HLA Allogene Expression in Multiple Myeloma Cells: Possible Use as Anti-tumor Vaccine

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Abstract

Multiple myeloma (MM) is a plasma cell malignancy with a high mortality rate. The current treatment options may prolong survival but none of them are curative. Allogeneic gene therapy is a novel vaccine strategy that can augment anti-tumor immune responses and cause remission in some non-hematologic cancers. Immuno-gene transfer has been successfully performed in human and murine myeloma cells using viral vectors. We used immuno-magnetic negative selection to purify myeloma cells from bone marrow (BM) specimens. We investigated the possibility of *in vitro* culture of bone marrow mononuclear cells (BMMC) and myeloma cell enrichments. We used liposomal and adenoviral vectors to transduce myeloma cell lines. We cloned the allogene HLA-B7 into a recombinant adenoviral transfer plasmid and, subsequently, produced a recombinant, HLA-B7-positive, adenoviral vector. We evaluated allogene expression by the adenoviral vector in myeloma cell lines. In three of four BM samples tested, 95-99% purification of primary myeloma cells was achieved. Poor expansion and considerable variability (6-22 days) in the duration of culture of BMMC (two samples) and purified myeloma cells (four samples) was observed. Adenoviral vectors expressing green fluorescent protein (GFP) were highly efficient (70-94%) for transduction of myeloma cell lines. Using the HLA-B7-adenoviral vector, a small population (13%) of HLA-B7-positive myeloma cells (RPMI-8226 cell line) was identified. These observations support the feasibility of creating a myeloma vaccine by transduction of primary human myeloma cells with an allogene.

Résumé

Le myélome multiple (MM) est une malignité des cellules plasmatiques dont le taux de mortalité est considérablement élevé. Les différents traitements actuels du MM permettent de prolonger la durée de vie sans toutefois être curatifs. Récemment, une nouvelle stratégie de vaccin, la thérapie de gène allogénique, a permis la rémission de certains cancers non-hématologiques en augmentant la réponse immune anti-tumorale. Aussi, le transfert immunogénique de gène a été appliqué avec succès autant chez les cellules du myélome murin qu'humain et cela utilisant des vecteurs viraux. Nous avons développé une technique de sélection négative immuno-magnétique pour purifier les cellules du myélome à partir d'échantillons de moelle osseuse. Par la suite, nous avons étudié la possibilité de cultiver *in vitro* des cellules mononucléaires de la moelle osseuse et des cellules purifiées du myélome. Nous avons utilisé les vecteurs liposomal et adénoviral pour la transduction des lignées cellulaires du myélome et nous avons cloné l'allogène HLA-B7 dans un plasmide recombinant de transfert adénoviral. Ainsi, nous avons produit un vecteur adénoviral exprimant HLA-B7 et évalué son expression allogénique chez des lignées cellulaires du myélome. Parmi quatre échantillons de moelle osseuses testés, nous avons obtenu une purification des cellules du myélome primaires variant entre 95 et 99%. Nous avons observé une faible expansion des cellules et une variabilité considérable (6 à 22 jours) de la durée des cultures des cellules mononucléaires de la moelle osseuse (deux échantillons) et des cellules purifiées du myélome (quatre échantillons). La transduction des lignées cellulaires du myélome était plus efficiente (70-94%) en présence des vecteurs adénoviraux exprimant la protéine fluorescente verte. Utilisant le vecteur adénoviral exprimant HLA-B7, une faible population (13%) des cellules du myélome positives pour le HLA-B7 (lignée cellulaire RPMI-8226) a été identifiée. Ces observations soutiennent l'hypothèse de développer un vaccin du myélome grâce à la transduction des cellules du myélome humain primaires avec un allogène.

Chapter 1

General Introduction

Introduction and Rationale

Multiple myeloma (MM) is a hematologic malignancy involving plasma cells that typically affects older individuals and accounts for approximately 10% of all hematologic tumors. In Canada between 1100 to 1300 people die of MM each year, most within 3 years of initial diagnosis. The causes of MM remain unknown. Plasma cells originate in the bone marrow from the B-lymphoblastoid lineage. Myeloma cells often produce large amounts of idiotype-specific immunoglobulin (Ig) that contribute to hyperviscosity syndrome, which is a major complication of this condition (Kyle, 1975). Tumor growth within the marrow accounts for the other major manifestations of this cancer such as immunosuppression, osteolytic lesions, anemia and bleeding (Bataille et al., 1997). The current treatment options for MM include varying degrees of chemotherapy as well as autologous or allogeneic bone marrow transplantation (BMT). Although these regimens can often prolong survival (Attal et al., 1996) they are generally not curative and most patients relapse within 1 to 3 years of therapy (Gregory et al., 1992; Barlogie et al., 1995). Hence there is a pressing need for alternative therapeutic strategies.

Numerous pre-clinical and clinical studies have demonstrated the feasibility of inducing an effective immune response against malignant cells by genetic modification of these cells to express cytokines, immuno-stimulatory molecules or antigens (Chen et al., 1998; DeBruyne, 1996). Allo-antigens are highly immunogenic and a disproportionately large percentage of the human immune repertoire appears to be directed against them (Droge 1979; Detour et al., 1999; Mendiratta et al., 1999;

Detours et al., 2000). Allo-antigen gene transfer in several murine and human tumors has been shown to induce an effective immune response against the modified cells and, more significantly, against unmodified tumor cells at distant sites (so called bystander killing). In several studies, such allo-antigen therapy has led to reduction of tumor load and even complete remission (Stopeck et al., 1997; Gleich et al., 1998; Ohno et al., 1997; Rubin et al., 1997; Nabel et al., 1996). In a systemic tumor such as MM, where *in vivo* access to the great mass of tumor cells is impossible, genetic modification of a small number of tumor cells with an allo-antigen and subsequent re-introduction of these cells to the patient could conceivably lead to enhanced immune surveillance and tumor killing throughout the body. Although MM is often a relatively slow growing tumor (Drewinko et al., 1981), the bone marrow of patients can contain large numbers of myeloma cells that are potentially accessible for isolation and genetic manipulation. It would be of considerable interest to evaluate the feasibility of introducing an allogene such as HLA-B7 into human myeloma cells.

Hypotheses

1) Primary myeloma cells from MM patients can be isolated using negative selection.
2) Myeloma cells can be genetically modified, through transfection or transduction with an appropriate vector, to express an allogeneic antigen.

Study Objectives

1) Maintain and possibly expand human myeloma cells in culture

2) Purify primary myeloma cells from MM patients using negative selection

3) Using a standard reporter gene, test transfection vectors for efficiency in the delivery of foreign genetic material in myeloma cell lines

4) Design, produce and test a recombinant adenoviral transfection vector that bears the gene encoding an allo-antigen, HLA B7, and evaluate its efficiency in transfection of human myeloma cell lines.

Literature Review

Epidemiology

MM is a neoplastic disorder belonging to the group of plasma cell dyscrasias. Related disorders include monoclonal gammopathy of undetermined significance (MGUS), Waldenstrom macroglobulinemia and Castleman's disease. MM accounts for 10% of all hematological malignancies and 1% of all cancer-related deaths in western countries. In the U.S., MM is the second most frequent malignancy of the blood afflicting ~14,000 people per year (American Cancer Society, 1995). The incidence of MM in the african-american population is twice that of the white population; and the average male to female incidence ratio is 3:2 (Hussein, 1994). Nearly 1 million people in the U.S. have been diagnosed with MGUS, a relatively benign disorder that is considered a precursor condition to MM (Kyle, 1984). Approximately 25% of patients with MGUS eventually develop MM (Kyle, 1993). MM is an age-dependent malignancy occurring most frequently between 60 and 70 years of age (Gautier et al., 1994). In Canada, 10 per 100,000 of the population are afflicted with MM and between 1100 to 1300 people die of MM each year (NCIC, 2001). The median survival period is 3 years after initial diagnosis, although many patients survive less than one year (Brunning et al., 1994). The year 2001 estimates for MM in Canada, according to the National Cancer Institute of Canada (NCIC), are as follows: 1700 new cases of MM will be identified, of which 940 will be male and 750 female; the number of deaths will be 1,250; the mortality ratio (deaths per total

number of cases) will be 0.73, which provides a crude indicator of disease severity, 1.0 indicating the worst prognosis (NCIC, 2001).

Overview of myeloma biology

Clinical features

MM is an immune cell disorder that is characterized by malignant proliferation of a clonal plasma cell that produces a specific immunoglobulin protein, also referred to as M-protein, that is of considerable diagnostic value. The M-protein is most commonly of IgG or IgA isotype although rare occurrences of IgD, IgM and IgE M-proteins have been observed (Brunning et al., 1994). Myeloma cells produce large amounts of M-protein which contribute to the major clinical features of this condition; hyperviscosity syndrome (e.g. stroke, renal failure) and immunosuppression (e.g. high antibody concentrations inhibit antibody production by normal B cells) (Kyle, 1975). Other clinical complications include osteolytic lesions and bone pain caused by increased osteoclast activity, anemia caused by suppression of erythropoiesis, and recurring infections resulting from immunosuppression. As the disease progresses and myeloma cells replace normal marrow cells, the degree of immunocompromise can become severe. The total mass of myeloma cells in the bone marrow accounts for the other major clinical manifestations of this cancer such as hypercalcemia, skeletal lesions, anemia and bleeding (Durie et al., 1980). Ultimately, the renal complications and infections are the direct causes of death in most MM patients (Kyle, 1975). A disease staging system using histological findings has been

established based on the plasma cell burden in bone marrow biopsies (Bartl et al., 1987). Stage I: <20% myeloma cell infiltration in the marrow; Stage II: 20 to 50%; Stage III: >50%. The histological enumeration of the percentage of myeloma cells in the marrow correlates well with the clinical stage of the disease (Bartl et al., 1987).

Role of Interleukin-6

Interleukin-6 (IL-6), a B-cell differentiation factor, plays a major role in stimulating and maintaining myeloma cell growth *in vitro* (Kawano et al., 1988; Klein et al., 1989). Several findings indicate that IL-6 has similar activity *in vivo* (reviewed in Nishimoto et al., 1997). Serum levels of IL-6 are highly correlated with disease severity (Bataille et al., 1989; Zhang et al., 1989). While there is strong evidence supporting paracrine regulation of myeloma cell growth by IL-6 (Klein et al., 1989), there is controversy concerning the precise role of autocrine IL-6-induced growth *in vivo* (Klein et al., 1995; Klein, 1995). Freshly isolated myeloma cells produce IL-6 constitutively, express the IL-6 receptor (IL-6R), and proliferate in response to exogenous IL-6 (Svenatsu et al., 1988). In normal individuals, bone marrow stromal cells and mesenchymal stem cells are both potent producers of IL-6 (Majumdar et al., 1998). Klein et al. (1989) have suggested that these cells are the major producers of IL-6 in myeloma patients, implicating a definitive paracrine growth mechanism. However, there is contrasting evidence of an autocrine growth loop with excess IL-6 production by the myeloma cells themselves (Kawano et al., 1988; Bataille 1989). Thus, it appears that both paracrine and autocrine mechanisms may contribute to the elevated levels of IL-6 in myeloma patients. Recently, human herpesvirus-8 (HHV-8) infection of bone marrow follicular dendritic cells (Rettig et al., 1997; Said et al.,

1997) and peripheral blood cells (Sjak-Shie et al, 1999; Berenson et al. 1999), has been implicated as a novel contributing factor in MM patients. These observations are strongly supported by the identification of a HHV-8 viral homologue of human IL-6 and the confirmation of its functionality in supporting the growth of an IL-6-dependent murine plasmacytoma cell line (Moore et al., 1996). These studies suggest that HHV-8 infection may also contribute to the increased levels of IL-6 in MM pateints. IL-6 is also produced by monocytes, T-cells and fibroblasts. Other cytokines such as interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), and tumor necrosis factor-alpha (TNF-α) act synergistically with IL-6 to stimulate proliferation and differentiation of myeloma cells (Michaeli et al., 1997). These effects are mediated primarily by cytokine-induced bone marrow stromal cell production of IL-6 (Carter et al., 1990). Overexpression of IL-6 in the marrow microenvironment is also implicated in the pathogenesis of bone lesions in MM patients since this cytokine is a potent activator of osteoclasts (Mundy, 1974; Pelliniemi et al., 1995; Nishimoto et al., 1997). The normal balance between osteoblast and osteoclast activity that exists in healthy individuals is disrupted by the over-production of osteoclast-stimulating factor (OSF) by bone marrow cells (Mundy, 1974) and IL-6-induced osteoclast-precursor formation (Kurihara et al, 1990). Taken together, these data confirm that the bone marrow microenvironment in myeloma patients is a favorable site for myeloma cell proliferation and that a key role in this process is played by abnormally high levels of IL-6 derived from several sources.

Etiology

The causes of MM remain largely unknown although several occupational and environmental factors (including exposure to chemicals, carcinogens and radiation) as well as certain human leukocytic antigen (HLA) types have been associated with the disease (Bergsagel, 2001; Pottern et al., 1992). Some of the critical oncogenic events and chromosomal abnormalities that may facilitate the transition from benign monoclonal gammopathy to malignant myeloma have been elucidated (Feinman et al, 1997). These authors discuss the involvement of key oncogenes (e.g. retinoblastoma gene product, c-myc and N-, K-ras), the high frequency of dysregulation of tumor-suppressor and apoptosis genes (e.g. p53, bcl-1 and bcl-2) as well as alterations in cytokine gene/promoter regulation (e.g. IL-6) in the developmental pathogenesis of MM. Abnormal somatic mutations within the immunoglobulin genes and the aberrant genotypes of myeloma cells account for their morphological immaturity and atypical differentiation (Bakkus et al., 1992; Battaile, 1997). Mature plasma cells develop in the bone marrow from the B-lymphoblastoid lineage originating in the germinal centers of lymphoid organs. Whether the precursor myeloma clone originates in the germinal centers or results from post-migration, somatic mutations of an intermediate B-cell in the bone marrow is an issue of debate (MacLennan, 1992). HHV-8 infection of bone marrow follicular dendritic cells and peripheral blood cells have emerged as potential etiologic factors in MM (as described in the previous section). However, due to contradictory reports, this association remains highly controversial at the current time (Tarte et al., 1999; Beck et al., 2000).

Current therapeutic strategies

Current treatment options for MM include varying intensities of anti-cancer chemotherapy, radiation therapy, interferon-alpha (IFN-α) and autologous or allogeneic bone marrow or stem cell transplantation. The 'standard' drug combination prescribed for the initial treatment of MM is melphalan and prednisone. Other combinations including glucocorticoids, cyclophosphamide, alkaloids or newer purine analogues have shown equal or less efficacy in inducing remission (reviewed in Bataille et al., 1997). Liver toxicity is occasionally associated with the standard chemotherapy in MM and the prolonged administration of these drugs can induce resistance or unresponsiveness in the tumor cells and, more importantly, worsening of certain myeloma-associated symptoms, such as renal malfunction, anemia and fatigue (Sheridan, 1996). Treatment with INF-α with or without chemotherapy can reduce plasma cell growth in MM patients (Mandelli et al., 1990) but at the cost of serious side-effects such as flu-like symptoms, fatigue, thrombocytopenia and neurologic toxicity (Sheridan, 1996). Furthermore, Klein et al. (1995) have demonstrated that INF-α can stimulate myeloma cell growth *in vitro*, thereby raising serious doubts about the therapeutic role of INF-α in MM. Allogeneic and autologous bone marrow transplantations (BMT) have become increasingly safe and efficient approaches to radically reduce myeloma tumor cell load, however even transplantation is seldom curative (Kovacsovics et al., 1997). Furthermore, BMT is accessible to only a very small proportion of patients at early stages of MM who have chemo-sensitive disease (Brunning et al., 1994; Harrouseau et al., 1997). The risks of graft-versus-host (GVH)

disease and graft-related toxicity are also serious limitations to BMT (Kovacsovics, 1997).

Recent immunotherapy-based experimental approaches such as antibody therapy (i.e. administration of monoclonal antibodies directed to tumor cell surface proteins) have shown some promise in treating lymphomas and myeloma (Maloney et al., 1999). In lymphoma, the tumoricidal effects of antibody treatment, mediated by complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity, have been documented *in vitro*. However, no such effect was seen *in vivo* (Stevenson et al., 1996) and, to date, the clinical benefits of this approach have been limited. Another immunologic approach, adoptive T-cell therapy, involves the transfer of immuno-competent cells derived either from allogeneic donors or by expansion of autologous, anti-tumor T-cells *ex vivo*. Clinical studies that have evaluated this approach in drug-resistant or post-BMT relapse MM patients report significant anti-tumor responses (Lokhorst et al., 1999). However, these studies also demonstrated that auto-immune and GVH-toxicity can occur frequently following such T-cell manipulations. Despite the limited successes to date, the immunologic data strongly support a central role for T-cells in an effective anti-myeloma response. Although all of the regimens outlined above can prolong survival, they are generally not curative. They are associated with significant side effects, toxicities and frequently with an overall decline in the quality of life. Furthermore, most MM patients relapse within 1 to 3 years of therapy (Brunning et al., 1994). Hence there is

a pressing need for the development and evaluation of alternative therapeutic strategies.

Gene therapy and cancer vaccines

Overview

In the past decade, the science of gene therapy (i.e. modifying a somatic cell by introduction of foreign genetic material for therapeutic benefit) has opened new avenues for the treatment of malignant, infectious and hereditary human diseases. The laboratory and clinical successes of this approach in defective-gene-replacement therapy of diseases such as adenosine deaminase deficiency, ALD (adrenoleukodystrophy), cystic fibrosis, Gaucher's disease and muscular dystrophy, has shown that a broad range of tissue types can be targeted using novel and efficient gene delivery systems (reviewed in Kerr et al., 1994). Particular emphasis has been given to the potential use of gene therapy in cancer, given the inadequacies of current cancer treatment strategies in general. The two main categories of anti-cancer gene therapy are a) suicide gene therapy, in which a destructive "suicide" gene is introduced leading to the expression of a "lethal" protein; and b) immunogene therapy, that aims to improve and/or stimulate the anti-tumor immune response. To date, the first strategy has focused on the genetic modification of tumor cells with genes that will confer susceptibility to specific pharmacologic agents. One example of this approach is the transfer of the herpes simplex virus (HSV)-thymidine kinase (tk) gene into tumor cells followed by treatment with the nucleoside analogue ganciclovir

(GCV) (Freeman et al., 1996). Monophosporylation of GCV by the viral tk gene and then subsequent di- and tri-phosphorylation by cellular enzymes produces compounds that are suitable substrates for DNA synthesis. The incorporation of triphosporylated GCV in growing strands of DNA results in chain termination and cell death (Pope et al., 1997). Incidently, this approach has been shown to induce killing in neighboring, unmodified tumor cells, a phenomenon refered to as 'bystander effect' (Freeman et al., 1993). While this effect has been credited mainly to the transfer of phosphorylated GCV between adjacent cells, there is also strong evidence for the involvement of the immune system (Pope et al., 1997).

Immunogene therapy

More than a century ago, New York Surgeon William Coley made the earliest recorded observations of the tumor-reducing potential of an activated immune system (Wells, 2000). Coley noticed marked regression of tumors in some of his patients in the aftermath of acute infections. He then attempted to induce a similar immune response experimentally by administering bacterial cell wall preparations to his patients and noticed a similar effect. We now know unequivocally of the central role played by cell-mediated immunity (CMI) in the clearance of tumor cells. Through extensive animal and human studies, it is now well-documented that a) many tumors are potentially highly immunogenic; b) many tumors develop immune-evasive mechanisms during oncogenesis; and c) if anti-tumor surveillance can be enhanced then cytokine-mediated and effector-cell-induced cytolytic elimination of tumor cells frequently occurs. Animal studies have also shown that the induction of tumor-

infiltrating lymphocytes (TIL), that include both CD4+ T helper cells and, more importantly, anti-tumor CD8+ cytotoxic T lymphocytes (CTL), is essential for immune-mediated tumor regression and long-lasting protective immunity against relapse (Gansbacher et al., 1990; Ley et al., 1991; Plautz et al., 1993). Gene transfer of CMI-stimulating cytokines (e.g. IL-2, IL-12, TNF-α, IFN-γ and GM-CSF), receptors (e.g. CD-40 ligand and Flt-3 ligand), or costimulatory molecules (e.g. B7-1 and B7-2) can generate anti-tumor immune responses in many solid tumors (e.g. adenocarcinoma, fibrosarcoma, colorectal carcinoma and melanoma), both in animal models and human clinical trials (reviewed in Chen at al., 1998). These techniques have recently been applied to hematologic cancers including MM. For example, in a mouse model of MM, autologous myeloma cells genetically modified to express cytokines (GM-CSF and IL-12) are capable of generating CTL that confer long-lasting protection against subsequent challenge with parental and drug-resistant tumors (Shtil et al., 1999). Also, recombinant adeno-associated virus (AAV)-mediated transfection of human myeloma cell lines with genes for the costimulatory molecules B7-1 and B7-2 has been shown to significantly enhance susceptibility to allogeneic CTL-mediated cytolysis (Wendtner et al., 1997). In a more recent study, Tarte et al. (1999) used primary myeloma cells from MM patients to demonstrate that retroviral-mediated transfer of B7-1 and B7-2 genes could amplify autologous, myeloma-specific CTL. These studies indicate that myeloma cells can be targeted for immunogene transfer to enhance the myeloma-specific CTL response.

