targeted the FLAG-V_LIgPl fusion protein to the cell surface of transfected Jurkat wild type cells via electroporation. The percentage of FLAG expression in transfected Jurkat cells increased by increasing cell number, amount of DNA, voltage and capacity and decreasing the final volume at electroporation. FLAG signals from transfected cells could be released by PI-PLC indicating that the retroviral vector targets the fusion protein to the cell surface via GPI-anchor. Xba1 and Cla1 restriction sites are not present in any type of human V genes. Thus, any type of the V_LIg can be directly cloned into this expression vector via Xba1 and Cla1 restriction sites. As a result, the vector pSFβ_S(FLAG)$_\omega$DAF without the V_LIgPl directs the FLAG to the plasma membrane and can be used as a control for immune response.

4.2 PMA-derived DC under serum-free conditions

DC are attractive cellular adjuvants for vaccination strategies due to their professional antigen-presenting capacity (Brossart *et al*., 2000; Klein *et al*., 2000). Currently, most *in vitro* culture systems for the production of these DC include serum. However, this is undesirable because serum contains growth factors that vary between individuals and could affect DC development (Luft *et al*., 1998). Unless the individual's own serum is used, serum adjuvants such as FCS will lead to a skewing of the immune response by foreign antigens. In addition, serum preparations contain a series of cytokines not yet identified which are possibly involved in DC differentiation. In order to study the requirements to differentiate a $CD34^+$ cell into DC, this study provides data that DC can differentiate out of $CD34^+$ cells from different origins in the presence of PMA without addition of serum. Here, CD34-derived dendritic cells (CD34-DC) were chosen because their preferential capacity to activate $CD8^+$ T cells compared to monocyte-derived DC (Ferlazzo *et al*., 1999). In addition, murine oncoretroviral vectors require cell division for integration into the nucleus (Glimm *et al*., 1998). Differentiation of monocytes to DC does not involve any cell division (Kiertscher and Roth, 1996; Zhou and Tedder, 1996; Ardeshna *et al*., 2000b). This is only provided by $CD34^+$ cells stimulated with cytokines before differentiation to DC.

Human mature DC with typical morphology (large and loosely adherent, and a subset developed long dendrites and hair-like cytoplasmic projections) and surface antigen phenotype ($CD1a^-$, $CD83^+$ and $CD86^+$) were obtained from CB- and PB-$CD34^+$ progenitor cells after one week of culture in serum-free medium upon stimulation with PMA alone. In addition, these cells were $CD1a^-$ throughout all experiments indicating that PMA induces DC differentiation without causing the generation of cellular intermediates. Morphological changes began within one day of stimulation, were fully manifested within three days, and were stable over at least 2 weeks in culture. The same result was obtained from *ex vivo* expanded BM-$CD34^+$ cells.

As described previously, there are two stages of DC differentiation from $CD34^+$ haematopoietic progenitor cells (HPC) using cytokines (Winzler *et al*., 1997). The first stage involves proliferation and differentiation of HPC to immature $CD1a^+$DC, cells exhibiting a high capacity for Ag uptake but relatively poor ability to activate T cells. This intermediate DC adhered to plastic surfaces, expressed Birbeck granules, and were negative for CD80, CD83 and CD86 co-stimulatory molecules. This differentiation is induced *in vitro* within 2 weeks by GM-CSF, TNF-α and other cytokines (Luft *et al*., 1998). The second stage involves maturation of immature $CD1a^+$DC to mature DC, cells that have decreased Ag uptake capability but are much more potent in activating T cells. This maturation involved increased expression of CD80, CD83, CD86, HLA-A, -B, -C, and -DR as well as down-regulation of CD1a and CD11b. Activated DC are characterized by the lack of adherence to plastic surfaces and the absence of Birbeck granules (Luft *et al*., 1998). *In vitro* maturation/activation can be induced by TNF-α ± other cytokines (Hart, 1997) or CD40 receptor cross-linking (Flores-Romo *et al*., 1997). Spontaneous maturation of intermediates into fully active DC expressing CD83 and co-stimulatory molecules occurred asynchronously over the ensuing 2-3 weeks. *In vivo* this second stage is triggered by bacteria or its components (LPS and DNA), viral infection and inflammatory cytokines (Banchereau *et al*., 2000).