Peptide or DNA-based, tumor-antigen-specific gene transfer has proven to be yet another efficient, CMI-stimulating, therapeutic strategy for cancers in which immunologically accessible tumor-associated antigens (TAA) have been identified (e.g. melanoma) (Chen et al., 1998). The idiotypic Ig component in MM is regarded by some as such a tumor-associated antigen and its targetting potential has been examined with some success (Kwak et al., 1999). However, the idiotypic antigen is specific to each individual patient's malignant plasma cell clone. Thus, a vaccine based upon this antigen would have to be "custom-made" for each patient, which limits the feasibility of this approach considerably. Some TAA common to both solid and hematologic tumors, including MM, have recently been identified (Van Baren et al., 1999; Treon et al., 1999). The messenger RNA levels of these TAAs have been determined in marrow samples from MM and MGUS patients using RT-PCR (reverse transcriptase-polymerase chain reaction) and immunocytochemistry (Van Baren et al., 1999). This study provides strong evidence that the expression of some of these TAAs was limited to the cytoplasm. They were not necessarily found in association with major histocompatibility (MHC) molecules at the cell surface, which is essential for antigen presentation to immune effector cells. Also, the expression of the various TAAs was found to vary significantly with the stage of disease. These observations could limit the possible benefits of a TAA-based gene transfer approach in MM. In summary, immuno-genetic manipulation of myeloma cells is emerging as a promising therapeutic approach for MM and is a strategy worthy of further inquiry.

Allogene expression and bystander effect

For reasons that are as yet unclear, the human immune system has a strong, pre-existing reactivity to allogeneic HLA (Droge 1979; Detour et al., 1999; Detours et al., 2000), as demonstrated in allogeneic-tissue graft rejection (Mendiratta et al., 1999). Based on this ability of allo-antigens to stimulate strong cellular immune responses, numerous animal and human studies have been performed using allo-antigen gene transfer in non-myeloma tumor models (Plautz et al., 1993; Egawa et al., 1995; DeBruyne, 1996; Nabel et al., 1996; Stopeck et al., 1997). Direct injection of an allogeneic HLA class I gene into murine colon adenocarcinomas and fibrosarcomas induces a CTL response against the modified cells and, more importantly, against unmodified tumor cells (Plautz et al., 1993). The immune response elicited can cause reduction in tumor growth and complete tumor regression in some animals. Similarly, allogene transfer in mice confers protection from nerve-fibrosarcoma challenge and resistance against the formation of metastases in the lung (Egawa et al., 1995). Success in mouse studies has led to widespread clinical evaluation of this strategy in problematic human cancers, such as squamous cell head and neck carcinoma (Gleich et al., 1998), colorectal carcinoma (Rubin et al., 1997) and metastatic melanoma (Nabel et al., 1996). Two independent Phase I clinical trials, both of which studied the safety, immune responsiveness and clinical efficacy of direct HLA-B7-allo-gene injection into metastatic melanoma nodules, have reported detectable levels of allogene (e.g. DNA) and allo-antigen expression in the tumor cells, inhibition of tumor growth and reduction in tumor size (Nabel et al., 1996;

Stopeck et al., 1997). Both studies found tumor remission in a significant proportion of patients, with only mild side-effects related to the procedures. The lack of autoimmunity, serum abnormalities, histopathology and gonadal infiltration following *in vivo*, allo-MHC, gene transfer in mice, pigs and rabbits provide further evidence of the safety of this approach (Nabel et al., 1992).

The precise mechanism by which the genetic modification of a relatively small number of tumor cells with allo-antigens elicits an effective anti-tumor immune response is currently unclear. Nonetheless, this bystander effect has been well documented in several tumors following suicide- or immuno-gene transfer (Miller et al., 1994; Pope et al., 1997; Culver, 1996). It is likely that the intense immunological activity (e.g. increased phagocytosis, upregulated gene expression of cytokines, adhesion molecules and other soluble factors) within the microenvironment in which the modified tumor cells are being destroyed permits enhanced immunologic surveillance (e.g. activated APC screen a broader range of antigens). This intense surveillance may lead to the recognition of previously inaccessible, 'cryptic' TAAs or break a state of tumor-induced tolerance towards TAAs (Chen, 1998; Fenton et al., 1997). There is also increasing evidence that systemic, tumor-specific immunity can develop following the treatment of localized tumors by gene therapy thus lending support to the existence of a distant bystander effect (Pope et al., 1997). In a systemic tumor such as MM, where access to the majority of tumor cells is impossible, allo-MHC-based genetic modification of a few tumor cells and their subsequent re-introduction to the patient at discreet sites could conceivably lead to the induction of

enhanced surveillance and tumor killing throughout the body. Hence, it would be of considerable interest to evaluate the feasibility of introducing an allogene such as an HLA Class-I molecule into human myeloma cells.

Recent progress in gene delivery systems and their implications for MM

Physical and chemical transfection systems

Traditionally, electroporation and calcium-phosphate-dependent transfection have been the preferred methods of delivering foreign DNA into mammalian cells (Andreason et al., 1988; Schenborn et al., 2000). Electroporation, which uses short electric pulses to create pore-like channels in the membrane lipid bilayer, has been used for *in vitro* transport of molecules across cell membranes (Weaver, 2000). The benefits of this method have even been considered for *ex vivo* and *in vivo* molecular transport in tissues and for possible applications in gene therapy (Dev et al., 2000; Weaver, 2000). However, with progress in membrane biochemistry research, these methods are rapidly becoming obsolete for clinical applications. Currently, the most promising means of gene delivery include cationic lipid complexes and recombinant viruses (Romano, 1998; Goedegebuure et al., 1997; Descamps et al., 1996). Cationic lipids have the dual capacity to bind to and form liposomal complexes with DNA as well as to interact with eukaryotic cell membranes facilitating the penetration of DNA into the cell (Plautz et al., 1994). Furthermore, many such formulations are non-toxic to cells (Nabel et al., 1993) and are readily metabolized *in vivo* (Gao et al. 1991). These attributes have been used widely in cancer gene therapy studies wherein

cytokine and allogeneic gene transfer into tumor cells has been accomplished safely and effectively (Stopeck et al., 1997; Rubin et al., 1997; Nabel et al, 1996; Osanto et al., 1993).

Adenoviral transduction vectors

As an alternate strategy, the innate ability of a virus to enter a cell and facilitate expression of its genome has been exploited in recombinant viral vector development. Viruses such as retroviruses, adenoviruses, vaccinia and herpesviruses have been modified to express exogenous, recombinant genes while their own ability to replicate has been limited by removal of key viral genes (Yeh et al., 1997; Romano et al., 1998). Replication-defective adenoviral vectors are particularly useful in gene therapy due to their ability to transduce non-replicating cells from a wide range of tissue types and their relatively large capacity to carry foreign genetic material. The first recombinant adenovirus vectors were developed for gene therapy studies in cystic fibrosis (CF) using type 5 adenovirus (Brody et al., 1994). The viral vectors produced for these studies were depleted of the adenoviral replication gene E1 and the therapeutic gene of interest was inserted in its place. These vectors are now known as "first generation", replication-deficient adenoviral vectors. Continued efforts have led to "second" (E1 and E3 gene-deleted) and "third" (E1, E2 or E3, and E4 gene-deleted) generation adenoviral vectors (Wang et al., 1996; Yeh et al., 1997). The adenovirus virion does not have an envelope and is comprised only of protein and linear, double stranded DNA. The genome of adenovirus is between 30-40 kb in size and, unlike retroviruses, it does not integrate into the host cell genome after

infection but exists extra-chromosomally in episomes. The coxsackie/adenovirus receptor (CAR) is the main cellular receptor required for adenoviral binding and entry into the cell (Bergelson et al., 1997). The alpha-v integrins, specifically $\alpha_v\beta_3$ and $\alpha_v\beta_5$, are also required for adenoviral internalization into mammalian cells (Mathias et al., 1994; Wickam et al., 1993). Adenoviral vectors are able to deliver genes to several different mammalian cell types due to the tissue polytropism of the virus. Vectors are typically amplified in the human embryonic kidney cell line, 293A, which contains the adenovirus replication gene E1 in its genome (Graham et al., 1977). Recombinant viral particles can be then extracted and purified (Yeh et al., 1997), making it fairly easy to produce high titres of engineered virus.

To date and for reasons that remain poorly understood, lymphocytes and malignant hematologic cells have posed a greater challenge in being transduced than solid tumor cells (Leon et al., 1998). Hence, a systemic evaluation of gene vector approaches (e.g. lipid and adenoviral) would be relevant for the strategy we are proposing in MM patients.

Accessibility, culture and isolation of myeloma cells

Unlike some solid tumors, many hematologic tumors are difficult to access due to their systemic distribution. They cannot be targeted for gene therapy *in vivo* without the obvious risk of affecting normal cells. As a result, genetic manipulation of these tumor cells is typically attempted *ex vivo* in enriched cell populations. Although MM is a slow growing tumor (Drewinko et al., 1981), the bone marrow of

many patients contains large numbers of myeloma cells at the time of diagnosis. A typical bone marrow aspirate contains 10^7 to 10^8 white blood cells (WBC) per ml of which between 10% to 90% may be malignant plasma cells, depending on the stage of disease (Brunning et al., 1994). As a result, myeloma cells are potentially accessible for isolation and gene manipulations.

The *in vitro* culture of primary myeloma cells is one of the main challenges in myeloma research. Several groups have attempted to expand myeloma cells in clonogenic colony assays that require complicated feeder cell cultures or conditioned media and monitoring of colony-forming units (Hamburger et al., 1977; Ludwig et al, 1984; Rhodes et al, 1990). The resulting colonies of progenitor myeloma cells are embedded in agar-based medium and hence are useful primarily for morphological analysis and drug-resistance assays. Furthermore, neither of the published methods have been easily reproduced by other laboratories (Pellat-Deceunynk et al., 1995; Barker et al., 1993). The cytokine IL-6, a major myeloma-stimulatory factor, has been used to culture myeloma cells in suspension cultures *in vitro* and to generate cell lines from long-term culture of primary marrow or pleural effusion cells from MM patients (Durie et al., 1985; Westendorf et al., 1996; Ohtsuki et al., 1989; Takahira et al., 1994). The lack of a reliable and consistent method for *in vitro* expansion myeloma cells, has led Pellat-Deceunynk et al. (1995) to emphasize the necessity of myeloma cell lines in MM research.

Despite the variable expression of a number of cell surface markers on myeloma cells from different individuals (Ruiz-Argüelles et al., 1994), moderately-specific plasma cell markers exist and are widely used to identify malignant myeloma cells (Tai et al., 2000; Stewart et al., 1997; Ohtsuki et al, 1989; Takahira et al, 1994; Rhodes et al., 1990). Among these are CD-38, PCA-1 and CD-138 (SYNDECAN). As a result, it has been possible to concentrate myeloma cells from BM aspirates using monoclonal antibodies and "positive" immunomagnetic selection (Borset et al., 1993). Another separation method involving multiple steps including density gradient fragmentation, rosetting and antibody treatments has also been described (Iwato et al., 1988). Both of these methods have drawbacks; they are complicated and involve several steps and the resulting enriched population of cells has antibodies bound to the surface of the cells, making further antibody staining and analysis problematic. On the other hand, an antibody-free enriched MM cell population can also be obtained by "negative" immunomagnetic selection (Tai et al., 2000; Fillola et al., 1996). This approach can yield a high percentage of viable, purified myeloma cells with minimal cell losses (Tai et al., 2000).

Chapter 2

Materials and Methods

Outline

Cells and transfection reagents

Patients and samples

Plasmids and viral vectors

Immuno-magnetic labelling and negative cell separation

Cloning HLA-B7 cDNA into adenoviral transfer plasmid

Plasmid transfections

Recombinant adenovirus vector

Adenovirus transductions

Flow Cytometry

Cell lines and transfection reagents

The cell lines RPMI-8226, U266 (human myeloma), 5637 (human bladder carcinoma), Vero (green monkey kidney) and 293A (human embryonic kidney) were purchased from ATCC (Manassas, VA). RPMI-8226 and U266 cells were maintained in RPMI 1640 culture media supplemented with 15% fetal bovine serum (FBS) and 10% FBS respectively, 2 mM L-glutamine, 100 μg/mL gentamicin, 10mM HEPES buffer and 4.5 g/L D-glucose. 5637 cells were maintained in RPMI 1640 supplemented with 10% FBS, 10mM HEPES buffer and 100 μg/mL gentamicin. Vero cells were maintained in MEM with 5% FBS. 293A cells were maintained in alpha-MEM media supplemented with 5% FBS, 100 μg/mL gentamicin and 10mM HEPES buffer. All cell cultures were incubated at 37°C, 5% CO_2. Transfection reagents Lipofectin, Lipofectamine, CellFectin, DMRIE-C (Gibco Life Sciences, Burlington, ON), Exogen (MBI Fermentas, Burlington, ON), DOTAP (Quantum Biotech Inc., Montreal, QC), Fugene 6 (Boehringer Mannheim, Mannheim, Germany) and Effectene (Qiagen, Mississauga, ON) were provided as test samples by the respective companies.

Patients and samples

Bone marrow (BM) specimen were obtained from diagnostic samples from multiple myeloma patients following informed consent. BM mononuclear cells (BMMC) were isolated by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation of BM aspirate. Cells were washed twice in Hank's buffered saline solution (HBSS, Gibco Life Sciences) and resuspended in either (a) RPMI-1640

(Gibco Life Sciences) supplemented with 10% fetal bovine serum (FBS) and 5-20 μg/ml recombinant IL-6, for cell cultures; or (b) HBSS without Ca++ or Mg++, with 5% FBS for magnetic sorting; or (c) Phospate Buffered Saline (Gibco Life Sciences) with 5% bovine serum albumin, BSA and 0.01% sodium azide for antibody staining and flow cytometric analysis.

Plasmids and viral vectors

Plasmid pRSVB7(k)T40 (Fig.2-1a), containing HLA-B7 cDNA, Rous Sarcoma Virus (RSV) promoter, and the ampicillin resistance gene, was kindly provided by Dr. Gary Nabel, Vector Core Laboratory, University of Michigan Medical Center, Ann Arbor, MI. The bicistronic adenoviral transfer plasmid pQBI-AdCMV5GFP (Fig.2-1b) was purchased from Quantum Biotech Inc. (Montreal, QC). The 1st generation replication-defecient(ΔE1)-adenovirus vector carrying green fluorescence protein (GFP) reporter gene was a gift from Dr. George Karpati, Montreal Neurological Institute, Montreal, QC. The 3rd generation replication-deficient(ΔE1/ΔE3/ΔE4)-adenovirus vector, AdCMV5GFP and the GFP-reporter plasmid pQBI-25, were provided as a test sample by Quantum Biotech Inc.

Immuno-magnetic labelling and negative cell separation

(Fig.2-2)

BMMC were resuspended in HBSS with 5% FBS at a concentration of 5 x 10^7 cells per mL. Cells were stained with a custom antibody cocktail containing monoclonal antibodies specific for human cell surface markers CD14, CD41, CD33, CD2 and

CD45RA (StemCell Tech, Vancouver, BC) followed by a colloid containing magnetic dextran iron particles (StemCell Tech). Cells were passed through a matrix column (StemCell Tech) placed in a magnetic field, under sterile conditions. The flowthrough containing unlabelled enriched cell population was collected, washed and resuspended in either (a) RPMI-1640 (Gibco Life Sciences) supplemented with 10% FBS and 5-20 μg/ml recombinant IL-6, for cell cultures; or (b) PBS with 5% BSA and 0.01% sodium azide, for flow cytometric analysis.

Cloning HLA-B7 cDNA into adenoviral transfer plasmid

(Fig.2-3)

1. Plasmid pRSVB7(k)T40 (Fig. 2-1a) was digested with Hind III and BamHI and the 2.1 Kb cDNA fragment containing the gene HLA-B7 was separated by agarose gel electrophoresis. The piece of agarose gel containing the fragment was cut out and the DNA was extracted using QIAquick Gel Extraction Kit (Qiagen Inc). The isolated 2.1 Kb DNA was treated with DNA Polymerase I, large (Klenow) fragment, to fill-in 5' deoxyribonucleotide overhangs created by restriction digest and DNA was precipitated using Qiaquick Nucleotide Removal Kit (Qiagen Inc).
2. The bicistronic adenoviral transfer plasmid vector, pQBI-AdCMV5GFP (Fig. 2-1b), was linearized by digestion with PmeI and dephosphorylated using calf-intestinal-alkaline-phosphatase (CIAP). Linearization was confirmed by agarose

gel electrophoresis and linear vector plasmid DNA was precipitated from the agarose gel using QIAquick Gel Extraction kit (Qiagen Inc.).

3. The 2.1 Kb insert DNA and linear vector plasmid DNA were ligated at 1:1 molar ratio, using the enzyme T4 DNA Ligase. The ligated DNA was transformed into competent *Escherichia coli* bacteria that were plated and cultured on LB agar with ampicillin. Ten bacterial colonies were picked and individually sub-cultured in liquid LB media containing ampicillin. Plasmid DNA was isolated from each sub-culture using QIAprep Spin Miniprep kit (Qiagen Inc.). Ten miniprep DNA isolates were digested with BglII followed by agarose gel electrophoresis and UV-autoradiography to determine presence and orientation of the 2.1 Kb HLA-B7-encoding insert in the bicistronic adenoviral transfer plasmid.

Plasmid transfections

1. *Transfection of myeloma cell lines with lipid and liposomal reagents*:

   For each transfection, 3 X 10^6 cells of the myeloma cell lines, U266 and RPMI 8226, were each plated in 6-well plates with 3 mL fresh culture media (described earlier). GFP-reporter plasmid pQBI-25 DNA, dissolved in Tris-EDTA buffer, was filter sterilized using SPIN-X microspin tubes (Corning Costar, Cambridge, MA). In separate tests, plated cells were transfected with 0.5, 1.0 or 2.0 μg of sterile pQBI-25 plasmid DNA, using eight lipid-based transfection reagents (Cellfectin, Lipofectamine, Lipofectin, DMRIE-C, DOTAP, Effectene, Exogen and Fugene-6) by following product-specific transfection protocols for each

reagent. Untransfected normal U266 or RPMI-8226 cells and 5637 cells transfected with pQBI-25 were used as negative and positive controls respectively. Following transfection, cells were incubated at 37°C, 5% CO_2. At 24, 48, 72 or 96 hours post-incubation, transfected and control cells were pelleted by centrifugation and resuspended in PBS with 1% paraformaldehyde for flow cytometric analysis of GFP expression. Percentage of cell viability was monitored by trpan blue exclusion and light microscopy.

2. *Transfection of 5637 cells with recombinant adenoviral transfer plasmids*

(Fig.2-4)

DNA from HLA-B7-gene-positive recombinant pQBI-AdCMV5GFP plasmid clones was isolated from liquid LB cell cultures of transformed *E. coli* using Strataprep Midiprep Kit (Stratagene, LaJolla, CA), dissolved in Tris-EDTA buffer, quantified in a UV-spectrophotometer (Becton Dickinson, San Jose, CA) and filter sterilized in SPIN-X microspin tubes (Corning Costar). Adherent 5637 bladder carcinoma cells were plated 24 hours prior to transfection in 100mm tissue culture (TC) plates. Using Fugene-6 transfection reagent and 2μg DNA/1x10^6cells, the plated cells were transfected with each of the HLA-B7 gene-positive plasmid clones, GFP-positive-control plasmid pQBIAdCMV5GFP or HLA-B7-positive-control plasmid pRSVB7(k)T40. Untransfected normal cells were used as the negative control. Following incubation for 48 hours at 37°C and 5% CO_2, control and transfected cells were washed twice with HBSS without Ca++ and Mg++, detached with 0.25% trypsin-EDTA, pelleted by centrifugation

and re-suspended in PBS with 5% bovine serum albumin (BSA) and 0.01% sodium azide, for flow cytometric detection of GFP and HLA-B7 expression. All transfections were repeated 3 times.

Recombinant adenovirus vector

1. *Production and amplification*

(Fig.2-5)

Plasmid DNA from two HLA-B7- and GFP-positive recombinant adenoviral transfer vectors were linearized by restriction digest with enzyme EcoRI, then separated from the enzyme with phenol-chloroform, precipitated with alcohol and resuspended in Tris-EDTA, filter sterilized in SPIN-X microspin tubes (Corning Costar) and quantified by UV-spectrophotometry. 293A cells were plated in 60mm TC plates with culture medium and incubated for 24 hours at 37°C and 5% CO_2. Using calcium-phosphate transfection, cells were co-transfected with 5 μg each of linearized recombinant vector DNA plus adenovirus-5 DNA (QBI-viral-DNA, Quantum Biotech Inc.) or control DNA, QBI-Transf+ plus QBI-carrier-DNA (Quantum Biotech Inc.). Co-transfected cells were incubated for 12-16 hours, washed with 1mM EGTA and PBS, detached with 0.25% trypsin-EDTA, split 1:4 into 60 mm TC plates and incubated for 6-8 hours. Plated cell monolayers were overlayed with 1.25% sterile Seaplaque agarose (Mandel Scientific, Guelph, ON) in culture medium and incubated for 5-21 days for viral plaque formation, with fresh agarose-media being added every 4-5 days. Forty-

two test and six control viral plaques were picked as agarose plugs from vector and control co-transfected plates, respectively, and viral particles from each were eluted into 5 mL alpha-MEM media for 24 hours. For the 1st round of viral amplification, 100μl of each viral eluate was used to infect 1 X 10^5 293A cells in 1mL culture media plated in 24-well TC plates. Infected cells were then incubated for 2-5 days until complete cytopathic effect (CPE) was achieved, after which cultures were frozen (-70°C, 24 hours) and thawed (37°C, 1-3 hours) once to allow release of viral particles from dead cells into the media. Cell debris was removed by centriguation to obtain ~1mL of culture supernantant (SN) containing viral particles. Subsequently, for the 2nd round of viral amplification, 0.5 mL each viral SN (from the 1st round of amplification) was used to infect 5 X 10^6 293A cells in 75cm^2 TC flasks with 10mL culture media, which were then incubated for 2-5 days until complete CPE, frozen, thawed and clarified by centrifugation to obtain 10mL of viral stock for each viral clone.