Because cytokine receptor stimulation activates complex signalling cascades that initiate multiple responses, differentiation to other lineages is almost simultaneously induced. Thus cytokine application not only generates DC but also macrophages and neutrophils as well (Santiago-Schwarz *et al*., 1992; Szabolcs *et al*., 1995; Bender *et al*., 1996). In contrast to cytokines or CD40 ligand, PMA alone induces DC differentiation, without causing cell

proliferation and the generation of cellular intermediates (Davis *et al*., 1998). In this regard, PMA-induced DC differentiation from $CD34^+$ cells more closely resembles cytokine-driven DC differentiation from monocytes. There is also no proliferation, and 20-90 % of input cells are lost during culture (Kiertscher and Roth, 1996; Zhou and Tedder, 1996; Ardeshna *et al*., 2000b). The observation that about 40-60 % of cells are lost during culture depending upon the source of $CD34^+$ progenitors is supported by the observation that PMA had a negative effect on $CD34^+$ cell survival that did not differentiate to DC (Davis *et al*., 1998). The ability to differentiate into DC in response to PMA is limited to $CD34^+$ cells, as PMA caused macrophage differentiation in $CD34^-CD15^+$ cells and cell death in $CD14^+$ monocytes (Davis *et al*., 1998). In addition, $CD34^+$ myeloid cell line KG1 differentiates into dendritic-like cells in response to PMA (with or without the calcium ionophore ionomycin or TNF-α). Comparison of KG1 to the PMA-unresponsive subline KG1a reveals differences in expression of TNF receptors (1 and 2), PKC isoforms (α, β1, β2 and μ) and RelB, suggesting that these components/pathways are important for DC differentiation (St. Louis *et al*., 1999). All receptor-mediated stimuli (GM-CSF, IL-4, TNF-α and CD40 cross-linking) that induce DC differentiation can also activate PKC as part of their intracellular signalling pathways (Arruda and Ho, 1992; Ren *et al*., 1994; Mufson, 1997). Direct activation of PKC with PMA alone is sufficient to induce differentiation of human BM-$CD34^+$ cells into mature and fully functional DC within 7 days only, suggesting a critical and specific role of this pathway for the progenitor lineage committed to DC (Davis *et al*., 1998). This specificity may facilitate further identification of signalling pathways involved in DC differentiation.

The rarity of $CD34^+$ HPC makes sufficient isolation for larger scale studies difficult. This problem could be overcome by *ex vivo* $CD34^+$ cell expansion. The effects of different cytokine combinations and culture conditions on BM-$CD34^+$ cell expansion were examined (Table 3). According to these data, the best conditions for expansion of $CD34^+$ cells are provided in serum-free medium (CellGro® SCGM) containing Flt-3L, IL-3 and SCF. In the case that 50 % of the cytokine-containing medium was exchanged after 3 days and the density of cells did not exceed 10^5 cells/ml, the increase of cells was 18.5-fold. The DC differentiation induced by PMA was not affected by pretreatment of the BM-$CD34^+$ cells with the above cytokine cocktail that induced $CD34^+$ cell expansion connected with myeloid but not with DC differentiation (Davis *et al*., 1998). The expansion of primitive progenitor cells in culture should facilitate retroviral transduction because retroviral vectors require target cell division for efficient integration into cellular genome (Glimm *et al*., 1998). Therefore, the

method described here may be used preferentially for introducing genes into DC by oncoretroviral vectors under serum-free conditions.