2. *Upscaling of HLA-B7/GFP positive viral clone*

(Fig.2-6b)

3 X 10^7 293A cells were infected with the viral stock of the HLA-B7/GFP positive recombinant adenoviral clone, at a concentration of 1mL viral stock/10^7 cells in 30mL culture media and 175cm^2 TC flasks. Following 2-5 days of incubation and complete CPE, each flask of infected cells were pelleted and resuspended in 1mL alpha-MEM media. Three freeze-thaw cycles were performed and cell debris was removed by centrifugation to obtain 3 mL of viral stock,

which was then diluted to 150 mL. The diluted viral stock was used to infect 30 X 10^7 293A cells in thirty 175cm^2 TC flasks (5 mL diluted viral stock/10^7 cells/30 mL culture media). After incubation and CPE, 30 mL viral stock was collected, as described above, and viral particles (VP) were purified and concentrated by double centrifugation through discontinous and continous cesium-chloride (CsCl) gradients. The resulting viral prep was titred by measuring OD_{260nm} of 1:5 and 1:10 dilutions of viral prep in virion lysis buffer (0.1% SDS, 10mM Tris-Hcl pH 7.4, 1mM EDTA) incubated for 10 min at 56°C; and multiplying the OD value by the dilution factor and extinction co-efficient (1.1 x 10^{12} virus per OD_{260} value).

Adenovirus transductions

1. *Transduction of myeloma cell lines with 1st generation adenovirus-GFP vector*

   3 x 10^6 cells each, in 1 mL culture medium, of 293A, U266 and RPMI-8226 cell lines were transduced with the 1st generation replication-deficient adenovirus, at a multiplicity of infection (MOI) of 1, 10, 100 or 200 viral particles (VP). Cells were incubated at 37°C and 5% CO_2, for 2 hours, after which cells were cultured in complete media (1 x 10^6/mL) for 24 hours. In parallel experiments, U266 and RPMI-8226 cells were starved (i.e. cultured in media devoid of FBS, glucose and L-glutamine) for either 24, 48, 72 or 96 hours prior to transduction with 1st generation adenovirus (MOI=200) and cultured in starvation media for 24 hours. Cell viability after starvation cells was assessed by trypan blue exclusion and light microscopy . For both sets of experiments, untransduced, normal U266 and

RPMI-8226 cells were used as negative controls and 293A cells transduced for 24 hours, with the same adenovirus (MOI = 1, 10 or 100 VP), were used as positive controls. GFP expression in all transduced and control cells was analysed by flow cytometry.

2. *Transduction of myeloma cell lines with 3rd generation adenovirus-GFP vector*

   2 x 10^6 cell each of U266 and RPMI-8226 were transduced with the 3rd generation adenovirus vector, AdCMV5-GFP, in 0.5 mL culture media, at MOI of 1, 10 or 100 plaque forming units (pfu)/mL, for 2 hours, at 37°C and 5% CO_2. Cells were then incubated in complete media for 24 or 48 hours, after which cell viabilty was measured by trypan blue exclusion and light microscopy, and GFP-expression was determined by flow cytometry. Transductions were repeated two times.

3. *Screening of recombinant adenoviral clones in 5637 cells*

   (Fig.2-6a)

   1 x 10^6 5637 cells, plated 24 hours in advance in 35mm TC plates, were transduced with 1 mL each of 42 recombinant-vector- and 6 control- viral stocks (from 2nd round of amplification in 293A cells, as described above), at an estimated MOI of 500 VP [titre of viral stocks as estimated by Adeasy recombinant-adenoviral-vector-production protocol (Quantum Biotech Inc.)]. Transduced cells were incubated at 37°C and 5% CO_2 for 24 hours following which 1 mL of complete medium was added to each plate and cells were incubated for an additional 12 hours. 5637 cells transfected with plasmid

pRSVB7(k)T40; recombinant HLA-B7/GFP-gene-positive plasmid clones; and 5637 cells transduced with adenoviral-GFP vector AdCMV5-GFP, were used as positive HLA-B7-, HLA-B7 plus GFP and GFP- expression controls. Untransduced normal 5637 cells were used as negative control. Viral-clones-transduced- and control- cells were then detached by 0.25% trypsin-EDTA, pelleted by centrifugation and re-suspended in PBS with 5% BSA and 0.01% sodium azide, for antibody staining and flow cytometric detection of GFP and HLA-B7 expression by the recombinant viral clones. Screening of clones was repeated two times.

4. *Transductions with HLA-B7/GFP-positive recombinant adenovirus clone* (Fig.2-6c)

3×10^6 each of U266, RPMI-8226 and Vero cells were transduced with either (a) the double CsCl-gradient-purified viral preps of the HLA-B7/GFP-positive recombinant adenovirus clone, at MOIs of 10^3, 10^4 or 10^5 VP (titre determined by spectrophotometry); and/or (b) viral stock (from 2^{nd} round of amplification in 293A cells) of HLA-B7/GFP-positive recombinant adenovirus clone, at an estimated MOI of 500 or 1000 VP [titre estimated by AdEasy recombinant-adenoviral-vector-production protocol (Quantum Biotech Inc.)]. Transduced cells were incubated for 2 hours, followed by 24 hours with complete media, at 37°C and 5% CO_2. Cells transduced with the AdCMV5-GFP vector were used as positive controls. Untransduced normal cells were used as the negative control. Vero cells were detached with 0.25% trypsin-EDTA and all cells were pelleted by

centrifugation and re-suspended in PBS with 5% BSA and 0.01% sodium azide, for antibody staining and flow cytometric detection of GFP and HLA-B7 expression.

Flow cytometry

Flow cytometric analysis and fluorescence detection in all experiments was performed using a FACScan Cytometer (Becton Dickinson).

1. *MM patient BMMC and myeloma cell enrichments :*

   Cells from BMMC and immuno-magenetic, negatively-selected flowthrough were stained with either (a) mouse anti-human primary antibody specific for PCA-1 (Coulter Inc, Fullerton, CA), washed with PBS 0.01% sodium azide, followed by FITC-conjugated goat anti-mouse secondary antibody, washed and finally PE-conjugated mouse anti-human CD38; or (b) FITC-conjugated mouse anti-human CD4 and PE-conjugated mouse anti-human CD8; or (c) FITC-conjugated mouse anti-human CD54 and PE-conjugated mouse anti-human CD14; or (d) controls--FITC-conjugated anti-mouse IgG1 and PE-conjugated anti-mouse IgG2a. Antibody staining was performed for 30 mins at 4°C, in the dark, after which cells were washed with PBS 0.01% sodium azide and pelleted. All cell preparations were then fixed in 150 µl PBS 1% paraformaldehyde. BMMC were gated to include the lymphoid cell population characterized by low side-scatter (granularity) and medium forward scatter (size) and to exclude monocytes and

other cell debris characterized by relatively higher forward- and side-scatter profiles; The enriched myeloma cells were gated to include the single, homogeneous concentration of lymphoid cells and to exclude all others.

2. Lipid-pQBI25GFP-transfected and 1st and 3rd generation adenovirus-GFP-transduced myeloma cell lines and control cells were washed with RPMI 1640 media, pelleted by centrifugation and fixed in 150 µl PBS 1% paraformaldehyde. Cells displaying a consistent pattern of medium forward- and side-scatter were gated.

3. (a) 5637 cells transfected with HLA-B7/GFP gene-positive recombinant plasmid clones; (b) 5637 cells transduced with viral stocks from 48 recombinant adenovirus clones; and (c) U266, RPMI-8226 and Vero cells transduced with viral stock and viral prep of the HLA-B7/GFP-positive recombinant adenoviral vector; and (d) all control, untransduced cells were stained with PE-conjugated mouse anti-human HLA-B7 antibody (Chemicon Intl., Temecula, CA) for 30 mins at 4°C, in the dark, after which the cells were washed with PBS 0.01% sodium azide, pelleted and fixed in 150 µl PBS 1% paraformaldehyde. For 5637 cells, the single homogeneous population of cells displaying medium forward- and side-scatter profiles were gated. U266, RPMI-8226 and Vero cells were gated to include the viable cells displaying medium forward-scatter and low side-scatter and to exclude dead cells and debris characterized by extreme (low or high) forward- or side-scatter profiles.

Fig. 2-1 :

Plasmid restriction maps of

a) HLA-B7-plasmid, pRSVB7(k)T40 *(Gary Nabel, Vector Core Lab., Univ. of Michigan)*

b) Adenoviral transfer plasmid, pQBI-AdCMV5GFP *(Quantum Biotech Inc.)*

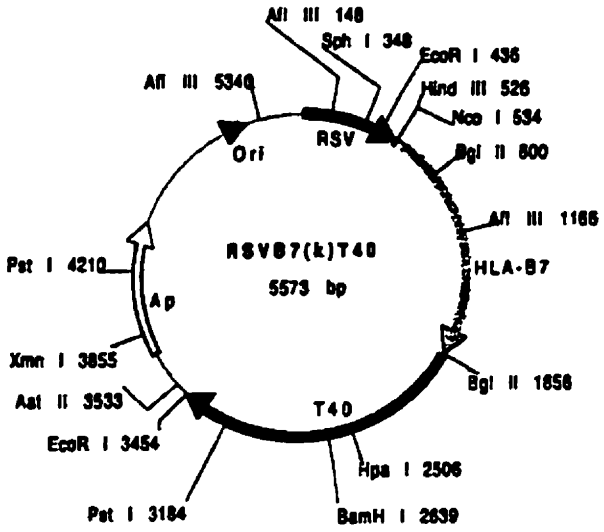


Fig. 2-1 (a)

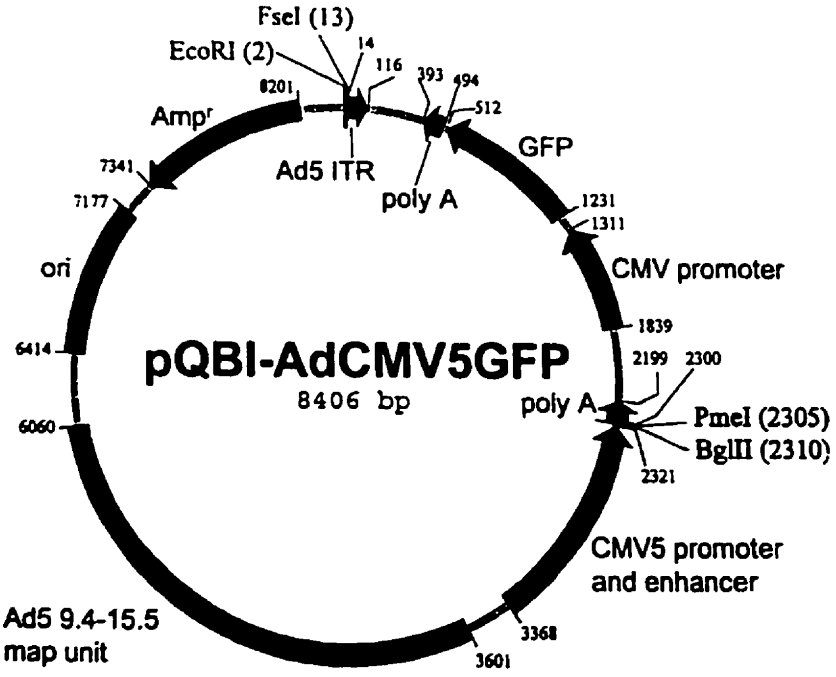


Fig. 2-1 (b)

Fig. 2-2 :

Diagram showing the steps involved in the purification of primary myeloma cells from BM aspirates using immuno-magnetic negative selection

Fig. 2-3 :

Schematic representation of cloning the HLA-B7 gene into the adenoviral transfer plasmid

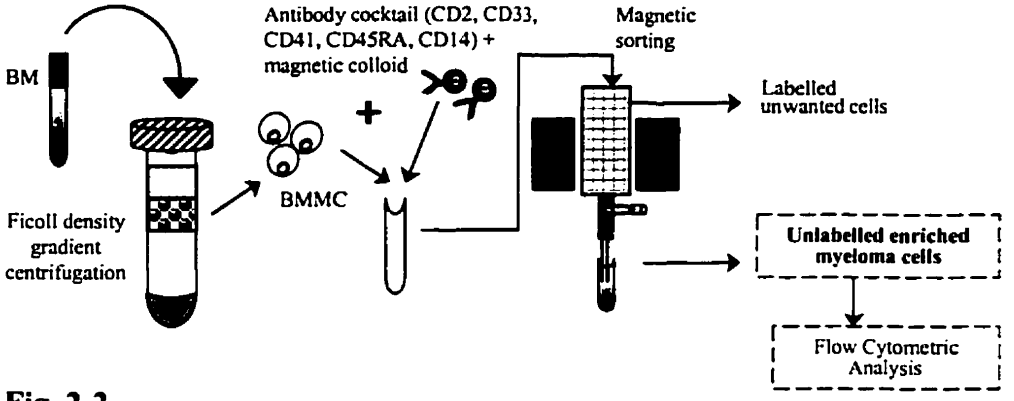

BM
Ficoll density gradient centrifugation
Antibody cocktail (CD2, CD33, CD41, CD45RA, CD14) + magnetic colloid
BMMC
Magnetic sorting
Labelled unwanted cells
Unlabelled enriched myeloma cells
Flow Cytometric Analysis

Fig. 2-2

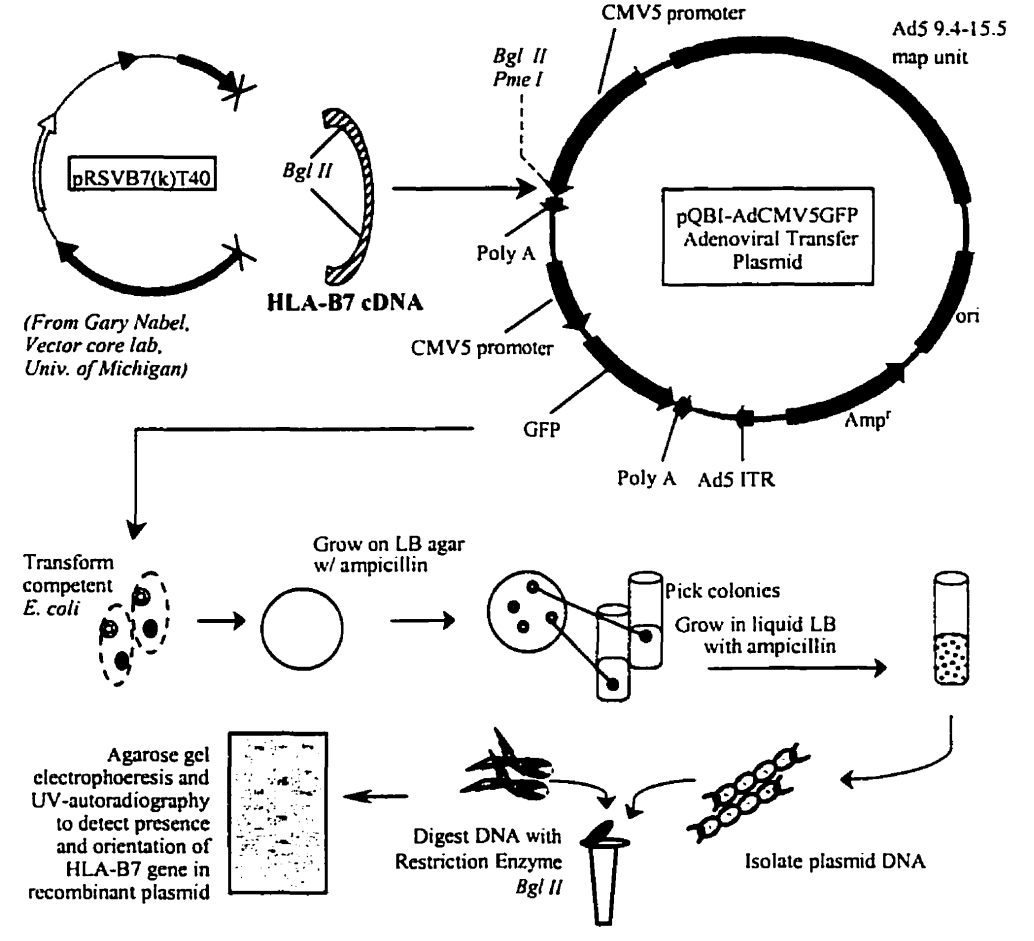

pRSVB7(k)T40
Bgl II
HLA-B7 cDNA
(From Gary Nabel, Vector core lab, Univ. of Michigan)
CMV5 promoter
Bgl II
Pme I
Ad5 9.4-15.5 map unit
Poly A
pQBI-AdCMV5GFP Adenoviral Transfer Plasmid
CMV5 promoter
ori
GFP
Ampr
Poly A
Ad5 ITR
Transform competent E. coli
Grow on LB agar w/ ampicillin
Pick colonies
Grow in liquid LB with ampicillin
Agarose gel electrophoeresis and UV-autoradiography to detect presence and orientation of HLA-B7 gene in recombinant plasmid
Digest DNA with Restriction Enzyme Bgl II
Isolate plasmid DNA

Fig. 2-3

Fig. 2-4 :

Diagramatic representation of studying HLA-B7 and GFP expression by recombinant adenoviral transfer plasmid clone(s) in 5637 cells

Fig. 2-5 :

Schematic diagram showing the steps involved in the production of a replication-defecient recombinant adenovirus

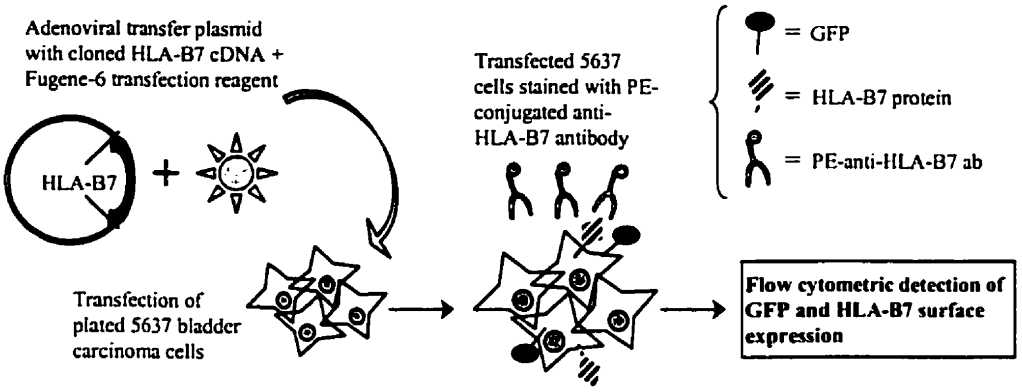

Adenoviral transfer plasmid with cloned HLA-B7 cDNA + Fugene-6 transfection reagent
HLA-B7
Transfection of plated 5637 bladder carcinoma cells
Transfected 5637 cells stained with PE-conjugated anti-HLA-B7 antibody
= GFP
= HLA-B7 protein
= PE-anti-HLA-B7 ab
Flow cytometric detection of GFP and HLA-B7 surface expression

Fig. 2-4

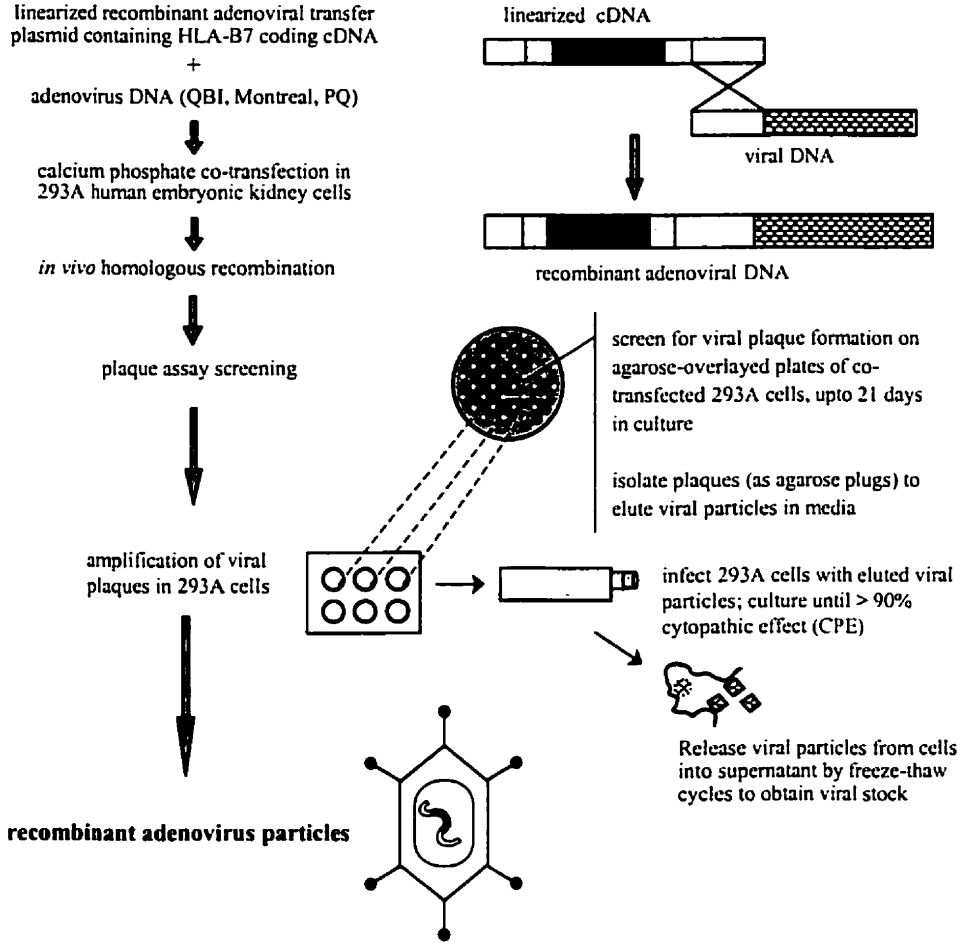

linearized recombinant adenoviral transfer plasmid containing HLA-B7 coding cDNA
+
adenovirus DNA (QBI, Montreal, PQ)
calcium phosphate co-transfection in 293A human embryonic kidney cells
in vivo homologous recombination
plaque assay screening
amplification of viral plaques in 293A cells
recombinant adenovirus particles
linearized cDNA
viral DNA
recombinant adenoviral DNA
screen for viral plaque formation on agarose-overlayed plates of co-transfected 293A cells, upto 21 days in culture
isolate plaques (as agarose plugs) to elute viral particles in media
infect 293A cells with eluted viral particles; culture until > 90% cytopathic effect (CPE)
Release viral particles from cells into supernatant by freeze-thaw cycles to obtain viral stock