To study the efficacy of PMA to generate mature DC in serum-free medium, PMA-derived DC compared with cytokine-derived DC were cultured using the same serum-free culture conditions. The maturation of cultured human blood DC was delineated by surface analysis of the co-stimulatory molecule B7-2 (CD86), because it is the most important ligand to induce CD28-mediated co-stimulation for $CD8^+$ T cell activation (Van Gool *et al*., 1999). It is well known that the cytokine requirements to differentiate CB- and PB-$CD34^+$ progenitor cells into DC are different (Garbe *et al*., 1998). Therefore, $CD34^+$ progenitors from CB and PB have been compared within the same experiment. $CD86^+$ yield under serum-free conditions was increased by PMA compared to cytokine cocktails in CB ($P = 0.071$) and PB ($P = 0.016$). Up-regulation of CD86 was most pronounced with addition of the calcium ionophore ionomycin. However, the output number of viable cells after differentiation was decreased by PMA plus ionomycin ($P < 0.05$) or plus TNF-α ($P > 0.05$) compared with that in PMA alone. The decrease of cell viability using PMA plus ionomycin or TNF-α compared to PMA alone may be due to apoptotic effect of these two stimuli. TNF-α and ionomycin appear to be using the same signalling pathway because the combination of PMA, ionomycin and TNF-α was no different from PMA plus TNF-α or ionomycin (St. Louis *et al*., 1999).

Functionally, PMA-generated DC were capable of stimulating autologous T cell proliferation by processing and presenting whole soluble Ag or SuperAg. The absence of autologous T cell proliferation when cultured with DC (no SEB or TT) demonstrates that DC themselves or the drug by which they have been treated do not stimulate responding T lymphocytes.

Taken together, it can be concluded that PMA is a potent activator for differentiation of human $CD34^+$ cells into mature DC in serum-free medium. The procedure described here may represent a method for generating pure populations of DC from freshly isolated or *ex vivo* expanded $CD34^+$ progenitor cells. This may facilitate an *in vitro* immune response using primed or genetically modified DC against infectious and tumor-associated antigens.

4.3 High titer virus production in serum-free medium

The use of retroviral vectors for human gene therapy requires the production of large quantities of high titer vector stocks (McTaggart and Al-Rubeai, 2000). The efficacy of GP+E86 ecotropic producer cell line to produce high-titer murine helper-free retroviruses (capable of delivering genes to murine cells) appears to be limited by the low transfectability of these packaging cells. The percentage of transfected cells expressing EGFP not exceeded 3 % after transfection with pSFβEGFP retroviral vector using electroporation or SuperFect^{TM} Transfection Reagent.

The infectious titer generated by such packaging cells derived from NIH/3T3 cells is generally low, necessitating identification of those clones that produce retroviruses at higher titer. Under optimal circumstances, this requires 1-2 months and must be repeated for each different construct. During prolonged selection process, the retroviral vector mRNA may inhibit growth of the packaging line, favouring the outgrowth of clones that produce low levels of virus; thereby, making it difficult to identify clones continuously producing retrovirus at a high titer (Pear *et al*., 1993). As a result of these deficiencies, transient production of retroviral supernatants has become the preferred method of producing retrovirus for most laboratory uses (Pear *et al*., 1993). In comparison to the production of stable retroviral producer cell lines, generation of high-titer retrovirus by transient production not only is less laborious, but also has allowed the production of high-titer retroviral supernatants from cDNAs that can't be produced by stable producer cell lines. Transient transfection has also increased the versatility of retrovirus-mediated gene transfer to include the rapid testing of different constructs, viral pseudotyping, and the construction of retroviral cDNA libraries (Pear *et al*., 1993).

Recently, the laboratory of Nolan created a new producer cell line [Phoenix Eco (ΦNX-Eco) and Phoenix Ampho (ΦNX-Ampho)] derived from the highly transfectable 293T cell line to generate high-titer helper-free retroviruses by transient transfection (Pear *et al*., 1993; Yang *et al*., 1999). The new producer cell line Phoenix was highly transfectable with SuperFect reagent than calcium phosphate precipitation method. The highest transfection efficiency (74.4 %) and a virus titer (4×10^6 CFU/ml) was obtained with the cells that were 2×10^6 at seeding, 24 h before transfection, using SuperFect reagent.