Fig. 2-5

Fig. 2-6 :

Schematic representation of –

a) Screening of recombinant viral isolates for HLA-B7- ad GFP-positive clones in 5637 cells

b) Upscaling of positive adenoviral vector

c) Testing efficiency of transduction and protein expression of positive vector in myeloma and non-myeloma cells

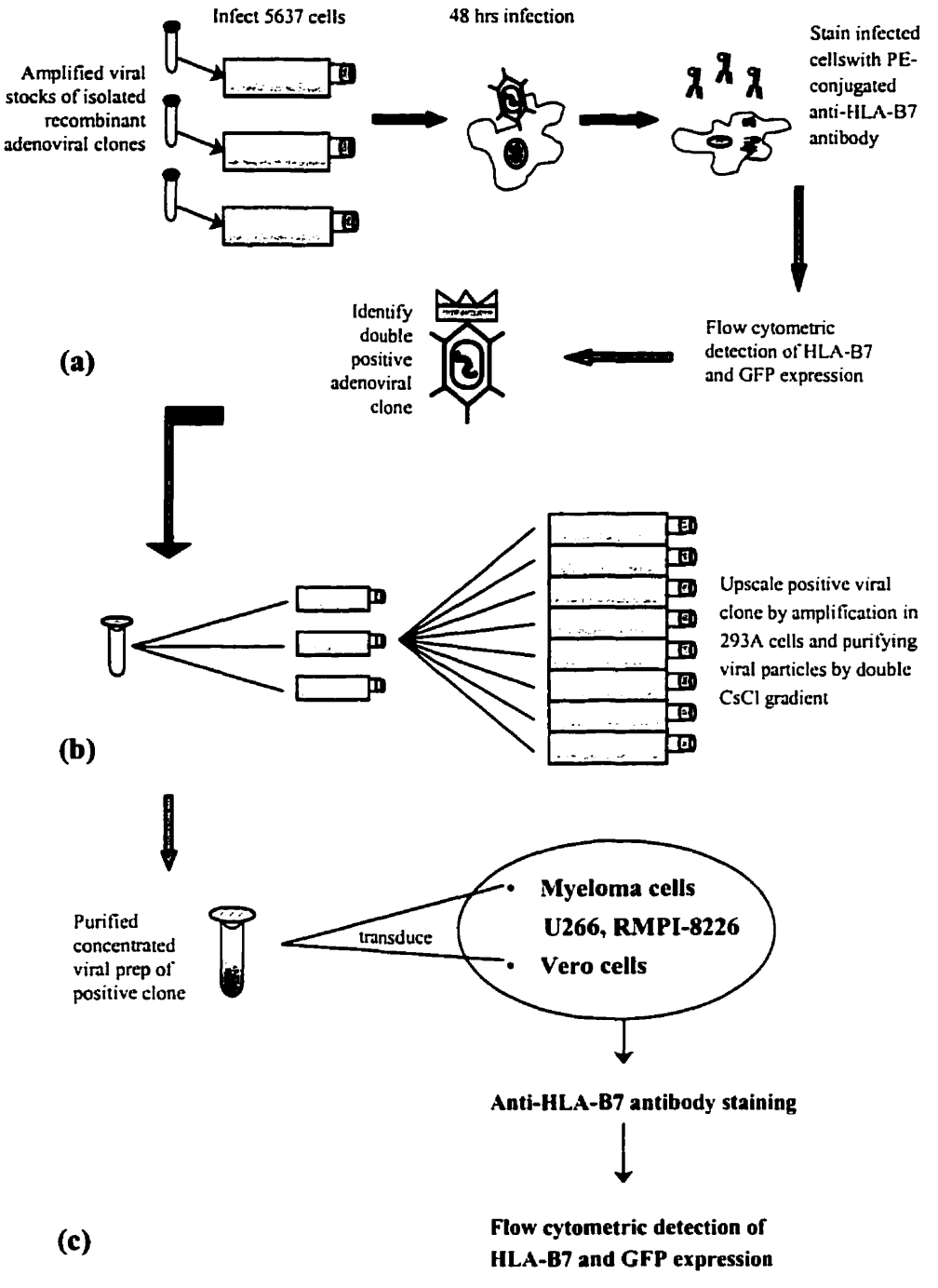

Infect 5637 cells
48 hrs infection
Amplified viral stocks of isolated recombinant adenoviral clones
Stain infected cellswith PE-conjugated anti-HLA-B7 antibody
Identify double positive adenoviral clone
Flow cytometric detection of HLA-B7 and GFP expression
(a)
Upscale positive viral clone by amplification in 293A cells and purifying viral particles by double CsCl gradient
(b)
Purified concentrated viral prep of positive clone
transduce
Myeloma cells
U266, RMPI-8226
Vero cells
Anti-HLA-B7 antibody staining
Flow cytometric detection of HLA-B7 and GFP expression
(c)

Fig. 2-6

Chapter 3

Results

Purification and culture of primary myeloma cells

We purified myeloma cells from BM samples of four patients (ID#'s MM3, MM5, MM6, MM8) using immunomagnetic negative selection. Myeloma cells were identified in the BMMC and the enriched cell population by the surface expression of plasma cell markers CD38+ and/or PCA-1+. Figure 3-1 shows cell-surface-marker analysis of bone marrow mononuclear cells (BMMC) from Patient-I.D# MM6 by flow cytometry. There was a diverse population of cells before enrichment with 10% expressing CD4+, 22% CD8+ (Figure 3-1, Panel-a); 60% with CD54+, 39% CD14+ (Figure 3-1, Panel-b); and finally 57% with CD38+ and/or PCA-1+ (Figure-3-1, Panel-c). Following purification, 95% of cells were CD38+ and/or PCA-1+ myeloma cells (Figure 3-1, Panel-e) while the remainder expressed CD4, CD8, CD54 or CD14 (1%, 2.5%, 3% and 23% respectively; Figure 3-1, Panel-d; data for CD54 and CD14 not shown). Table 3-2 summarizes the initial and final purity (% of CD38+ and/or PCA-1+ cells) from all four patient BM samples. The initial percentage of myeloma cells in the four BMNC (MM3, MM5, MM6, and MM8) populations was 14%, 67%, 57% and 85%, respectively. High level of purification was obtained from two other samples, MM5 and MM8, in which 99% of the cells in the enriched flowthrough were CD38+ and/or PCA-1+ myeloma cells and less than 1% of cells had either CD4+ or CD8+ (not shown). In contrast, for the first sample, MM3, only 51% of cells following enrichment were myeloma cells. Therefore, in three out of four BM samples that showed high initial numbers of myeloma cells (i.e. >50%), we were able to obtain >95% purification by negative selection (Table 3-2). The yield (% of

myeloma cells recovered after magnetic sorting) for all four samples is also summarized in Table 3-2. In three out of four samples, we were able to recover at least 65% of the myeloma cells present in the initial BMNC population. The highest yield obtained was 75% from two samples, MM3 and MM6. The yield of myeloma cells was considerably higher in those samples that had an initial BMNC population of greater than 10×10^6 cells (shown in Table 3-1).

To study the *in vitro* proliferation of myeloma cells, MNCs from 2 patients (MM1, MM2) and myeloma cell enrichments from 4 patient samples (MM3, MM5, MM6, MM8) were cultured in the presence of 5-20 ng/mL IL-6. Table 3-1 shows the initial volume and number of MNCs obtained from each sample, the maximum number of days in culture and the proportion of myeloma cells in each sample. There was considerable variability in the initial percentage of myeloma cells and the duration of culture for the total MNC and the myeloma cell enrichments, in all six pateint samples. MNCs from sample MM1 were able to survive in culture for a maximum of 22 days. During this period, the number of cells dropped by 100-fold. Similar observations were made for the other patient samples, none of which survived for more than 10 days in culture and all of which lost significant numbers of cells (50- to 100-fold) during this time. These results show that primary myeloma cells, in either the total MNC population or a purified enrichment, are difficult to maintain, let alone expand, i*n vitro* for longer than 1-3 weeks, even in the presence of the myeloma-cell growth factor, IL-6. However, it is interesting to note that the culture with total

MNCs and higher initial myeloma-cell content survived longer in culture than the enriched myeloma cell preparations.

Transfection of myeloma cell lines using chemical transfection reagents

We attempted to introduce plasmid the pQBI25, carrying the GFP reporter gene, using six lipid-based transfection reagents into two myeloma cell lines, RPMI-8226 and U266. Figure 3-2, Panel-a shows the population of U266 cells that was gated for analysis; GFP-expression in control, Fugene6, Cellfectin, Lipofectamine, Lipofectin, DMRIE-C transfected U266 cells are shown in Figure 3-2, Panels-b, c, d, e, f and g respectively. No GFP-expression was noted in the transfected U266 cells 24 hours following transfection whereas our positive control, pQBI+Fugene6 transfected 5637 bladder carcinoma cells showed >95% GFP expression (Figure 3-2, Panel-h). Similar results were obtained for the myeloma cell line RPMI-8226. The transfections were repeated two times for each reagent and, each time, no GFP-expression was detected at 24, 48, 72 or 96 hours post-transfection, in U266 or RPMI-8226 cell lines. These results clearly indicate that chemical transfection reagents are ineffective at transferring foreign DNA into myeloma cells.

Transduction of myeloma cell lines using 1st generation adenovirus

We transduced RPMI-8226 and U266 cells with ΔE1-adenovirus encoding a GFP reporter at an MOI of 100 and 200 VP. No GFP expression was detected at 24, 48 and

72 hours post-transduction in either cell line. Transductions were performed three times and similar poor results were obtained each time (data not shown). In 293A cells transduced with the ΔE1-adenovirus, GFP-expression was observed to be 34% at MOI of 10 VP, and 92% at MOI of 100 VP, 24 hours post-transduction (data not shown). Based on these observations we then tested the effect of starvation (e.g. no FBS, glucose or L-glutamine) on the transfection efficiency of ΔE1 adenovirus-GFP at an MOI of 200 VP, in RPMI-8226 and U266 cells. Cells were starved for 24, 48, 72 or 96 hours prior to transduction with the vector. Prior to flow cytometry, the viability, at 24, 48, 72 and 96 hours of starvation, of RPMI-8226 cells was observed to be 78%, 76%, 60% and 40% and that of U266 cells to be 95%, 90%, 84% and 82%, respectively. In the transduced U266 cells, GFP-expression was observed to be 40%, 62%, 71% and 73% at 24 hr, 48 hr, 72 hr and 96 hr starvation (Figure 3-3, Panel c, d, e and f) respectively, as opposed to 0.3% (Figure 3-3, Panel-b) in the gated cell population (Figure 3-3, Panel-a) with no transduction. GFP-expression in transduced RPMI-8226 cells ranged from 5% at 24 hours of starvation to 9% at 96 hours of starvation (not shown). These experiments were repeated once and similar results were obtained. These results suggest that starvation of cells may be an effective method to improve the transduction efficiency when using a first generation adenovirus vector. However, the negative effects of starvation on cell viability, make this approach unsuitable for primary myeloma cells, which, as our previous results show, grow poorly in culture even with the addition of recombinant IL-6.

Transduction of myeloma cell lines using 3rd generation adenovirus

To test the efficacy of the improved 3rd generation, RPMI-8226 and U266 cells were transduced with AdCMV5-GFP, at MOIs of 1, 10 and 100 pfu for 24 or 48 hours. GFP-expression was detected at 24 hr in 80%, 90% and 94% of U266 cells (Figure 3-4, Panels-a, b and c) and in 73%, 85% and 88% of RPMI-8226 cells (Figure 3-4, Panels-c, d and f), at MOIs of 1,10 and 100, respectively. GFP-expression in both cell lines was sustained for up to 48 hours post-transduction. The experiments were repeated two times and similar high levels of GFP-expression were observed in both cell lines. These results strongly support the use of a 3rd generation adenovirus to obtain high level expression of a foreign gene in myeloma cell lines and its potential applications in gene-delivery to primary myeloma cells.

HLA-B7 and GFP protein expression of recombinant plasmids in 5637 bladder carcinoma cells

Having shown that the 3rd generation adenovirus is an efficient transduction vector for myeloma cell lines, we cloned the gene encoding the allogene, HLA-B7, into the adenoviral transfer plasmid, pQBI-AdCMV5GFP. Following cloning and screening, two plasmids, designated #9 and #10, were identified as HLA-B7-gene-positive. Figure 3-5, panel-b shows the restriction enzyme BglII digest, gel-electrophoresis and UV-autoradiograph of ten isolated recombinant plasmid clones. Based on the resulting 274-bp DNA fragment in the recombinant plasmid (illustrated in Figure 3-5, panel-a), we clearly identified clones #9 and #10 as positive. The two HLA-B7/GFP-gene-positive plasmids were then used to transfect 5637 bladder carcinoma cells to

test for surface expression of the HLA-B7 protein. By 24 hours post transfection, 35% of cells transfected with plasmid #9 and 45% of cells transfected with plasmid #10 were positive for HLA-B7/GFP expression (Figure 3-6, Panels-e and f respectively). Figure 3-6, Panels-a and -b show the gated cell population and GFP/HLA-B7 expression by control normal 5637 cells stained with anti-HLA-B7 antibody, respectively; Panel-c shows GFP expression by positive-control plasmid-pQBI-AdCMV5GFP, and Panel-d shows HLA-B7 expression by positive-control plasmid pRSVB7(k)T40. All plasmid transfections were repeated three times and similar levels of HLA-B7 and GFP protein expression were seen each time. These results indicate that the two HLA-B7 and GFP positive adenoviral transfer plasmids produced suitable surface levels of proteins for the production of a recombinant adenoviral vector.

Confirmation of HLA-B7 and GFP expression by recombinant adenovirus clone in 5637 bladder carcinoma cells

Based on previous results, plasmid DNA of clones #9 and #10 was co-transfected in 293A cells, with adenovirus5-DNA, to produce recombinant viral particles. Forty-two vector and six control adenoviral clones were isolated from individual viral plaques. Typically, the next step is amplification and protein-expression analysis of the isolated viral clones in 293A cells. We encountered significant difficulties at this stage that were subsequently resolved with the demonstration that 293A cells are (by serendipity) HLA-B7 positive (confirmed by PCR analysis and positive antibody reactivity of HLA-B7, data not shown). Therefore, 5637 cells were transduced with

viral stocks of the isolated and amplified 42 vector- and 6 control- viral clones, to detect HLA-B7 and GFP expression. One positive recombinant viral clone, designated Ad#8, was identified and confirmed as HLA-B7 and GFP positive. Figure 3-7, Panel-a shows the negative control for protein expression, untransduced 5637 cells with anti-HLA-B7 antibody; Panel-b shows 5637 cells transduced with a GFP-positive control virus, AdCMV5-GFP, with 98% expressing GFP; Panel-c shows 5637 cells transfected with a HLA-B7 positive control, plasmid pRSVB7(k)T40, with 10% expressing HLA-B7; Panel-d shows 41% HLA-B7 and GFP expression on Ad#8-transduced 5637 cells. These experiments were repeated three times and each time we observed similar levels of HLA-B7 and GFP expression. The results of this section show that the isolated viral clone, Ad#8, expresses both proteins of interest at significant levels and is a potential vector for allo-gene delivery in myeloma cells.

Transduction of myeloma cell lines with recombinant adenovirus Ad#8

We amplified the HLA-B7/GFP-positive adenovirus, Ad#8, in 293A cells and purified viral particles. The viral titre of the double CsCl-purified prep was measured as 1.5×10^{12} VP/mL. Viral stock containing viral particles and the double CsCl-purified viral prep were used to transfect RPMI-8226, U266 and Vero green monkey kidney cell lines. Figure 3-8, Panel-a shows the gated, viable RPMI-8226 population; Panel-b shows the negative control for protein expression, untransduced RPMI-8226 cells stained with anti-HLA-B7 antibody; Panel-c shows that 13% of RPMI-8226 cells transduced with CsCl-purified viral prep at MOI of 10^4 VP, after 24 hours,

expressed HLA-B7 and GFP; Panels-d and e show that 6% of RPMI-8226 cells transduced with Ad#8-viral stock at estimated MOI of 500 VP and 12% at estimated MOI of 1000 VP, respectively, expressed HLA-B7 and GFP. Cell line U266 transduced with Ad#8-viral stock at an estimated MOI of 500 VP showed 6% positive GFP expression (Figure 3-9, panel-d). However, the expression of HLA-B7 by Ad#8 viral clone could not be confirmed since the control U266 cells were (by coincidence) HLA-B7-positive (Figure 4-9, panel-b). In addition, HLA-B7 and GFP expression by Vero cells transduced with the CsCl-viral prep was observed to be 10% at an MOI of 10^3 and 19% at an MOI of 10^4 (Figure 3-9, panels-h and i, respectively). These results demonstrate that despite low levels of protein expression, a recombinant adenovirus expressing HLA-B7 and GFP can be used to transduce myeloma cell lines. The data also shows that our recombinant viral vector preparation has significant infectivity, as seen in the higher levels of protein expression in Vero cells. Up to 13% of recombinant-vector transduced RPMI-8226 cells were positive for protein expression, implying that this approach has the potential to be effective in generating an allogene-modified myeloma cell population.