The range of cells that are infectable is determined primarily by the envelope protein of the virus and the presence of appropriate receptors for this protein on the surface of infected cells (Miller *et al*., 1991). One restriction of retroviral gene transfer into haematopoietic stem cells is the low level of amphotropic virus receptor (von Laer *et al*., 2000). The virions produced by the murine pseudotyped packaging cells PG13 are hybrids between the *gag-pol* proteins of Molony murine leukemia virus (M-MLV) and the *env* protein of gibbon ape leukemia virus (GaLV). These hybrid GaLV pseudotype virions are able to infect cells from higher mammals and human better than amphotype virions (Miller *et al*., 1991).

Retroviral vectors have proven useful in a variety of gene transfer applications. A key feature of their utility is the availability of retrovirus packaging cells that allow production of retroviral vectors in the absence of helper virus. The GaLV-based packaging cells (PG13) have been constructed on the basis of murine cells that don't express the receptor for GaLV. Thus, infection of packaging cells and the potential for generation of recombinant vectors is supposed to be very low (Miller *et al*., 1988, 1991).

In this study, transient transfection of human ecotropic packaging cells, ΦNX-Eco using a superFect reagent was utilized to produce high-titer murine recombinant retroviral vectors ($> 1 \times 10^6$ CFU/ml) used for transduction of murine pseudotyped packaging cells PG13 to generate high-titer ($> 5 \times 10^5$ CFU/ml) retroviruses pseudotyped with gibbon ape leukemia *env* protein (to increase the transduction efficiency to dividing human progenitor cells). The pseudotype retrovirus-containing supernatants were harvested under serum-free conditions (CellGro® SCGM), to avoid skewing the resulting immune response by foreign antigens present in FCS, at 37°C as described previously (Glimm *et al*., 1998). The temperature to produce viral particles was not decreased because PG13 rapidly looses viral titer when grown at the lower temperature (Reeves *et al*., 2000).

4.4 V_LIgPl gene expression in dendritic cells

Gene transfer and expression of exogenous genetic information coding for an immunogenic protein in DC can promote an immune response (Chischportich *et al*., 1999). Mobilized $CD34^+$ cells represent an optimal source for the generation of mature and functional DC suitable for cancer immunotherapy (Ratta *et al*., 1998). Despite advantages of both retroviral

vectors and hematopoietic stem cells as tools for genetic therapy, retroviral gene transfer into primitive stem cells of large animals has been problematic (MacNeill *et al*., 1999). This is due to multiple different factors, including low level expression of amphotropic retroviral receptors, G_o/G_1 cell cycle status of progenitors and possible induction of differentiation of primitive haematopoietic progenitors on the attempt to induce cell proliferation (Richardson and Bank, 1996; Chu *et al*., 1998; von Laer *et al*., 2000). These problems could be overcome by using the GaLV pseudotype virions with SFFVp/MESV hybrid retroviral vector backbone and the cytokine cocktail induced $CD34^+$ cell expansion without DC differentiation. Other factors include low viral titer, short half-life of the retroviral vector and Brownian movement of the retrovirus (Andreadis *et al*., 1997; Palsson and Andreadis, 1997). To overcome these limitations, high titer retroviruses produced by transient transfection were used. However, increased viral titer does not always correlate with enhanced gene transfer efficiency due to elevated levels of inhibitors and metabolic toxins secreted by the packaging cells. Serum-free viral supernatant may reduce the level of inhibitors and inactivators (Liu *et al*., 2000).

One of the factors required for successful retroviral transduction is contact between viral particles and target cells. Combining agents that improve virus-target cell interaction via different mechanisms will increase transduction efficiency (Liu *et al*., 2000). Polycations such as polybrene and protamine sulphate increase the infection efficacy of the retroviral vector by reduction of electrostatic repulsion between virus and target cells (Liu *et al*., 2000). Concentrations of 5-10 μg/ml of protamine sulphate provided essentially the same infection efficiency as polybrene with low toxicity on a range of cell types (Cornetta and Anderson, 1989). Efficient retroviral gene transfer into haematopoietic stem and progenitor cells can also be achieved by co-localizing retrovirus and target cells on specific adhesion domains of recombinant fibronectin (FN) fragments (Hanenberg *et al*., 1997).

Here, different methods were used for retroviral transduction of $CD34^+$ haematopoietic progenitor cells in serum-free medium to determine the optimal transduction conditions. The best result was obtained in presence of the polycation protamine sulphate combined with centrifugation at 1000 x g for 2 h at room temperature. Cell surface expression of FLAG by $pSF\beta_S(FLAG\text{-}V_LIgPl)_\omega DAF$-transduced $CD34^+$ progenitor cells was 45.9 % ± 4.0 % one week after three rounds of consecutive transductions. It could be released by PI-PLC that specifically cleaves proteins bound to the membrane via GPI-anchor (Moran *et al*., 1991). Surface antigen phenotype characterization of $CD34^+$ progenitor cells after expansion and

transduction under serum-free conditions revealed that the cells were negative for CD1a, CD15 and CD14. In addition, only 3.9 % ± 0.3 % were $CD86^+$. On the other hand, the level of CD34 expression was 78.9 % ± 4.7 %. These data indicated that the used cytokine cocktail induced $CD34^+$ cell expansion without DC differentiation.

The ability of cytokine- and PMA-derived DC obtained from transduced-$CD34^+$ progenitor cells under serum-free conditions to express V_LIgPl and co-stimulatory molecules important to activate T cells was compared in this thesis. It was found that, in spite of all cytokine-derived DC were positive for MHC class I and II, only subpopulation was $CD1a^+$, $CD40^+$, $CD83^+$ and $CD86^+$ (Table 5). On the other hand, the entire population of PMA-derived DC was negative for CD1a and positive for other co-stimulatory molecules. In addition, co-expression of V_LIgPl gene and CD86 co-stimulatory molecule increased in PMA-derived DC compared with cytokine-derived DC. These data pointed out that PMA-derived DC are more homogeneous and mature than cytokine-derived DC.

4.5 An *in vitro* T cell response against V_LIgPl

The existence of separate processing pathways for presentation of exogenous and endogenous antigens provided a suitable model for understanding how MHC class II-restricted $CD4^+$ helper T-cell responses are generated against extracellular antigens while MHC class I-restricted $CD8^+$ cytotoxic T-cell responses are directed against cytosolic antigens. Exogenous antigens are internalized by APC, degraded in vesicular intracellular compartments, and loaded on MHC class II molecules in a post-Golgi compartment. In contrast, peptides derived from cytosolic antigens by the action of proteasomes are transported into the endoplasmic reticulum lumen by an adenosine triphosphate-dependent transporter associated with antigen presentation (TAP). In the endoplasmic reticulum lumen, a chaperone-mediated assembly generates a stable complex containing MHC class I heavy chain, β_2-microglobulin, and an antigenic peptide. This complex traffics to the cell surface, where it can be recognized by $CD8^+$ T cells. The strict dichotomy was challenged by several studies that have shown that peptides generated from exogenous proteins can gain access to the cytosol and therefore be presented on class I MHC molecules (Brossart and Bevan, 1997).

MHC/peptide complexes by themselves do not initiate immune responses. An immune response follows a two-signal pattern, one corresponding to the MHC/peptide complex (signal 1) and another to a series of co-stimulatory molecules (signal 2), in particular B7 molecules (Tarte *et al*., 1999; Ribas *et al*., 2000). A high density of B7 molecules is needed to trigger a full T cell response (Tarte *et al*., 1999). Presentation of tumor antigen by malignant cells not expressing co-stimulatory molecules is considered to be a major cause of the failure of the host's immune response against tumors (Brown *et al*., 1998). Since antigen presentation is the deciding element between response or tolerance, a key player is the APC, which has the greatest concentration of MHC/peptide and co-stimulatory molecules. When an antigen is presented by non-APC cells (tumor cells), the antigen is presented without co-stimulation (first signal without second signal), therefore a different T lymphocyte function such as anergy results (Guinan *et al*., 1994; Schwartz, 1996). If the same antigen is presented by APC, both first and second signals are present, the immune system senses danger to that antigen and triggers a full T cell response (Ribas *et al*., 2000).