Fig. 3-1:

Cell-surface marker analysis, by flow cytometry, of primary BMMC and myeloma cell enrichment by immunomagnetic negative selection from patient MM6:

(a) CD4 and CD8 expression in BMMC;
(b) CD54 and CD14 expression in BMMC;
(c) CD38 and PCA-1 expression in BMMC;
(d) CD4 and CD8 expression in myeloma cell enrichment
(e) CD38 and PCA-1 expression in myeloma cell enrichment

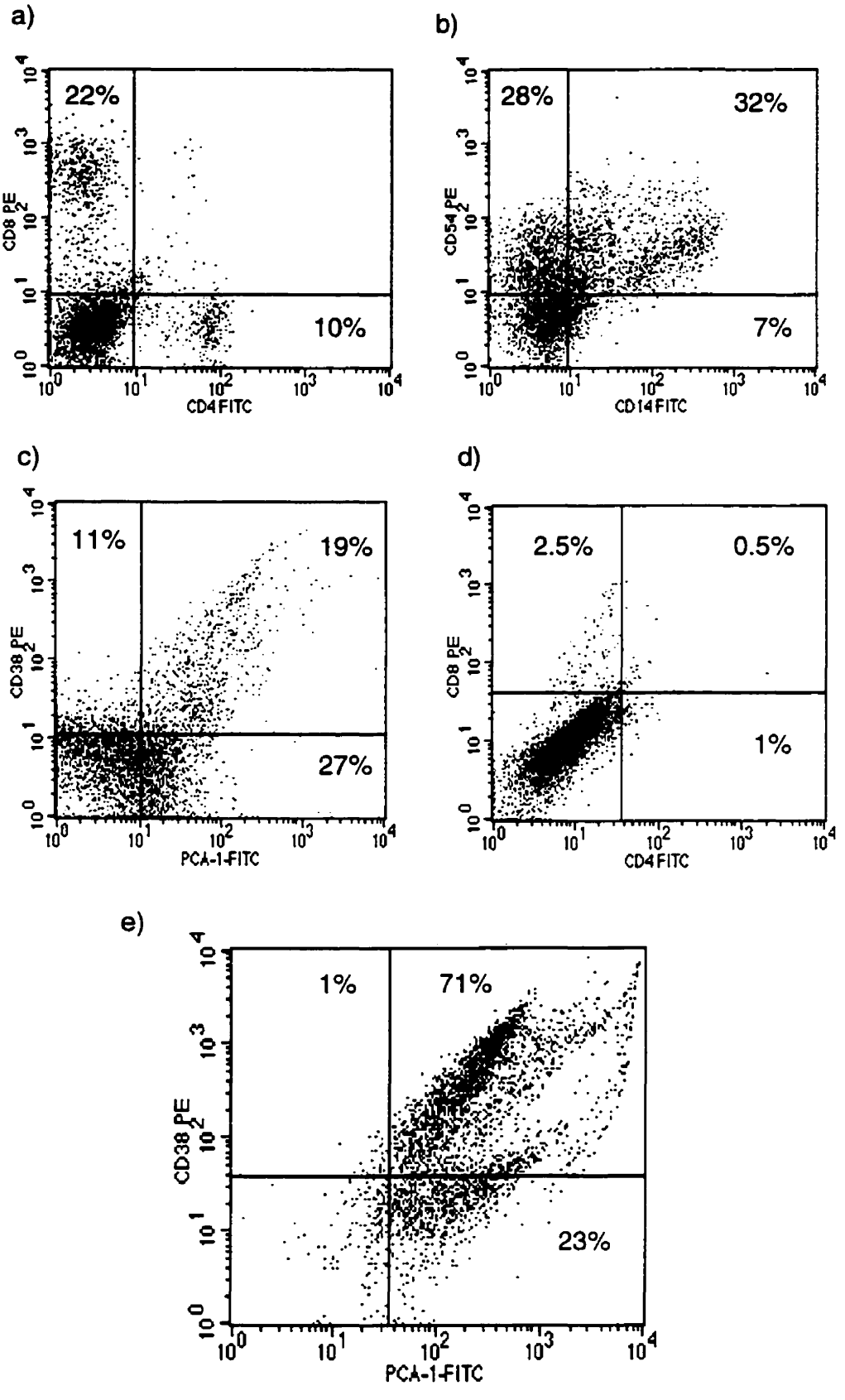

a)
22%
10%
CD8 PE
CD4 FITC
b)
28%
32%
7%
CD54 PE
CD14 FITC
c)
11%
19%
27%
CD38 PE
PCA-1-FITC
d)
2.5%
0.5%
1%
CD8 PE
CD4 FITC
e)
1%
71%
23%
CD38 PE
PCA-1-FITC

Table 3-1 :

Summary of BM samples, culture data and myeloma cell percentages from seven MM patients

Table 3-2 :

Summary of purity and yield of myeloma cell enrichments from four BM samples following immuno-magnetic negative cell separation

Patient Sample ID #	Initial Sample Volume	Initial Sample # of MNC	% of myeloma cells in sample	Negative cell separation performed	Max. # of days in culture w/IL-6
MM 1	5 mL	2×10^7	85 %	No	22*
MM 2	5 mL	7×10^6	22%	No	8*
MM 3	6 mL	10×10^7	14%	Yes	8**
MM 5	4 mL	10×10^6	67%	Yes	10**
MM 6	6 mL	6×10^7	57%	Yes	8**
MM 7	3 mL	2×10^6	--	No	--
MM 8	5 mL	2.5×10^7	85%	Yes	6**

Sample ID#	Initial Purity	Final Purity	Yield
MM 3	14%	51%	75%
MM 5	67%	**99%**	32%
MM 6	57%	**95%**	75%
MM 8	85%	**99%**	65%

Purity = % of CD38+ and/or PCA-1+ cells

Yield = % of myeloma cells recovered by separation

* = All MNC cultured

** = myeloma cell enrichment cultured

Fig. 3-2 :

Transfection efficiency of 5 lipid-based transfection reagents in U266 cells using GFP-reporter plasmid pQBI-25

(a) gated cell population;
(b) negative control, untransfected;
(c) Fugene-6;
(d) Cellfectin;
(e) Lipofectamine;
(f) Lipofectin;
(g) DMRIE-C;
(h) positive control, 5637 cells transfected with transfected with Fugene6 and plasmid pQBI-25

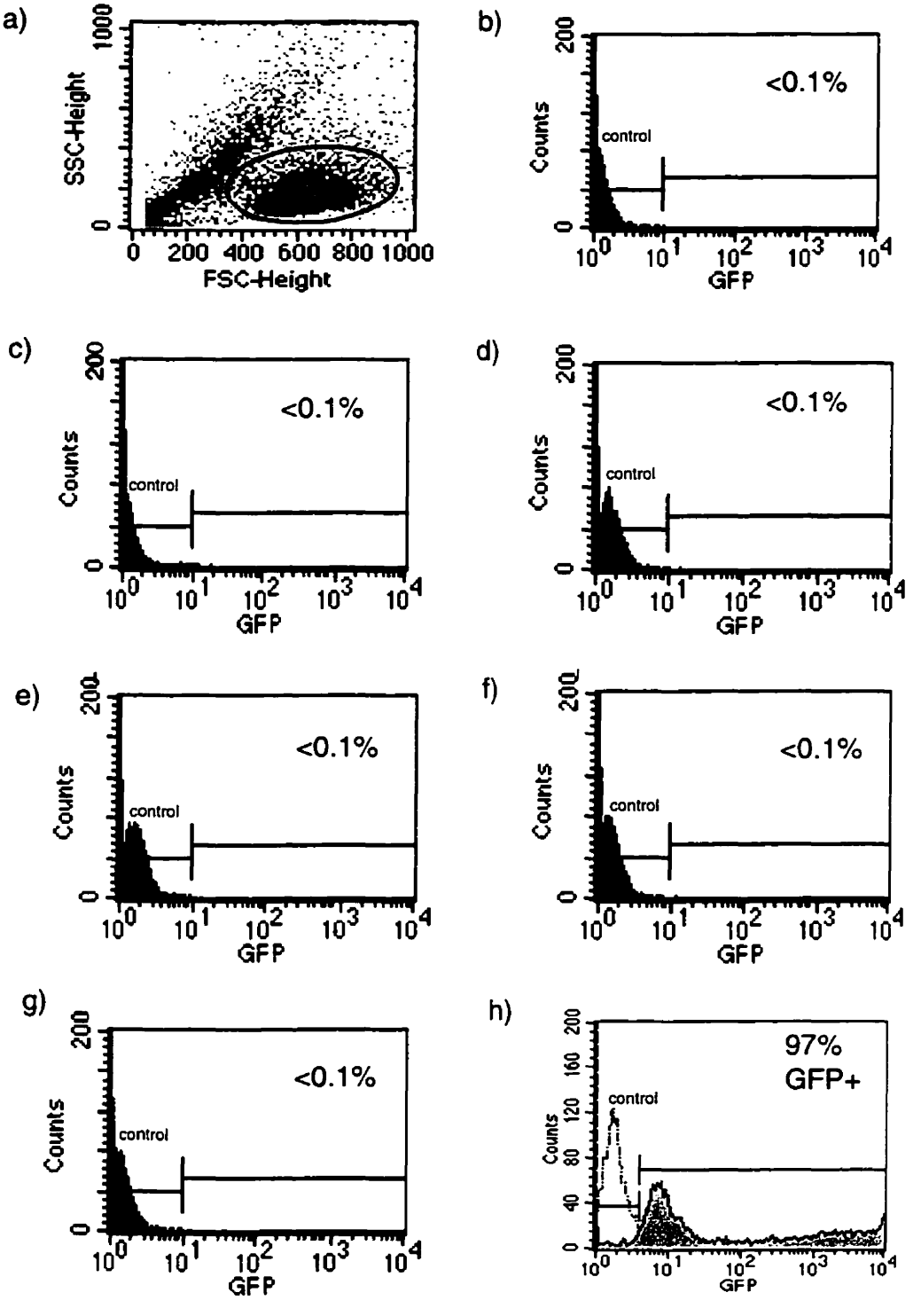

a)
SSC-Height
FSC-Height
0
200
400
600
800
1000
b)
<0.1%
control
Counts
GFP
c)
<0.1%
control
Counts
GFP
d)
<0.1%
control
Counts
GFP
e)
<0.1%
control
Counts
GFP
f)
<0.1%
control
Counts
GFP
g)
<0.1%
control
Counts
GFP
h)
97%
GFP+
control
Counts
GFP

Fig. 3-3:

Effect of starvation on transduction efficiency of 1st generation adenovirus vector expressing GFP in U266 cells

(a) gated cell population;
(b) control untransduced U266 cells;

GFP-expression in adenovirus-transduced U266 cells cultured in starvation media for
(c) 24 hours,
(d) 48 hours,
(e) 72 hours and
(f) 96 hours

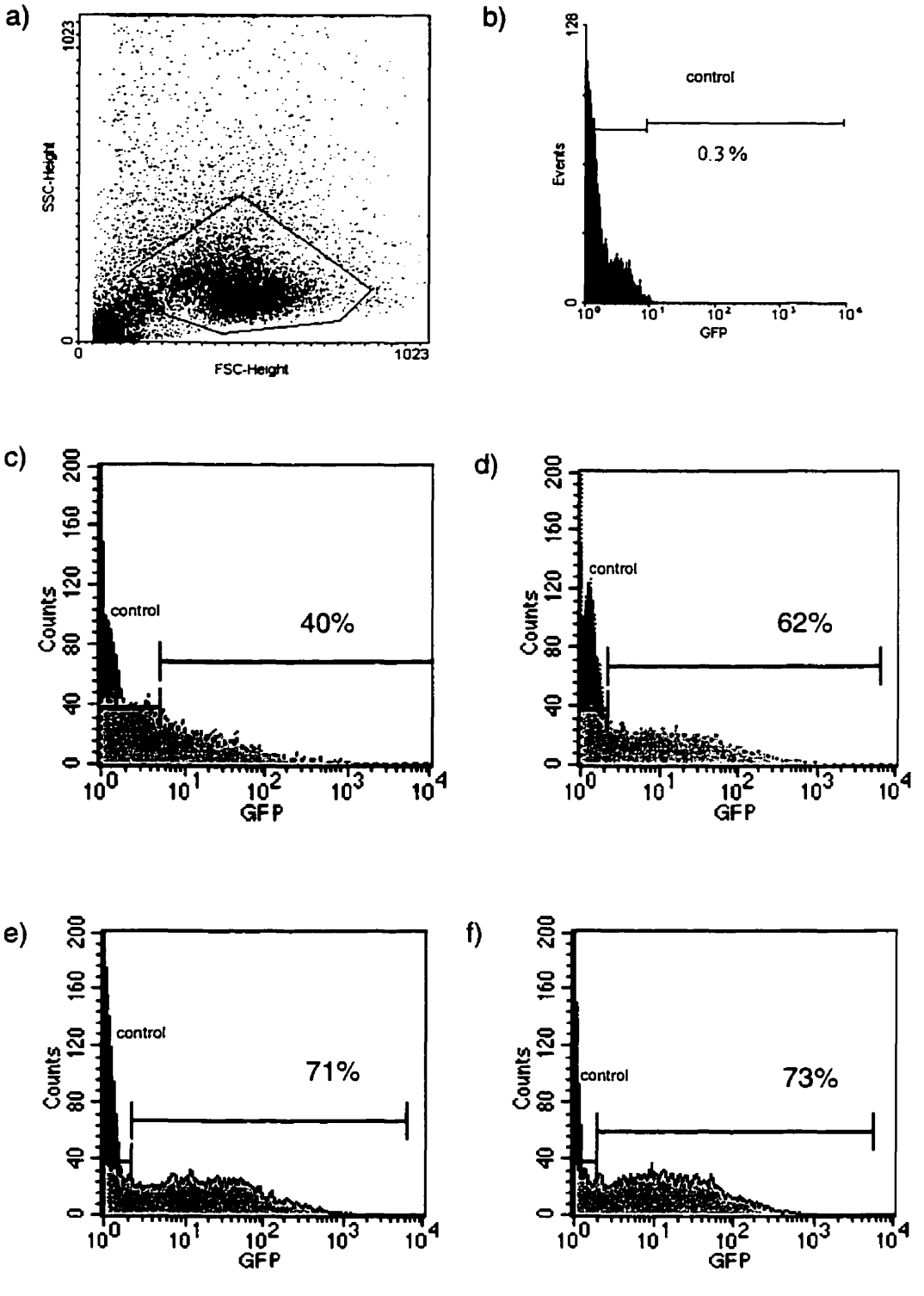

a)
SSC-Height
FSC-Height
0
1023
b)
control
0.3%
Events
GFP
c)
control
40%
Counts
GFP
d)
control
62%
Counts
GFP
e)
control
71%
Counts
GFP
f)
control
73%
Counts
GFP

Fig. 3-4 :

Transduction efficiency of 3rd generation adenovirus-GFP vector in U266 cells transduced at MOI of 1-(a), 10-(b) and 100-(c) PFU; and RPMI-8226 cells transduced at MOI of 1-(d), 10-(e) and 100-(f) PFU

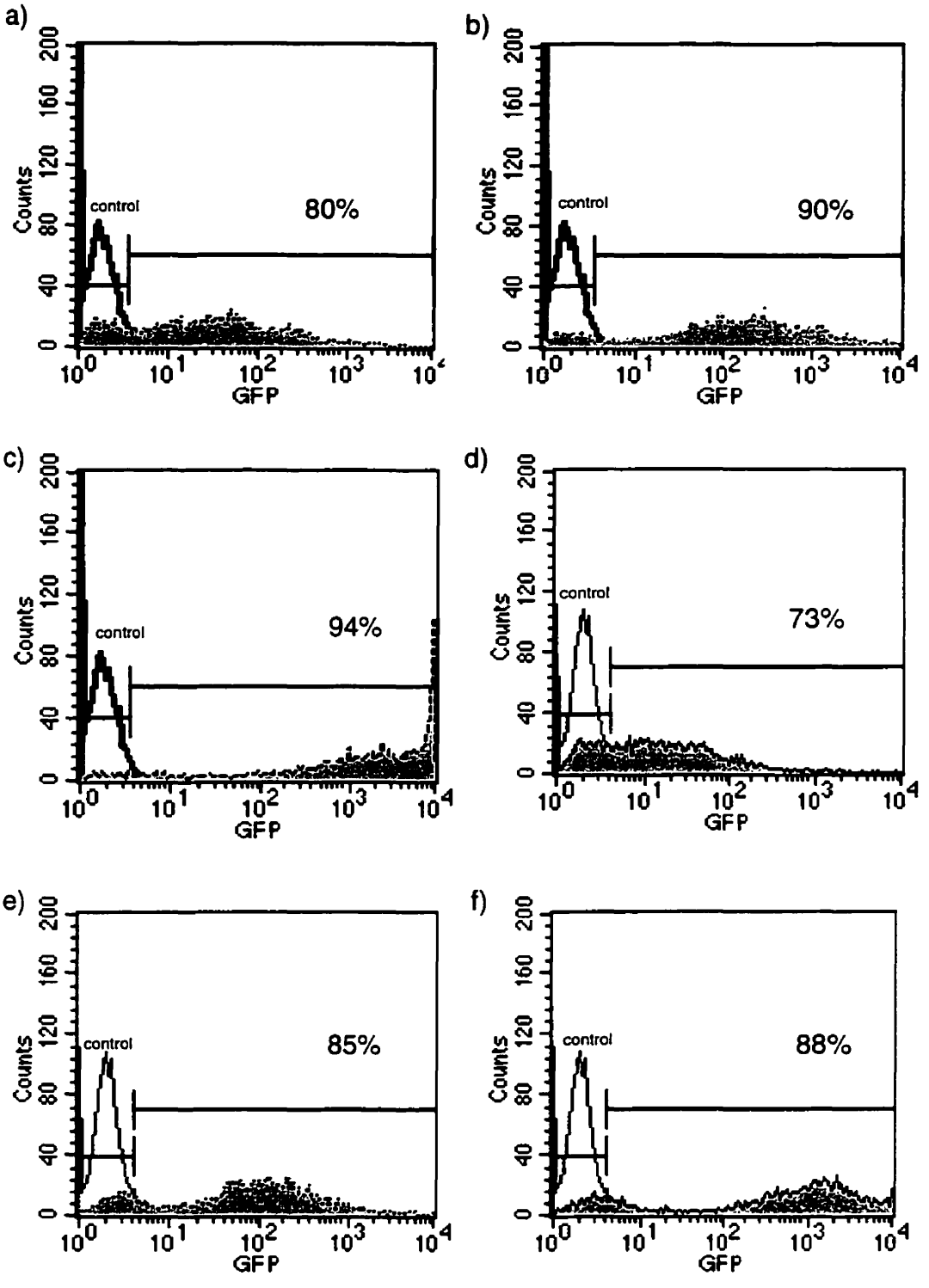

a)
b)
c)
d)
e)
f)
Counts
GFP
control
80%
90%
94%
73%
85%
88%

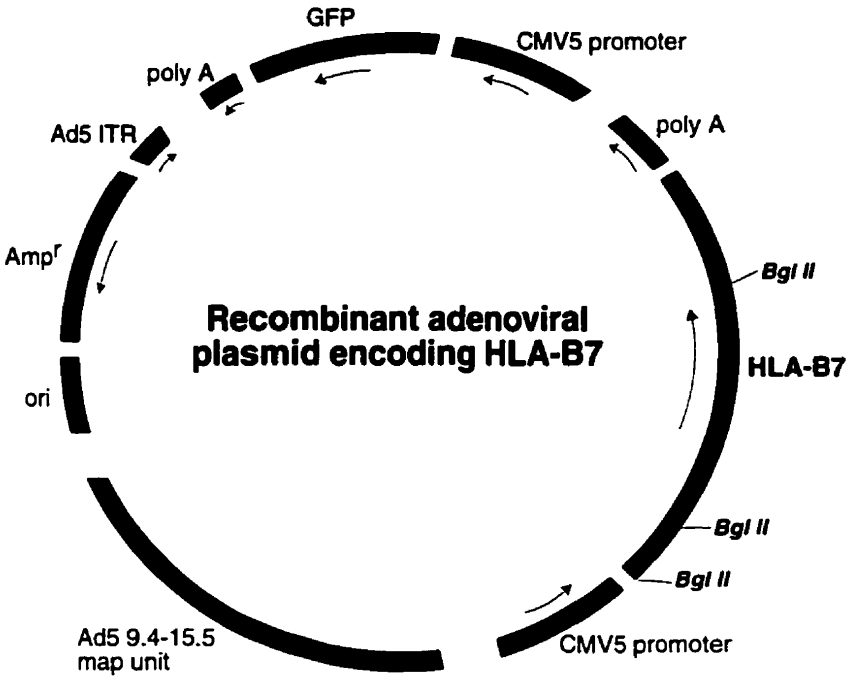
Fig. 3-5 :

Cloning of HLA-B7 gene into recombinant adenoviral transfer plasmid, pQBI-AdCMV5GFP

(a) construct showing map and orientation of recombinant plasmid containing HLA-B7 encoding cDNA;

(b) UV-autoradiograph confirming presence and orientation HLA-B7 encoding cDNA in isolated recombinant plasmid clones- 2,3,5,7, 9 and 10 (from miniprep plasmid DNA-isolates of transformed E-coli cells) digested with enzyme BglII and analysed by agarose gel electrophoresis; results confirmed by presence of 274-bp fragment resulting from Bgl-II digest of plasmid clones

a)


GFP
CMV5 promoter
poly A
poly A
Ad5 ITR
Amp^r
Bgl II
Recombinant adenoviral plasmid encoding HLA-B7
HLA-B7
ori
Bgl II
Bgl II
Ad5 9.4-15.5 map unit
CMV5 promoter

b)

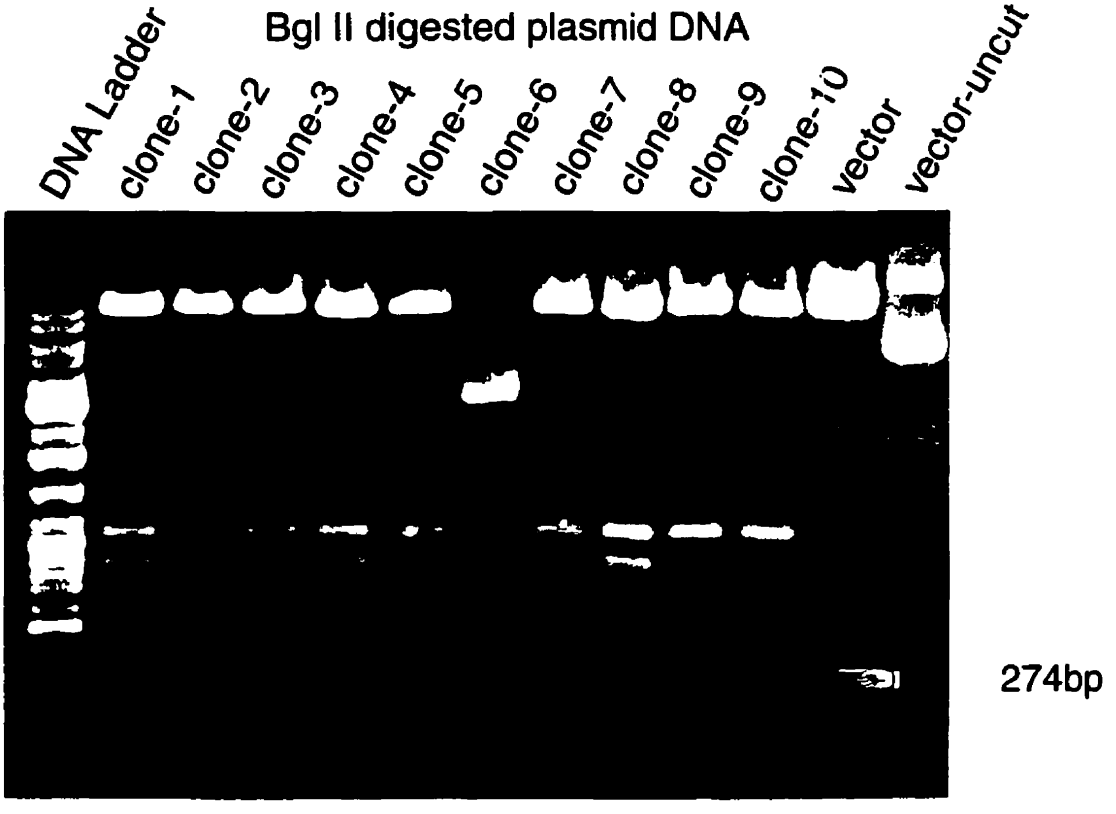
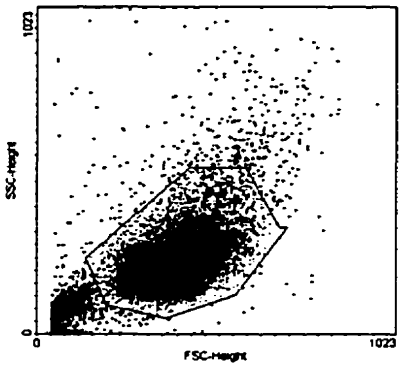