In multiple myeloma CD28 expression was present on most CD4 cells but lower on CD8 cells especially from those patients who also showed evidence of expanded T cell clones immunity (Brown *et al*., 1998). In addition, B7-1 (CD80) and B7-2 (CD86) are generally absent or only marginally present on tumor cells (Brown *et al*., 1998; Tarte *et al*., 1999; Pope *et al*., 2000). These results are consistent with the concept that engagement of the T cell receptor by tumor antigen on B7-1 deficient malignant plasma cells would result in T cell anergy rather than productive immunity (Brown *et al*., 1998). An activated $CD8^+$ T cell that finds its specific MHC/peptide complex on the surface of a target cell triggers the killing mechanism without requiring the presence of signal 2 (Ribas *et al*., 2000). Therefore, vaccination of multiple myeloma patients with an *in vitro* activated autologous CTL against the tumor-associated antigen is needed.

In order to proof that transduced DC are functional by eliciting an immune response, T cell proliferation against V_LIgPl has been tested. It was found that pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF-transduced DC successfully activated proliferation of autologous T cells. This proliferative response is more prominent in PMA-derived DC (PI = 328.54) compared to cytokine-derived DC (PI = 143.29) indicating that PMA-derived DC are more potent to activate autologous T cell proliferation. The absence of autologous T cell proliferation in the presence of mock- or pSFβ_S(FLAG)$_\omega$DAF-transduced DC indicates that the proliferation induced by

pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF-transduced DC is specific for V_LIg of plasmocytoma. The proliferative response was augmented by increase of the percentage of pSFβ_S(FLAG-V_LIgPl)$_\omega$DAF-transduced DC and predominantly inhibited by antibodies against MHC class I or CD86. These data indicate that the elicited proliferative response is not directed against the constructed whole GPI-linked fusion protein present at the surface of transduced DC. Proliferation is rather stimulated by fragments of the transduced peptide presented on MHC class I molecules.

On the other hand, moderate proliferation in the presence of autologous DC transduced with pSFβN1 (containing Neo^R) or pSFβEGFP (containing EGFP) has been observed. These data support the idea that neomycin and EGFP by themselves are immunogenic.

In conclusion, the data suggest that autologous T cells recognize immune epitopes within multiple myeloma variable region of paraprotein light chain, which had been presented by DC on MHC class I molecules. In addition, CD86 co-stimulation plays a critical role in activation of naive T lymphocytes. Thus a system was developed *in vitro*, allowing further studies *in vivo* to determine the MHC restriction pattern of different V_LIg peptides in different HLA-type multiple myeloma patients. Such results could provide the basis of a putative V_LIg-based immunotherapy in multiple myeloma.

5 List of abbreviations

7AAD	7-amino-actinomycin D
a.a	Amino acids
Ag	Antigen
APC	Antigen-presenting cells
ATCC	American Type Culture Collection
BM	Bone marrow
Bp	Base pairs
BSA	Bovine serum albumin
CB	Cord blood
CB-DC	Cord blood-derived dendritic cells
CD	Cluster of differentiation
CD34-DC	CD34-derived dendritic cells
CDRIII	Third complementarity-determining region
CFU/ml	Colony forming units per ml
CPM	Count per minute
CTL	Cytotoxic T lymphocytes
D	Diversity gene segment
DAF	Decay-accelerating factor
DC	Dendritic cells
DEPC-water	Diethyl pyrocarbonate treated-water
DMEM	Dulbecco´s modified Eagle´s medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
Env	Envelope
EP	Electrophoresis
FCS	Fetal calf serum
FGFR3	Fibroblast growth factor receptor 3
FITC	Fluorescein isothiocyanate