Bgl II digested plasmid DNA
DNA Ladder
clone-1
clone-2
clone-3
clone-4
clone-5
clone-6
clone-7
clone-8
clone-9
clone-10
vector
vector-uncut
274bp

Fig. 3-6 :

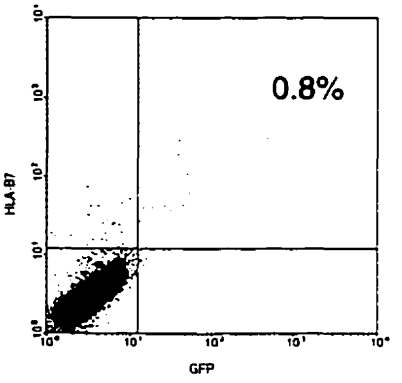
Positive HLA-B7 and GFP expression in 5637 cells transfected with recombinant plasmid clones #9 and #10 using Fugene6 reagent:

(a) gated cell population;
(b) negative control normal 5637 cells with PE-conjugated anti-HLA-B7 antibody;
(c) 5637 cells transduced with GFP-positive control, plasmid-pQBI-AdCMV5-GFP;
(d) 5637 cells transduced with HLA-B7 positive control plasmid-pRSVB7(k)T40;
(e) 5637 cells transduced with clone #9
(f) 5637 cells transduced with clone #10

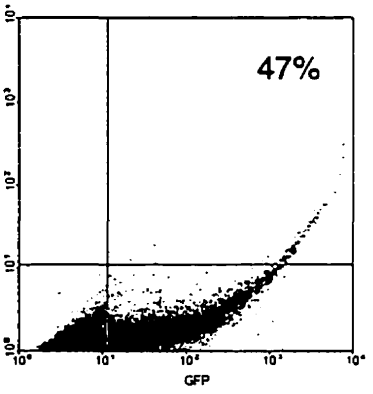
a)


SSC-Height
1023
0
FSC-Height
0
1023

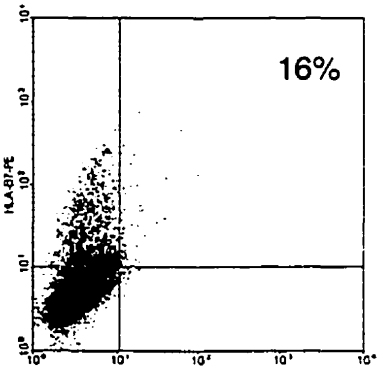
b)


0.8%
HLA-B7
GFP

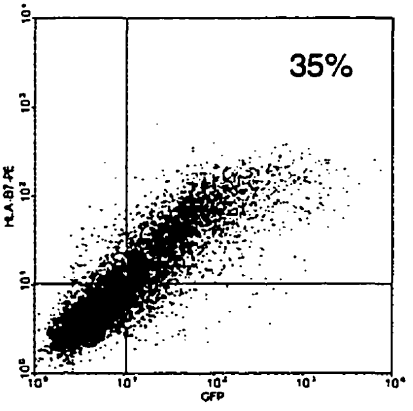
c)


47%
GFP

d)


16%
HLA-B7-PE

e)


35%
HLA-B7-PE
GFP

f)

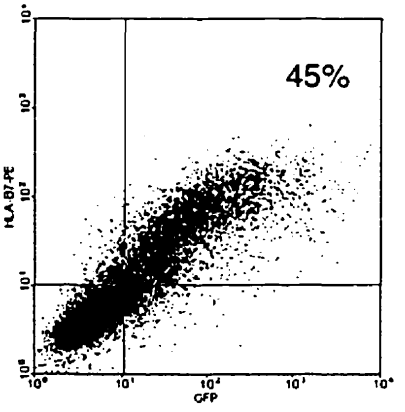
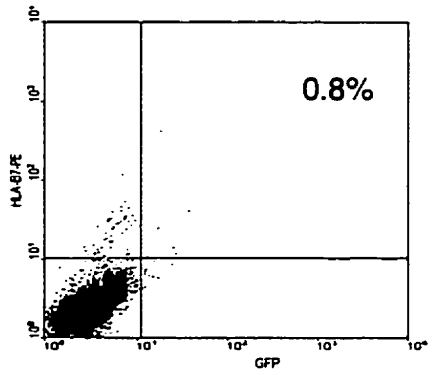

45%
HLA-B7-PE
GFP

Fig. 3-7 :

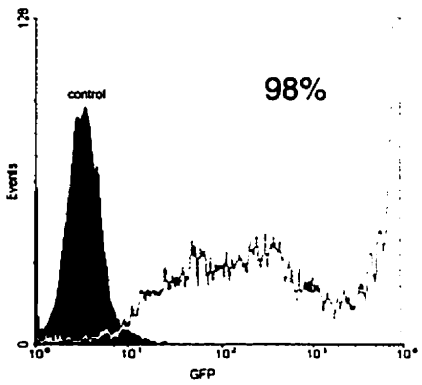
Confirmation of HLA-B7 and GFP expression by recombinant adenovirus clone- Ad#8 in 5637 cells:

(a) control untransduced 5637 cells with PE-anti-HLA-B7 antibody;
(b) 5637 cells transduced with GFP-control virus, AdCMV5-GFP;
(c) 5637 cells transfected with HLA-B7 positive control plasmid-pRSVB7(k)T40;
(d) 5637 cells transduced with viral stock of recombinant clone Ad#8

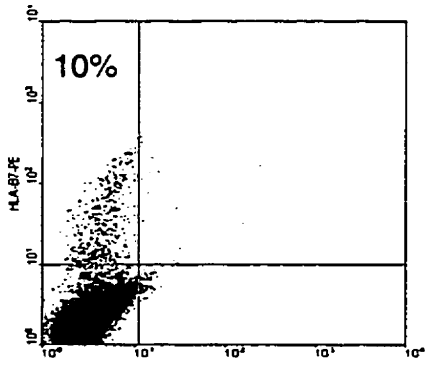
a)


0.8%
HLA-B7-PE
GFP

b)


control
98%
Events
GFP

c)


10%
HLA-B7-PE

d)

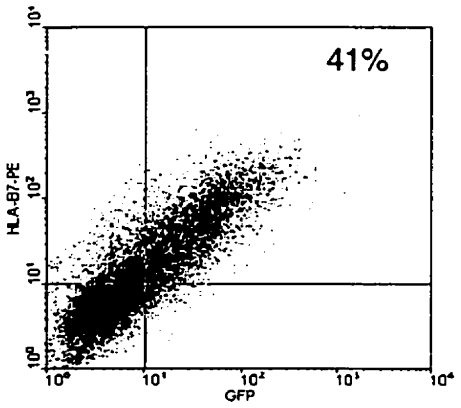

41%
HLA-B7-PE
GFP

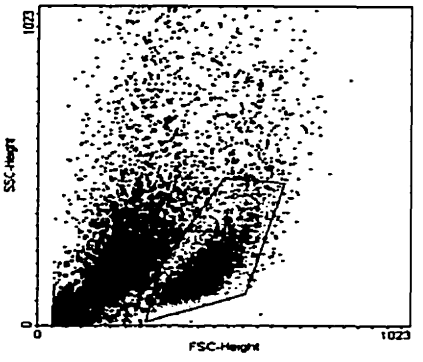
Fig. 3-8 :

Transduction efficiency of recombinant adenoviral clone Ad#8 in myeloma cell line RPMI-8226;

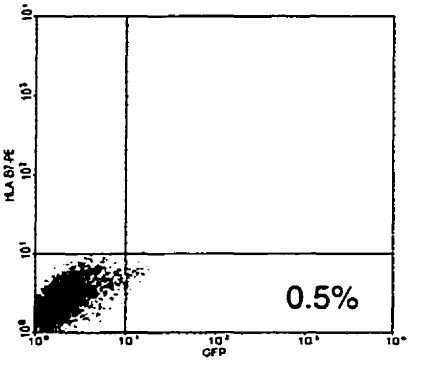
(a) gated cell population;
(b) control RPMI-8226 cells with PE-anti-HLA-B7;
(c) RPMI-8226 cells transduced with double-CsCl-gradient-purified Ad#8 viral prep, MOI of 10^4 VP;

293A-cells-amplified Ad#8 viral stock -
(d) MOI of 500 VP, and
(e) MOI of 1000 VP

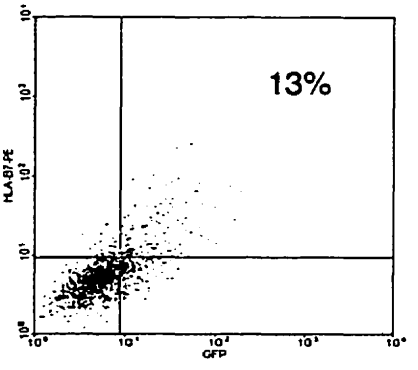
a)


SSC-Height
FSC-Height
0
1023

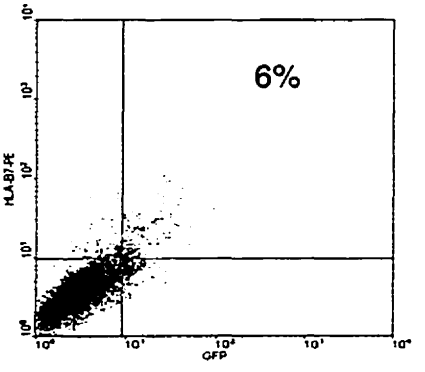
b)


HLA-B7-PE
GFP
0.5%

c)


HLA-B7-PE
GFP
13%

d)


HLA-B7-PE
GFP
6%

e)

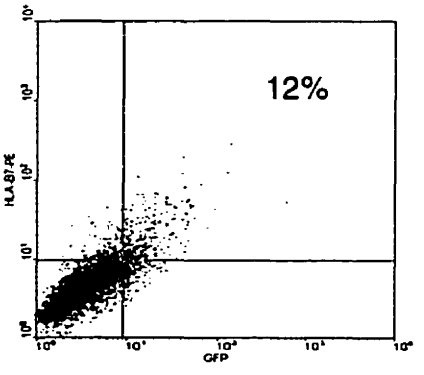

HLA-B7-PE
GFP
12%

Fig. 3-9 :

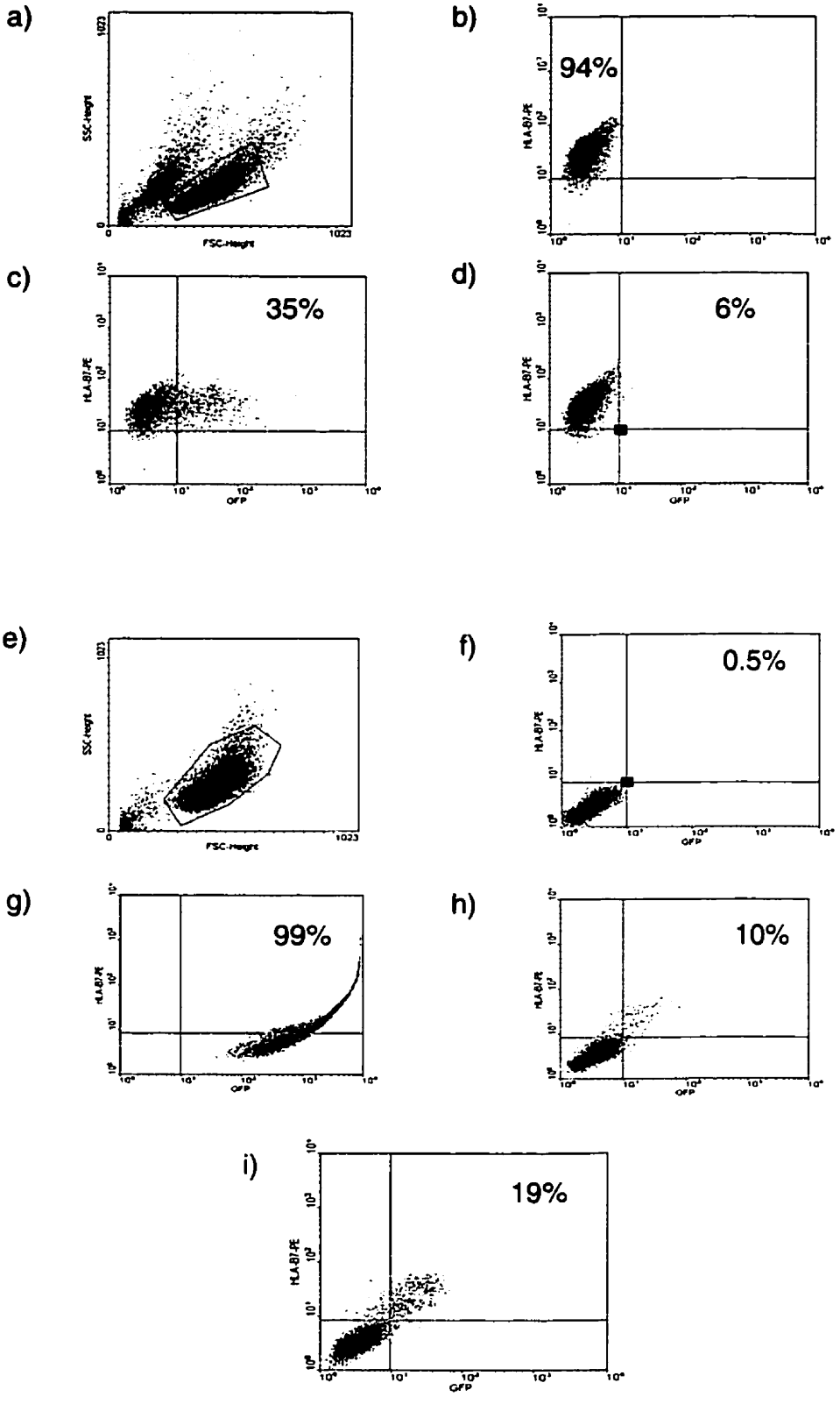
Transduction efficiency of recombinant adenoviral clone Ad#8 in myeloma cell line-U266 and Vero cells:

(a) gated U266 cell population;
(b) control U266 cells with PE-anti-HLA-B7 antibody;

U266 transduced with –
(c) GFP-control virus-AdCMV5GFP;
(d) Ad#8 viral stock, MOI of 500 VP

(e) gated Vero cell population;
(f) control Vero cells with PE-anti-HLA-B7 antibody;

Vero cells transduced with –
(g) GFP-control virus-AdCMV5GFP and
(h) Ad#8 viral prep at MOI of 10^3 VP
(i) Ad#8 viral prep at MOI of 10^4 VP


a)
b)
94%
c)
35%
d)
6%
e)
f)
0.5%
g)
99%
h)
10%
i)
19%
SSC-Height
FSC-Height
HLA-B7-PE
GFP

Chapter 4

Discussion and conclusions

The possibility that allogeneic MHC-expressing myeloma cells can enhance anti-tumor immune responses has vital implications for the therapy of MM. MM remains an incurable disease. The immune system in MM patients is weakened and, despite the presence of natural killer cells and tumor-specific T-cells, there is insufficient cytolytic activity directed against the malignant cells (Munshi, 1997). Thus, a strategy that isolates myeloma cells and targets them for immunogene therapy offers an attractive alternative to the current treatment options. Methods that yield purified and homogenous populations of primary myeloma cells are of prime importance to this approach.

In the first part of this study, we describe a simple method of purifying primary myeloma cells from BM aspirates of MM patients. Using differential-density-gradient centrifugation and a cocktail of magnetically-labeled monoclonal antibodies (CD14, CD41, CD33a, CD2 and CD45RA) followed by negative cell separation, we were able to obtain greater than 95% purification of myeloma cells in three of four BM samples. Indeed, extraordinary purity (99%) was achieved in two of four samples, as determined by CD38 and/or PCA-1 positive phenotype. The yield of myeloma cells following purification was quite variable, however, ranging from 32% to 75%. As might be expected, the lowest purity (51%) was achieved from the sample (MM1) that had the lowest initial percentage of myeloma cells. The lowest total recovery or yield (32%) was achieved for the sample (MM5) that had the lowest initial number of total BMNC. There was notable variability in both the total number of BMNC and the percentage of myeloma cells in the seven samples available for this

study. These preliminary observations suggest that the yield and level of purity achievable using this method may be limited by the total number of BMNC harvested and the percentage of myeloma cells, respectively, in the original samples. The irregular and infrequent supply of primary BM samples made further optimization and testing of this method difficult. During the course of our study, a concurrent report was published in which the investigators used negative selection with immunomagnetic beads to isolate primary myeloma cells (Tai et al., 2000). Despite variable initial myeloma cell percentages (6-85%), they also report high yield (>95%) and high purity (85-99%) using this approach. Fillola et al. (1996) have tested a similar method of primary myeloma cell purification. These investigators also reported substantial variability in purity achievable (2% to 100%) and an average yield of 20% in their patient samples. These discrepant observations may be due to the fact that plasma cells from MM patients display significant variability in cell surface marker expression (Helfrich et al., 1997; Ruiz-Argüelles et al., 1994). This variability has led some researchers to suggest the concurrent use of two or more antibodies for the definitive identification of myeloma cells (Ruiz-Argüelles et al., 1994; Tai et al., 2000), as was done in our study (CD38 and PCA-1). Currently, little is known about the phenotypic differences in myeloma cells or the proportion of pre-B- and B-cells, at the different stages of disease. Such differences may also affect the yield and efficiency of negative cell separation for myeloma cells, and would provide useful information for optimization of the antibody cocktail required to enrich the myeloma cell clone at various stages of differentiation.

Some investigators have used positive selection (with immunomagnetic beads) to purify primary myeloma cells from MM patients (Portier et al., 1991; Borset et al., 1993). The monoclonal antibody used in these studies (B-B4), has recently been found to specifically recognize a plasma-cell marker, SYNDECAN-1 (Wijdenes et al., 1996). Although, positive cell separation can produce samples with high purity (>98%) (Borset et al., 1993; Portier et al., 1991), the resulting cell population has surface-bound antibodies, that can complicate further antibody staining or other manipulations. Moreover, it is not currently known if positive selection targets myeloma cells at all stages of maturation, or if antibody-receptor signaling induces functional changes in the myeloma cells (e.g. activation, viability).

The complications in cell identification and purification are not unique to MM but extend to other lymphoid malignancies as well. Studies in chronic myeloid leukemia (CML) have shown that multiple phenotypic parameters (e.g. CD34+, CD71+, HLA-DR-, CD38-) are necessary for identification and isolation of the malignant cells in BM or leukapheresis samples (Maguer-Satta et al., 1996; Martin-Henao et al., 1996; Verfaillie et al., 1992). Furthermore, the Ph+ (Philadelphia chromosome positive) genotype, which represents the gene translocation unique to the malignant hematopoietic stem cells in CML, is also required for positive selection of primary CML cells (Tefferi et al., 1995; Maguer-Satta et al., 1996; Verfaillie et al., 1992). As a result, these investigators have used a combination of two distinct methods (e.g. fluorescence-activated cell sorting (FACS) and fluorescence *in situ* hybridization (FISH)) in their studies to satisfy both genotypic and phenotypic

requirements of positive CML cell isolation. In other hematologic malignancies such as chronic lymphocytic leukemia (CLL) and non-hodgkin's lymphoma (NHL), multi-step detection procedures have also typically been required for positive/negative selection or purging of the malignant cells (Murphy et al., 1987; Kvalheim et al., 1989; Farley et al., 1994; Pugh et al., 1998; Wells et al., 1998; Dreger et al., 2000). Vilpo et al. (1998) emphasized the negative effect of non-specific antibody binding on the yield and purification of leukemic B-cells in CLL, presumably due to the variability in surface marker expression. These studies corroborate the challenges of tumor cell purification in hematologic malignancies in general.

In order to address the issues of gene-delivery to myeloma cells, it is essential to have a primary myeloma cell population of maximum purity. Our results show that negative selection can be an effective purification method for primary myeloma cells in some patients. The enriched cell population does not have bound antibodies and, as a result, can be used immediately for other manipulations such as cell-surface marker analysis and gene therapy.

In addition to myeloma cell isolation, another major issue in MM research is the *in vitro* expansion and long-term culture of primary myeloma cells, which have been serious obstacles for most investigators. Between 1970 and 1990, a small number of groups demonstrated that primary myeloma cells could be propagated *in vitro* through short-term clonogenic bioassays of malignant plasma cells (Hamburger

et al. 1977; Izaguire et al.,1980; Ludwig et al., 1984; Millar et al., 1988). In all of these studies, the methods used were complicated (e.g. complex tissue culture systems with multiple feeder cell cultures and conditioned media). Thus, it is not surprising that none of these methods was widely adopted by other laboratories. Furthermore, the cells derived using these methods were in the form of colonies in solid agar medium, and thus, were used primarily for drug-sensitivity assays and characterization of malignant myeloma clone.