FLAG	An artificial antibody-binding site
Flt-3L	Flt-3 ligand
GaLV	Gibbon ape leukemia virus
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPI	Glycosylphosphatidylinositol
H	Hour
HBSS	Hank’s buffer saline solution
HHV-8	Human herpesvirus-8
HLA	Human leukocyte antigens
HPC	Haematopoietic progenitor cells
Id	Idiotypic protein
Ig	Immuoglobulin
IgH	Immuoglobulin heavy chain
IL	Interleukin
IMDM	Iscove´s modified Dulbecco´s medium
IPTG	Isopropyl-β-D-thiogalactoside
J	Joining gene segment
Kb	Kilo base pairs
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria-Bertani
LTR's	Long terminal repeats
M	Monoclonal immunoglobulin
M1, M2 and M5	Anti-FLAG monoclonal antibodies
Mabs	Monoclonal antibodies
MACS	Magnetic cell separator
MESV	Murine embryonic stem cell virus
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
MHH	Hannover medical school
Min	Minute
MLRs	Mixed lymphocyte reactions
MM	Multiple myeloma
M-MLV	Molony murine leukemia virus

MMRF	Multiple myeloma research foundation
MNC	Mononuclear cells
Mo	Monocytes
mRNA	Messenger ribonucleic acid
Neo	Neomycin phosphotransferase gene
Neo^R	Neomycin resistance
NGFR	Nerve growth factor receptor
OD	Optical density
P	Probability
PB	Peripheral blood
Pbs	Primer binding site
PBS	Phosphate buffered saline solution
PCMV	PCC4-cell-passaged mutant virus
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Proliferative index
PI-PLC	Phosphatidylinositol-specific phospholipase C
PKC	Protein kinase C
PMA	Phorbol ester
Ppt	Polypurine tract
Rb	Retinoblastoma
RE	Endoplasmic reticulum
RNA	Ribonucleic acid
Rpm	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
RZPD	Resource Centre of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics
SB	Sample buffer
SCF	Stem Cell Factor
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEB	Staphylococcal enterotoxin B
Sec	Second

SFFVp	Polycythemic strain of the spleen focus-forming virus
TAA	Tumor-associated antigens
TAP	Transporter associated with antigen presentation
TGF-β	Transforming growth factor-β
Th	T helper cells
TNF-α	Tumor Necrosis Factor-Alpha
TT	Tetanus toxoid
UV	Ultra violet
V	Variable gene segment
V_L	Variable region of paraprotein light chain
V_LIgPl	Plasmocytoma variable region of immunoglobulin light chain
v/v	Volume/volume
ω	Cleavage attachment site
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

6 List of producers

-Amersham Pharmacia Biotech, Braunschweig, Germany

-Amgen, Thousand Oaks, California, USA

-Applichem GmbH, Darmstadt, Germany

-ATCC, American Type Culture Collection, Manassas, Virginia, USA

-Baker Company, Inc., Sanford, Maine, USA

-B. Braun, Melsungen, Germany

-Beckmann, Muenchen, Germany

-Becton Dickinson GmbH, Heidelberg, Germany

-Bender and Hobein AG, Zurich, Switzerland

-Biochrom KG, Berlin, Germany

-Bio-Rad Laboratories GmbH, Muenchen, Germany

-Calbiochem-Novabiochem GmbH, Schwalbach, Germany

-Cambridge Technology, Inc., Watertown, Massachusetts, USA

-CellGenix, Freiburg, Germany

-Cinna Biotecx Labs Inc., Houston, Texas, USA

-Clontech GmbH, Heidelberg, Germany

-Cytogen®, Lahmer, Germany

-DAKO, Hamburg, Germany

-Difco Laboratories, Detroit, USA

-Eppendorf, Hamburg, Germany

-Eurogentec, Seraing, Belgium

-Falcon, New Jersey, USA

-Gelaire, Meckenheim, Germany

-Gibco BRL, Neu-Isenburg, Germany

-Greiner GmbH, Frickenhausen, Germany

-Hannover medical school (MHH), Hannover, Germany

-Hawksley & Sons Limited, Sussex, UK

-Heidolph, Schwabach, Germany

-Heraeus, Hanau, Germany

-H. Juergens & Co., Bremen, Germany

-ICN Biomedicals GmbH, Eschwege, Germany

-Immunex Corp, Seattle, Washington, USA

-Immunotech GmbH, Hamburg, Germany

-International Biotechnologies Inc, New Haven, Connecticut, USA

-Invitrogen BV, Groningen, Netherlands

-Knick, Berlin, Germany

-Landgraf, Hannover, Germany

-Leitz, Oberkochen, Germany

-Life Technologies, Gaithersburg, Maryland, USA

-LKB, Wallac, UK

-Macherey-Nagel GmbH, Dueren, Germany

-MBI Fermentas Molecular Biology GmbH, St. Leon-Rot, Germany

-Merck KGaA, Darmstadt, Germany

-Millipore GmbH, Eschborn, Germany

-Miltenyi Biotec, Bergisch Gladbach, Germany

-MWG-Biotech, Ebersberg, Germany

-Nalge Nunc International, Roskilde, Denmark

-New Brunswick Scientific Co. Inc., Edison, New Jersey, USA

-New England BioLabs, Beverly, Massachusetts, USA

-Nikon, Surrey, UK

-PAA Laboratories GmbH, Coelbe, Germany

-Pharmacia, Freiburg, Germany

-PharMingen GmbH, Hamburg, Germany

-Promega, Mannheim, Germany

-Qiagen GmbH, Hilden, Germany

-Roche Diagnostics GmbH, Mannheim, Germany

-Savant, Farmingdale, New York, USA

-Seromed, Berlin, Germany

-Serotec, Oxford, UK

-Serva, Heidelberg, Germany

-Shimadzu Deutschland GmbH, Duisburg, Germany

-Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany

-Stratagene GmbH, Heidelberg, Germany

-RZPD, Resource Center of the German Human Genome Project at the Max-Planck-Institute for Molecular Genetics, Berlin, Germany

-Takara, Otsu, Japan

-Tebu GmbH, Frankfurt/M., Germany

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9 Curriculum vitae

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Date and place of birth	25.06.1967 in Cairo, Egypt
Marital status	Married, 2 children
1973 - 1978	Primary school in Cairo
1978 - 1981	Preparatory school in Cairo
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1984	Begin of biological studies in Faculty of Science, Ain Shams University, Cairo
Mai 1988	B.Sc. in Zoology-Chemistry
Oct. 1988 - Nov. 1989	Military service
Dec. 1989	Demonstrator in Division of Physiology, Department of Zoology, Faculty of Science, Ain Shams University
Dec. 1989-Oct. 1990	M.Sc. preparation courses in physiology
Nov. 1990	Registration for M.Sc. degree in Division of Physiology, Department of Zoology, Faculty of Science, Ain Shams University on “Effect of oral contraceptives on some metabolic activities of female rats” under supervision of Prof. Dr. Nadia El-Beih
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1996	Ph.D. Grant from the Egyptian Administration of Mission
Aug. 1997 - Nov. 1997	German language course in Goethe Institute, Bremen

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List of publications

G. Ramadan, R. E. Schmidt and J. Schubert (2000): Retroviral expression of FLAG-$V_LIg_{plasmocytoma}$ fusion protein in autologous dendritic cells via a GPI-anchor. In A. Mackiewicz, M. Kurpisz, and J. Zeromski (Eds): 14th European Immunology Meeting-EFIS 2000, Poznan, Poland, September 23-27, 2000. *Monduzzi Editore, Bologna,* 455-459.

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03/2000 **16. Frühjahrstagung der Deutschen Gesellschaft für Immunologie, Köln, Deutschland (Oral presentation).**

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