In the current study, we made a limited attempt to expand primary myeloma cells from total MNC (two BM samples) and from the enriched myeloma cell populations obtained by immunomagnetic negative selection (four BM samples), using IL-6 supplementation. The duration of these cultures ranged from 6 to 22 days, during which there was a significant loss in the number of cells in all cases. In no case were cell numbers even maintained and no significant expansion was observed. Interestingly, the culture of total MNC from one sample survived significantly longer than the cultures of enriched myeloma cells, implying that the non-myeloma cells present in the BM aspirates play an important role in the maintenance of the malignant cells. Stewart et al., (1997) have also reported similar duration (21 days) of total BMNC cultures from MM patient samples. Indeed, the best evidence for *in vitro* growth of myeloma cells has been derived from studies in which the BM stromal cells provided paracrine support (Caligaris-Cappio et al, 1992; Lokhorst et al., 1994). Furthermore, it is well established that the BM stromal cells and BMNC are vital feeder cells for the establishment of myeloma cell lines (Takahira et al., 1994;

Shimizu et al., 1989; Scibienski et al., 1992). However, little is known about the effects of long-term marrow cultures and cell-line establishment on the myeloma clone. Several cytokines and growth factors other than IL-6 produced in the BM microenvironment (e.g. IL-3, TNF, IL-1, GM-CSF, IL-11) are known to be involved in the *in vivo* pathogenesis and proliferation of myeloma cells (Klein, 1995; Nishimoto et al., 1997). The expression of these cytokines is known to vary widely at different stages of the disease (Klein, 1995). As a result, it is likely that the stage of disease in the MM patients from which the BM samples are obtained can also influence the cytokine-supplementation "cocktail" necessary to establish long-term myeloma cultures. Full characterization of the optimal growth conditions may yield improved systems for long-term culture of myeloma cells in the future.

For immuno-genetic modification of myeloma cells, the vector for gene-delivery is a crucial element that determines the efficiency of gene transfer and protein expression. We evaluated the transfection efficiency of six lipid-based reagents in two myeloma cell lines. In both cell lines, these reagents were ineffective for the transfer of the GFP-reporter gene over a wide range of concentrations. Although we had no success in myeloma cell lines, transfection of related cells has been achieved by several groups. Simoes et al. (1999) used lipid and liposome based vectors to transfect some B-lymphocytic cell lines with the use of a targeting ligand (e.g. transferrin) or an endosome-disrupting peptide, in ternary complexes. Keller et al. (1999) have demonstrated that liposome-mediated transgene expression but not gene delivery is possible in murine hematopoiteic cells through adhesion-assisted

lipofection, using an adherent monolayer of fibroblast cells. In B-cell lymphoma cells, it is possible to deliver a liposome-entrapped anti-cancer drug by coupling of the liposome-drug complex to B-cell specific antibodies (Lopes de Menezes et al., 1998). These data suggest that cationic liposome-mediated gene delivery to hematologic cells is achievable only with complicated modifications and cell-specific targeting. There is evidence to suggest that B-cells endocytose liposome-antibody complexes (Grivel et al., 1994), and that exogenous antigen (from DNA-liposome complexes) can be processed and presented to T-cells through both MHC-class I and II pathways, by antigen-presenting cells (Rao et al., 2000). Myeloma cells are known to have the capacity for antigen-presentation (Yi et al., 1997). Based on these data, it may be possible to induce liposome-mediated allogene-expression in myeloma cells by idiotype-specific targeting of the liposome-DNA complexes. However, for our purpose, it would be important to evaluate the changes exerted by such complexes in myeloma cells (e.g. apoptosis, activation), and to ensure MHC-class I, but not class II, allo-antigen presentation to CTL. To date, lipid-based transfection vectors have been most effective for gene-delivery by direct injection into solid tumors (e.g. melanoma and carcinoma) (Stopeck et al., 1997; Nabel et al., 1996). In contrast, malignant cells in MM are systemically distributed and generally only form solid tumor masses (i.e. plasmacytomas) in very advanced stage disease. Whether or not the direct injection of lipid-based vector constructs could be effective in immunogene transfer to plasmacytomas requires further study. Our data strongly suggest that lipid-based vectors are unlikely to be a suitable option for *ex vivo* gene-modification of myeloma cells.

An attractive alternative to lipid-based gene delivery systems is offered by virus-based vectors. The adenovirus-GFP-reporter model is of particular interest since GFP expression can be easily detected by flow cytometry or fluorescence microscopy and is useful for optimizing and studying the kinetics of transduction efficiency in different cell types (Martin et al., 1997). We used a first-generation (ΔE1) and a third-generation (ΔE1/ΔE3/ΔE4) replication-deficient adenovirus vector to deliver the GFP-reporter gene into myeloma cell lines. In our hands, the first-generation adenoviral vector was inefficient in transducing the two myeloma cell lines under normal culture conditions. Since this vector was produced with moderate stringency and selectivity (personal communication with George Karpati, MNI, Montreal), it is possible that the low transduction efficiency we observed was the result of variability in the infectivity of the viral vector (e.g. high concentrations of defective-interfering particles in the viral stock (Alemany et al., 1997)). However, this trivial explanation is unlikely since high GFP-expression in 293A cells could be achieved using the same vector. We have also seen that starving myeloma cell lines of essential nutrients could significantly increase the transduction efficiency of this vector (e.g. 73% in U266 cells). The mechanism by which starvation of cells allows increased transgene uptake and expression is not clearly understood. Moreover, the increase in transduction efficiency achieved by starvation is accompanied by significant losses in cell viability. As a result, this approach is not a suitable option for increasing the adenoviral-mediated transduction of primary myeloma cells, that have limited growth potential and require additional growth factors and nutrient supplementation for survival *in vitro*. In contrast, the third generation adenoviral vector we used was

highly effective for transducing myeloma cell lines. GFP-reporter expression ranging from 70% to 94% was observed and could be maintained for up to 96 hours. Wattel et al. (1996) have reported moderate efficacy (20%-64%) using a first-generation adenoviral vector in thirteen hematologic cell lines, including three myeloma cell lines (two of which were used in our study; i.e. U266 and RPMI-8226). Meeker et al. (1997) have reported similar efficiencies for adenoviral-mediated gene-transfer in malignant lymphocytic cell lines (40-80%). Together, these data establish the potential utility of adenovirus vectors for the central objective of our study; that is allogene-expression in myeloma cells.

Based on the high efficacy of the adenovirus-GFP, we cloned the HLA-B7 gene into an adenoviral transfer plasmid and observed significant HLA-B7 and GFP expression (35-45%) by two recombinant plasmids. These plasmids were used to construct recombinant adenoviral particles. Coincidentally, we found that 293A cells are HLA-B7-positive and express this molecule constitutively. This finding caused serious difficulties in the evaluation of HLA-B7 protein expression by the bicistronic plasmids and recombinant adenoviral clones. To our knowledge, no other attempts to construct an HLA-B7-adenoviral vector have been reported. 293A cells are particularly useful for studying recombinant adenoviral protein expression since the viral particles are typically produced and amplified in these cells. One way in which viral-HLA-B7 (episomal) expression could theoretically be distinguished from the 293A cellular-HLA-B7 is through PCR-amplification (i.e. detection of intron sequences that are characteristic of genomic DNA). However, such an approach

would be of little help in evaluating differences in the cell-surface expression of HLA-B7. As a result, we had to evaluate protein expression by both the cloned plasmids and the recombinant viral clones in 5637 cells instead. Using these cells, we identified one particularly promising adenoviral vector clone (Ad#8) that was double positive (41% expressing both HLA-B7 and GFP). These results showed that we could produce a recombinant adenoviral vector with significant expression of both proteins.

Next, we evaluated the transduction efficiency of the HLA-B7/GFP-positive adenoviral vector in two myeloma cell lines over a wide range of MOI. Our results suggested that a small population of genetically modified, HLA-B7-positive myeloma cells was achievable (13% in RPMI-8226 cells). Although some transduction in U266 cells was measurable (6% GFP-expression), we were unable to confirm HLA-B7-expression by this vector due to strong reactivity of the HLA-B7 antibody to U266 cells. We could also demonstrate that the recombinant viral vector preparation had significant infectivity, as reflected by the relatively high levels of HLA-B7 protein expression in Vero cells (19%).

There have been contradictory reports of adenoviral-mediated transduction efficacy in cell lines *versus* primary cells from lymphoid malignancies, including MM, which underline the inconsistencies of this approach (Wattel et al., 1996; Meeker et al., 1997; Leon et al., 1998; Cantwell at al., 1988; Prince et al., 1998). Factors that are likely to influence the resistance of myeloma cells to transduction

include variability in integrin and CAR receptor levels and the possibility that, despite viral internalization, transgene expression may be hindered at the level of transcription. Teoh et al., (1998) have demonstrated that human myeloma cell lines (including RPMI-8226) and primary cells express both CAR and the other molecules required for viral-internalization (i.e. α_5 integrins). Furthermore, using a tumor-selective promoter, DF3/Muc1, these authors have shown that it is possible to achieve high (80-100%) transduction efficiency in myeloma cell lines but not in normal BM mononuclear cells or primary myeloma cells. Subtle variations in CAR-adenoviral interactions may underlie these observations. It has recently been demonstrated that specific amino acid residues are involved in the CAR-adenoviral-fiber-protein interactions for the different adenovirus subgroups (Nemerow, 2000) and that altered CAR-viral binding can result from specific amino-acid substitutions (Roelvink et al., 1999).

Together, these observations suggest the potential for improvement of adenoviral vectors based on CAR-binding properties. Evaluation of CAR/integrin expression in primary myeloma cell samples prior to adenoviral-mediated transduction would be needed to predict the success of this approach. While the cytomegalovirus (CMV)-promoter used in this study has been successfully applied in retroviral and adenoviral based gene therapy protocols (Romano, 1998), it might also be worthwhile to consider more B-cell-specific promoters, such as DF3/Muc1 or immunoglobulin promoters, to achieve higher protein expression in myeloma cells. Clearly, further manipulation of our recombinant adenoviral vector will be required to achieve satisfactory levels of cell binding, internalization and transgene expression in

myeloma cells. An improved viral vector preparation could then be used to transduce the more challenging primary myeloma cells.

Although adenoviral vectors have come under close scrutiny, following the report of a fatality in an adenoviral-based gene therapy trial (Marshall, 1999), there is strong evidence to support virus-mediated gene-transfer in myeloma cells for the purpose of enhancing the T-cell mediated anti-tumor response in humans (Tarte et al., 1999; Wendtner et al., 1997)). This approach is strongly supported by the murine myeloma model as well (Shtil et al., 1999; Ramarathinam et al., 1994; Cayeux et al., 1995). Myeloma cells have been shown to express high levels of MHC Class I molecules and, more importantly, several tumor-specific antigens, such as MUC1, MAGE, GAGE and BAGE, at varying levels (Van Baren et al., 1999; Treon et al., 1999). Autologous, MHC I-restricted, myeloma-specific CTL exist and can be identified in MM patients (Pellat-Deceunynck et al., 1999). However, the presence of these immune-effector elements does not seem to correlate with the clinical state of the average MM patient. The disease is known to generate a state of immune compromise in most patients, which becomes more severe as the disease progresses. It seems that these "obvious" TAAs are not recruiting or activating sufficient CTL in MM patients. This deficiency could be due (in part) to the down-regulation or lack of co-stimulatory signals, such as B7-1, B7-2, or the over-production of the B-cell stimulatory factor, IL-6, that is characteristic of this disease. Van Barren et al. have also shown that certain TAAs are expressed only in the cytoplasm of the malignant plasma cells, implying that these antigens may be cryptic. Tumor-associated antigens

can also fail to generate an effective T-cell response if there is inhibition of dendritic cell maturation or activation in the tumor environment (Troy et al., 1997).

To date, allogene-based immuno-therapy has proven to be highly effective in enhancing anti-tumor CTL responses directed against some tumors (e.g. melanoma). In some cases there has been an extension of this response to unmodified tumor cells throughout the body; the so-called distant bystander effect (Stopeck et al., 1997). Allogene-expressing tumor cells can be highly immunogenic and are very likely to be killed by pre-existing allo-reactive T-cells, releasing tumor-specific antigens (cryptic and otherwise) into their microenvironment. Presentation of these antigens in the resulting inflammatory milieu may provide the necessary cytokine and cellular signals for the recruitment and activation of a critical mass of tumor-specific CTL (illustrated in Fig. 4). This proliferating CTL population would then seek out and kill other tumor cells bearing the newly recognized TAAs. Applied to earlier stages of MM or during remission, when the tumor load is still relatively low, such an approach could theoretically 'vaccinate' the patient against further tumor expansion. Alternatively, if allogene-therapy can be performed safely in patients with advanced myeloma (e.g. high tumor load, drug-resistant cells, plasmacytoma formation), then vectors could be designed to target the more drug-resistant tumor cells, or for direct injection into plasmacytomas. These possibilities are supported by the observation that, as the tumor progresses, the cells that proliferate most rapidly are the ones that have accumulated drug-resistance and oncogenic potential, giving them a growth advantage over the less aggressive tumor clones (Feinman et al., 1997). Thus,

allogene-therapy in conjunction with chemotherapy could theoretically facilitate tumor clearance at all stages of disease. In the case of plasmacytomas, it may be possible to isolate local tumor-infiltrating lymphocytes (TIL) following the allogene-induced immune response and expand these TIL *ex vivo*, to be used in subsequent treatment cycles, as has been reported in one case of advanced melanoma (Nabel et al., 1996).

In conclusion, we have provided strong evidence for the efficiency of negative immuno-magnetic selection for *ex vivo* purification of primary myeloma cells. In a limited study of the culture characteristics of BMMC and enriched myeloma cells, we found poor survival and considerable variability even among a small number of samples. These observations highlight the difficulties and challenges related to *in vitro* maintenance and expansion of primary myeloma cells. We have demonstrated that adenoviral vectors can be used to deliver a gene of interest into myeloma cell lines while chemical transfection reagents are ineffective. Finally, we constructed a recombinant adenovirus that was capable of delivering an allogene into myeloma cell lines. Further studies to improve the viral construct, optimize viral production and purify MM cells will be required to assess this approach in primary myeloma cells. In general, our results support the long-term objective of this strategy; the development of an allogene-expressing myeloma cell population that can eventually be used as a cancer "vaccine" in MM patients.

Fig. 4 :

Schematic representation of the applications and potential benefits of allogene-expression in myeloma cells: Allo-reactive CTL-mediated killing of allogene-modified myeloma cells; Immunologic interactions in an allo-antigen induced immune response and the resulting bystander effect

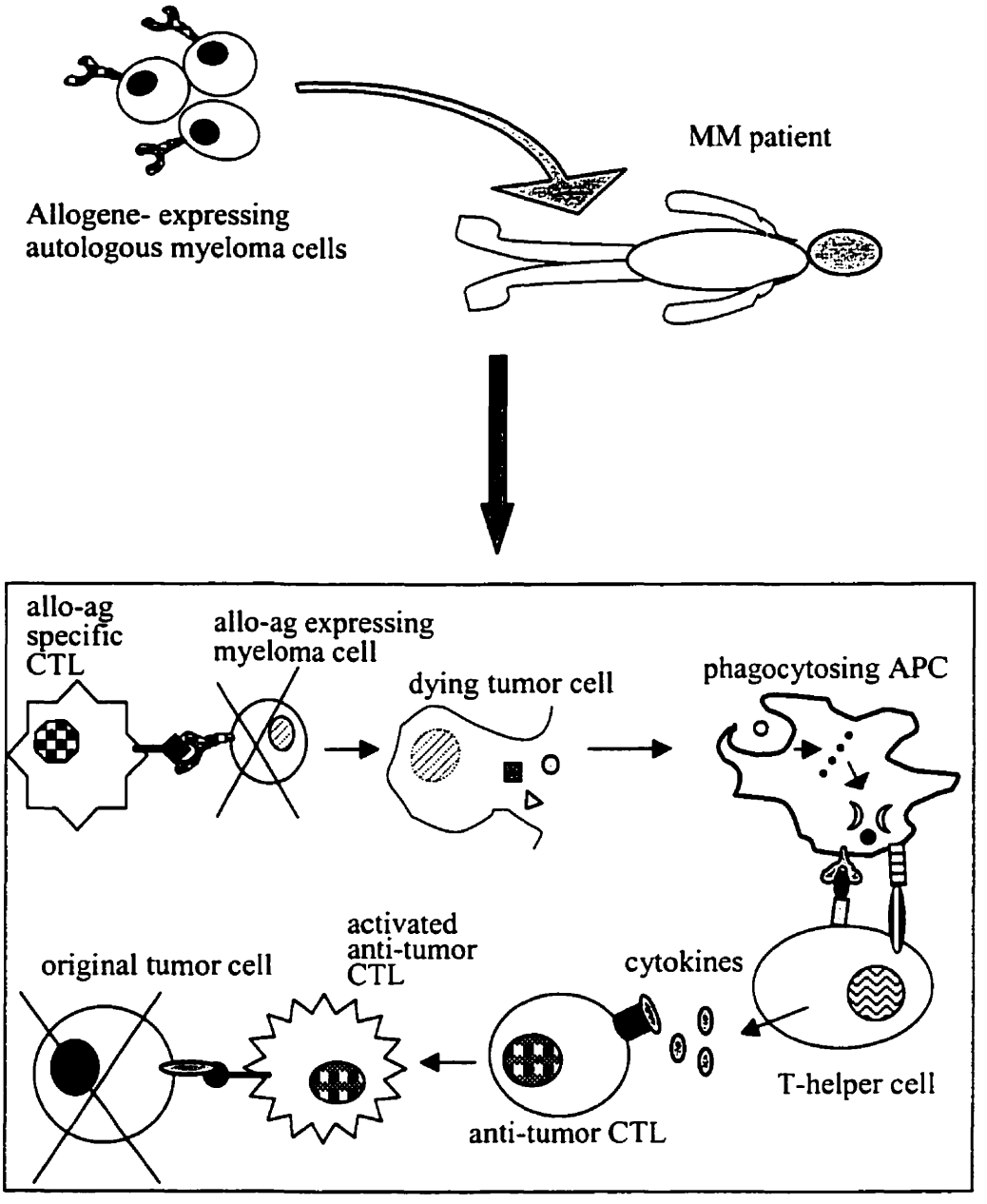

MM patient
Allogene- expressing
autologous myeloma cells
allo-ag
specific
CTL
allo-ag expressing
myeloma cell
dying tumor cell
phagocytosing APC
activated
anti-tumor
CTL
original tumor cell
cytokines
T-helper cell
anti-tumor CTL

Fig. 4

Chapter 5

Bibliography

Alemany R, Dai Y, Lou YC, Sethi E, Prokopenko E, Josephs SF, Zhang WW. Complementation of helper-dependent adenoviral vectors: size effects and titer fluctuations. *J Virol Methods* 1997; 68: 147-59

Andreason GL, Evans GA. Introduction and expression of DNA molecules in eukaryotic cells by electroporation. *Biotechniques* 1988; 6 :650-60.

American Cancer Society: Cancer facts and figures, Atlanta, GA, 1995.

Attal M, Harousseau JL, Stoppa AM. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. *N Engl J Med* 1996; 335: 91-97

Bakkus MHC, Heirman C, Van Riet I, Van Camp B, Thielemans K. Evidence that multiple myeloma Ig heavy chain VDJ genes contain somatic mutations but show no intraclonal variation. *Blood* 1992; 80: 2326-2335

Barker HF, Ball J, Drew M, Franklin IM. Multiple myeloma: the biology of malignant plasma cells. *Blood Rev* 1993; 7: 19-23

Barlogie B, Jagannath S, Vesole D. Autologous and allogeneic transplants for multiple myeloma. *Semin Hematol* 1995; 32: 31-44

Bartl R, Frisch B, Fateh-Moghadam A, kettner G, Jaeger K, Sommerfeld W. Histologic classification and staging of multiple of myeloma. A retrospective and prospective study of 674 cases. *Am J Clin Pathol* 1987; 87: 342-55

Bataille R, Jourdan M, Zhang XG. Serum levels of interleukin-6, a potent myeloma cell growth factor, as a reflection of disease severity in plasma cell dyscrasias. *J Clin Invest* 1989; 84: 2008-2011

Bataille R, Harousseau J-L. Multiple myeloma: review. *N Engl J Med* 1997; 336:1657-1664

Beck R, Neipel F, canji B, Hebart H, Kanz L, Jahn G, Einsele H. Absence of HHV-8 DNA sequences in leucapheresis products and bone marrow samples of patients with advanced multiple myeloma. *Br J Haematol* 2000; 109: 676-677

Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997; 275: 1320-3

Berenson JR, Vescio R. HHV-8 is present in multiple myeloma patients. *Blood* 1999; 93: 3157-3159

Bergsagel D. The known causes of multiple myeloma. *MM Res Web Server* 2001; Jan-7: 1-11

Borset M, Helseth E, Naume B, Waage A. Lack of IL-1 secretion from human myeloma cells highly purified by immunomagnetic separation. *Br J Haematol* 1993; 85: 446-451

Brody SL, Crystal RG. Adenovirus-mediated *in vivo* gene transfer. *Ann N Y Acad Sci* 1994; 716: 90-101

Brunning RD and McKenna RW. *Tumors of the bone marrow.* Washington, D.C. Armed Forces Institute of Pathology (Available from the American Registry of Pathology, Armed Forces Institute of Pathology), 1994: 323-367

Caligaris-Cappio F, Gregoretti MG, Ghia P, Bergui L. *In vitro* growth of human multiple myeloma: implications for biology and therapy. *Hematol Oncol Clin North Am* 1992; 6: 257-271

Cantwell MJ, Sharma S, Friedmann T, Kipps TJ. Adenovirus vector infection of chronic lymphocytic leukemia B cells. *Blood* 1996; 88: 4676-

Carter A, Merchav S, Silvian-Draxler I, Tatarsky I. The role of interleukin-1 and tumor necrosis factor-α in human multiple myeloma. *Br J Haematol* 1990; 74: 424-431

Cayeux S, Beck C, Aicher A, Dorken B, Blankenstein T. Tumor cells co-transfected with interleukin-7 and B7.1 genes induce CD25 and CD28 on tumor-infiltrating T lymphocytes and are strong vaccines. *Eur J Immunol* 1995; 25: 2325-

Chen H-C, Wu T-C. Experimental vaccine strategies for cancer immunotherapy. *J Biomed Sci* 1998; 5: 231-252

Chen L. Immunologic ignorance of silent antigens as an explanation of tumor evasion. *Immunol Today* 1998; 19: 27-29

Culver KW. Gene therapy for malignant neoplasms of the CNS. *Bone Marrow Transplantation* 1996; 18 (suppl 3): S6-9

DeBruyne L. Treatment of malignancy by direct gene transfer of a foreign MHC class I molecule. *Cancer Immunol Immunother* 1996; 43: 180-189

Descamps V, Duffour M-T, Mathieu M-C, Fernandez N, Cordier L, Abina MA, Kremer E, Perricaudet M, Haddada H. Strategies for cancer gene therapy using adenoviral vectors. *J Mol Med* 1996; 74: 183-189

Detours V, Perelson AS. Explaining high alloreactivity as a quantitative consequence of affinity-driven thymocyte selection. *Proc Natl Acad Sci USA* 1999; 96:5153-5158

Detours V. Perelson AS. The paradox of alloreactivity and self MHC restriction: quantitative analysis and statistics. *Proc Natl Acad Sci USA* 2000; 97: 8479-83

Dev SB, Rabussay DP, Widera G, Hoffman GA. Medical applications of electroporation. *IEEE Transac Plasma Sci* 2000; 28: 206-223

Dreger P, Viehmann K, von Neuhoff N, Kruss D, Glass B, Kneba M, Mitsky P, Jopp P, Rautenberg P, Mills B, Schmitz N. A prospective study of positive/negative *ex vivo* B-cell depletion in patients with chronic lymphocytic leukemia. *Exp Hematol* 2000; 28: 1187-96

Drewinko B, Alexanian R, Boyer H. The growth fraction of human myeloma cells. *Blood* 1981; 57: 333-338

Droge W. Hypothesis on the origin of the strong alloreactivity. *Immunobiology* 1979; 156: 2-12.

Durie BGM, Salmon SE, Moon TE. Pretreatment tumor mass, cell kinetics and prognosis in multiple myeloma. *Blood* 1980; 55: 364-372

Durie BGM, Vela E, Baum V. Establishment of two new myeloma cell lines from bilateral pleural effusions. Evidence for sequential *in vivo* clonal change. *Blood* 1985; 66: 548-555

Egawa K, Seo N, Tanino T, Tsukiyama T. Protection against metastasis by immunization with an allogeneic lymphocyte antigen. *Cancer Immunol Immunother* 1995; 41: 384-388

Farley TJ, Preti RA, Ahmed T, Ciavarella D. A two-phase approach to B lymphocyte purging of autologous bone marrow grafts for patients with malignant lymphoma contaminated bone marrow. *Prog Clin Biol Res* 1994; 389: 105-9

Feinman R, Sawyer J, Hardin J, Tricot G. Cytogenetics and molecular genetics in multiple myeloma. *Hematol Oncol Clinics North Am* 1997; 11: 1-25

Fenton RG, Longo DL. Danger versus tolerance: paradigms for future studies of tumor-specific cytotoxic T lymphocytes. *J Natl Cancer Inst* 1997; 89: 272-275

Fillola G, Muller C, Bousquet R, Fontanilles AM, Laharrague P, Corberand JX. Isolation of bone marrow plasma cells by negative selection with immunomagnetic beads. *J Immunol Methods* 1996; 190: 127-31

Freeman SM, Abboud CN, Whartenby KA, Packman CH, Koeplin DS, Moolten FL, Abraham GN. The "bystander effect": tumor regression when a fraction of the tumor mass in genetically modified. *Cancer Res* 1993; 53: 5274-5283

Freeman SM, Whartenby KA, Freeman JL, Abboud CN, Marrogi AJ. *In situ* use of suicide genes for cancer therapy. *Sem Oncol* 1996; 23: 31-45

Gao X, Huang L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem Biophys Res Commun* 1991; 179: 280-285

Gansbacher B, Zier K, Daniels B, Cronin K, Bannerji R, Gilboa E. Interleukin-2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J Exp Med* 1990; 172: 1217-1224

Gautier M, Cohen HJ. Multiple myeloma in the elderly. *J Am Geriatr Soc* 1994; 42: 653-664

Gleich LL, Gluckman JL, Armstrong S, Biddinger PW, Miller MA, Balakrishnan K, Wilson KM, Saavedra HI, Stambrook PJ. Alloantigen gene therapy for squamous cell

carcinoma of the head and neck: results of a phase-1 trial. *Arch Otolaryngol Head Neck Surg* 1998; 124: 1097-1104

Goedegebuure PS, Eberlein TJ. Vaccine trials for the clinician: prospects for viral and non-viral vectors. *The Oncologist* 1997; 2: 300-310

Graham FL, Smiley J, Russel WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type-5. *J Gen Virol* 1977; 36: 59-72

Gregory WM, Richards MA, Malpas JS. Combination chemotherapy versus melphalan and prednisone in the treatment of multiple myeloma: An overview of published trials. *J Clin Oncol* 1992; 10: 334-342

Grivel JC, Crook K, Leserman L. Endocytosis and presentation of liposome-associated antigens by B cells. *Immunomethods* 1994; 4: 223-8

Harrousseau JL, Attal M. The role of autologous hematopoietic stem cell transplantation in multiple myeloma. *Sem Hematol* 1997; 34: 61-66

Hamburger A, Salomon SE. Primary bioassay of human myeloma stem cells. *J Clin Invest* 1977; 60: 846-854

Helfrich MH, Livingston E, Franklin IM, Soutar, RL. Expression of adhesion molecules in malignant plasma cells in multiple myeloma: comparison with normal plasma cells and functional significance. *Blood Rev* 1997; 11: 28-38

Hussein M. Multiple myeloma: An overview of diagnosis and management. *Clev Clin J Med* 1994; 61: 285-298

Iwato K, Kawano M, Asaoku H, Tanabe O, Tanaka H, Kuramoto A. Separation of human myeloma cells from bone marrow aspirates in multiple myeloma and their proliferation and M-protein secretion in vitro. *Blood* 1988; 72: 562-566

Izaguirre CA, Minden MD, Howatson AF, McCulloch EA. Colony formation by normal and malignant human B-lymphocytes. *Br J Cancer* 1980; 42: 430-437

Kawano M, Hirano T, Matsuda T. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myeloma. *Nature* 1988; 332: 83-85

Keller H, Yunxu C, Marit G, Pla M, Reiffers J, Theze J, Froussard P. Transgene expression, but not gene delivery, is improved by adhesion-assisted lipofection of hematopoietic cells. *Gene Ther* 1999; 6: 931-8

Kerr WG, Mule JJ. Gene therapy: current status and future prospects. *J Leuk Biol* 1994; 56: 210-214

Klein B, Zhang X-G, Jourdan M. A Paracrine but not autocrine regulation of myeloma cell growth and differentiation by interleukin-6. *Blood* 1989; 73: 517-526

Klein B, Zhang X-G, Lu Z-Y. Interleukin-6 in human multiple myeloma. *Blood* 1995; 85: 863-872

Klein B. Cytokine, cytokine receptors, transduction signals, and oncogenes in human multiple myeloma. *Sem Hematol* 1995; 32: 4-19

Kovacsovics TJ, Delaly A. Intensive treatment strategies in multiple myeloma. *Sem Hematol* 1997; 34: 49-60

Kurihara N, Bertolini D, Suda T. IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. *J Immunol* 1990; 144: 4226-

Kvalheim G, Fjeld JG, Phil A, Funderud S, Ugelstad J, Fodstad O, Nustad K. Immunomagnetic removal of B-lymphoma cells using a novel mono-sized magnetizable polymer bead, M-280, in conjunction with primary IgM and IgG antibodies. *Bone Marrow Transplant* 1989; 4: 567-74

Kwak LW, Thielemans K, Massaia M. Idiotypic vaccination as therapy for multiple myeloma. *Semin Hematol* 1999; 36(suppl): 34-37

Kyle RA. Multiple myeloma: review of 869 cases. *Mayo Clin Proc* 1975; 50: 29-40

Kyle RA. Benign monoclonal gammopathy: A misnomer ? *JAMA* 1984; 251: 1849-1854

Kyle RA. Benign monoclonal gammopathy-- After 20 to 35 years of follow-up. *Mayo Clin Proc* 1993; 68: 26-36

Leon RP, Hedlund T, meech Sj, Li S, Schaack J, Hunger S, Duke RC, DeGregori J. Adenoviral-mediated gene transfer in lymphocytes. *Proc Natl Acad Sci USA* 1998; 95: 13159-64

Ley V, Langlade-Demoyen P, Kourilsky P, Larsson-Sciard E-L. Interleukin-2-dependent activation of tumor-specific cytotoxic T lymphocytes *in vivo*. *Eur Immunol* 1991; 21: 851-854

Lokhorst HM, Lamme T, de Smet M. Primary tumor cells of myeloma patients induced interleukin-6 secretion in long-term bone marrow cultures. *Blood* 1994; 84: 2269-2277

Lokhorst HM, Leibowitz D. Adoptive T-cell therapy. *Sem Hematol* 1999; 36: 26-29

Lopes de Menezes DE, Pilarski LM, Allen TM. In vitro and in vivo targeting of immunoliposomal doxorubicin to human B-cell lymphoma. *Cancer Res* 1998; 58: 3320-30

Ludwig H, Fritz E, Peest D. A plasma clot culture system for growing and antiproliferative drug sensitivity testing of myeloma stem cells. *Leuk Res* 1984; 8: 702-711

MacLennan ICM. In which cells does neoplastic transformation occur in myelomatosis ? *Curr Topics Microbiol Immunol* 1992; 182: 209-213

Maguer-Satta V, Petzer AL, Eaves AC, Eaves CJ. BCR-ABL expression in different subpopulations of functionally characterized Ph+ CD34+ cells from patients with chronic myeloid leukemia. *Blood* 1996 Sep 1;88(5):1796-804

Majumdar MK, Thiede MA, Mosca JD, MoormanM, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cellul Physiol* 1998; 176: 57-66

Maloney DG, Donovan K, Hamblin TJ. Antibody therapy for treatment of multiple myeloma. *Sem Hematol* 1999; 36: 30-33

Mandelli F, Avvisati G, Amadori S. Maintenance therapy with recombinant interferon-alpha-2b in patients with multiple myeloma responding to conventional induction chemotherapy. *N Engl J Med* 1990; 322: 1430-1434

Marshal E. Clinical trials: Gene therapy death prompts review of adenovirus vector. *Science* 1999; 286: 2244-45

Martin R, Raidl M, Hofer E, Binder BR. Adenovirus-mediated expression of green fluorescent protein. *Gene Ther* 1997; 4: 493-495

Martin-Henao GA, Ingles-Esteve J, Cancelas JA, Garcia J. Isolation of CD34+ hematopoietic progenitor cells in chronic myeloid leukemia by magnetic activated cell sorting (MACS). *Bone Marrow Transplant* 1996; 18: 603-9

Mathias P, Wickam T, Moore M, Nemerow G. Multiple adenovirus serotypes use alpha-V integrins for infection. *J Virol* 1994; 68: 6811

Meeker TC, Lay LT, Wroblewski JM, Turturro F, Li Z, Seth P. Adenoviral vectors efficiently target cell lines derived from selected lymphocytic malignancies, including anaplastic large cell lymphoma and Hodgkin's disease. *Clin Cancer Res* 1997; 3: 357-364

Mendiratta SK. Kovalik JP. Hong S. Singh N. Martin WD. Van Kaer L. Peptide dependency of alloreactive CD4+ T cell responses. *Intl Immunol* 1999; 11: 351-60

Michaeli J, Choy CG, Zhang X. The biological features of multiple myeloma. *Cancer Invest* 1997; 15: 76-84

Millar BC, Bell JBG, Lakhani A, Ayliffe MJ, Selby PJ, McElwain TJ. A simple method for culturing myeloma cells from human bone marrow aspirates and peripheral blood *in vitro*. *Br J Haematol* 1988; 69: 197-203

Miller AR, McBride WH, Hunt K, Economou JS. Cytokine-mediated gene therapy for cancer. *Ann Surg Oncol* 1994; 1: 436-450

Moore PS, Boshoff C, Weiss R, Chang Y. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* 1996; 274: 1739-

Mundy GR. Evidence for the secretion of an osteoclast stimulating factor in myeloma. *N Engl J Med* 1974; 291: 1041-

Munshi NC. Immunoregulatory mechanisms in multiple myeloma. *Hematol Oncol Clinics North Am* 1997; 11: 51-69

Murphy JJ, Tooze J, Millard RE, Hudson L. Analysis and isolation of leukaemic and residual normal B lymphocyte populations from patients with chronic lymphocytic leukaemia. *Clin Exp Immunol* 1987; 68: 669-76

Nabel EG, Gordon D, Yang Z-Y, Xu L, San H, Plautz GE, Wu BY, Gao X, Huang L, Nabel GJ. Gene transfer in vivo with DNA-liposome complexes: Lack of autoimmunity and gonadal localization. *Hum Gen Ther* 1992; 3: 649-656

Nable GJ, Nable EG, Yang ZY, Fox BA, Plautz GE, Gao X, Huang L, Shu S, Gordon D, Chang AE. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biological activity, and lack of toxicity in humans. *Proc Natl Acad Sci USA* 1993; 90: 11307-11311

Nabel GJ, Gordon D, Bishop DK, Nickoloff BJ, Yang Z-Y, Aruga A, Cameron MJ, Nabel EG, Chang AE. Immune response in human melanoma after transfer of an allogeneic class I major histocompatibility complex gene with DNA-liposome complexes. *Proc Natl Acad Sci USA* 1996; 93: 15388-15393.

NCIC (National Cancer Institute of Canada): Canadian Cancer Statistics 2001, Toronto, Canada, 2001

Nenerow GR. Adenoviral vectors – new insights. *Trends Microbiol* 2000; 8: 391-393

Nishimoto N, Yoshihito S, Yoshizaki K, Kishimoto T. Myeloma biology and therapy. *Hematol Oncol Clinics North Am* 1997; 11: 159-172

Ohno T, Yang Z, Ling X, Jaffe M, Nabel EG, Normolle D, Nabel GJ. Combination gene transfer to potentiate tumor regression. *Gene Ther* 1997; 4: 361-366.

Ohtsuki T, Yawata Y, Wada H, Sugihara T, Mori M, Namba M. Two human myeloma cell lines, amylase-producing KMS-12-PE and amylase-non-producing KMS-12-BM, were established from a patient, having the same chromosome marker, t(11;14)(q13;q32). *Br J Haematol* 1989; 73: 199-204

Osanto S, Brouwenstyn N, Vaessen N. Immunization with interleukin-2 transfected melanoma cells. A phase I-II study in patients with metastatic melanoma. *Hum Gene Ther* 1993; 4: 323-330

Pellat-Deceunynk C, Amiot M, Bataille R, Van Riet I, Van Camp B, Omede P, Boccadoro M. Human myeloma cell lines as a tool for studying the biology of multiple myeloma: a reappraisal 18 years after. *Blood* 1995; 86: 4001-2

Pellat-Deceunynk C, Jego G, Harousseau JL, Vie H, Bataille R. Isolation of human lymphocyte antigens class I-restricted cytotoxic T lymphocytes against autologous myeloma cells. *Clin Cancer Res* 1999; 5: 705-709

Pelliniemi TT, Irjala K, Mattile K. Immunoreactive interleukin-6 and acute pahse proteins as prognostic factors in multiple myeloma. *Blood* 1995; 85: 765-

Plauzt GE, Yang Z-Y, Wu B-Y, Gao X, Huang L, Nabel GJ. Immunotherapy of malignancy by in vivo gene transfer into tumors. *Proc Natl Acad Sci USA* 1993; 90: 4645-4649

Plautz GE, Nable EG, Fox B, Yang ZY, Jaffe M, Gordon D, Chang A, Nabel GJ. Direct gene transfer for understanding and treatment of human disease. *Ann N Y Acad Sci* 1994; 716: 144-153

Pope IM, Poston GJ, Kinsella AR. The role of bystander effect in suicide gene therapy. *Eur J Cancer* 1997; 33: 1005-1016

Portier M, Rajzbaum G, Zhang XG, Attal M, Rusalen C, Wijdenes J, Mannoni P, Maraninchi D, Piechaczyk M, Bataille R. *In vivo* interleukin 6 gene expression in the tumoral environment in multiple myeloma. *Eur J Immunol* 1991; 21: 1759-1762

Pottern LM, Gart JJ. HLA and multiple myeloma among black and white men: Evidence of a genetic association. *Cancer Epidemiol Biomarkers Prev* 1992; 1: 177-182

Prince HM, Dessureault S, Gallinger S, Krajden M, Sutherland DR, Addison C, Zhang Y, Graham FL, Stewart AK. Efficient adenovirus-mediated gene expression in malignant hurnan plasma cells: relative lymphoid resistance. *Exp Hematol* 1998; 26: 27-36

Pugh RE, Bitter MA, Shpall EJ, Hami LS, Wolf DM, Franklin WA. CD19 selection improves the sensitivity of B cell lymphoma detection. *J Hematother* 1998; 7: 159-68

Ramarathinam L, Castel M, Wu Y, Liu Y. T-cell costimulation by B7/BB1 induces CD8 T cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. *J Exp Med* 1994; 179: 1205-

Rao M, Alving CR. Delivery of lipids and liposomal proteins to the cytoplasm and Golgi of antigen-presenting cells. *Adv Drug Deliv Rev* 2000; 41: 171-88

Rettig MB, Ma HJ, Vescio RA, Pold M, Schiller G, Belson D, Savage A, Nishikubo C, Fraser J, Said JW, Berenson JR. KSHV infection of bone marrow dendritic cells from multiple myeloma patients. *Science* 1997; 276: 1851-1854

Rhodes EGH, Colleen O, Flynn MP. A serum-free culture method for myeloma progenitors *in vitro*: proliferative and immunophenotypic characteristics. *Exp Hematol* 1990; 18: 79-83

Roelvink PW, Mi Lee G, Einfeld DA, Kovesdi I, Wickham TJ. Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science* 1999; 286: 1568-71

Romano G, Pacilio C, Giordano A. Gene transfer technology in therapy: Current applications and future goals. *The Oncologist* 1998; 3: 225-236

Rubin J, Galanis E, Pitot HC, Richardson RL, Burch PA, Charboneau JW, Reading CC, Lewis BD, Stahl S, Akporiaye ET, Harris DT. Phase I study of immunotherapy of hepatic metastases of colorectal carcinoma by direct gene transfer of an allogeneic histocompatibility antigen, HLA-B7. *Gene Ther* 1997; 4: 419-425.

Ruiz-Argüelles G, San Miguel JF. Cell surface markers in multiple myeloma. *Mayo Clin Proc* 1994; 69: 684-690

Said JW, Rettig MB, Heppner K, Vescio RA, Schiller G, Ma HJ, Belson D, Savage A, Shintaku IP, Koeffler HP, Asou H, Pinkus G, Pinkus J, Schrage M, Green E, Berenson R. Localization of KSHV in bone marrow biopsy samples from patients with multiple myeloma. *Blood* 1997; 90: 4278-4282

Schenborn ET, Goiffon V. Calcium phosphate transfection of mammalian cultured cells. *Methods Mol Biol* 2000; 130:135-45.

Scibienski RJ, Paglieroni T, Caggiano V, Lemongello D, Gumerlock PH, Mackenzie MR. Factors affecting the *in vitro* evolution of a myeloma cell line. *Leukemia* 1992; 6: 940-

Sheridan CA. Multiple myeloma. *Sem Oncol Nurs* 1996; 12: 59-69

Shimizu S, Yoshioka R, Hirose Y, Sugai S, Tachibana J, Konda S. Establishment of two interleukin-6 (B cell stimulatory factor 2/interleukin β2) –dependent human bone marrow-derived myeloma cell lines. *J Exp Med* 1989; 169: 339-

Shtil AA, Turner JG, Durfee J, Dalton WS, Hua Y. Cytokine-based tumor cell vaccine is equally effective against parental and isogenic multidrug-resistant myeloma cells: The role of cytotoxic T lymphocytes. *Blood* 1999; 93: 1831-1837

Simoes S, Slepushkin V, Gaspar R, Pedroso de Lima MC, Duzgunes N. Successful transfection of lymphocytes by ternary lipoplexes. *Biosci Rep* 1999; 19: 601-9

Sjak-Shie NN, Vescio RA, Berenson JR. HHV-8 infection and multiple myeloma. *J Leuk Biol* 1999; 66: 357-60

Stevenson GT, Glennie MJ, Hamblin TJ. Antibody treatment of lymphoma: Experience and prospects, in Tourraine JL(ed), Cancer in transplantation: Prevention and Treatment. Amsterdam, the Netherlands, Kluwer. 1996: 289-297

Stewart AK, Prince HM, Cappe D, Chu P, LutzkoC, Sutherland DR, Dube I. *In vitro* maintenance and retroviral transduction of human myeloma cells in long-term marrow cultures. *Cancer Gene Ther* 1997; 4: 148-156

Stopeck AT, Hersh EM, Akporiaye ET, Harris DT, Grogan T, Unger E, Warneke J, Schluter SF, Stahl S. Phase I study of direct gene transfer of an allogeneic

histocompatibility antigen, HLA-B7, in patients with metastatic melanoma. *J Clin Oncol* 1997; 15: 341-349.

Svenatsu S, Hibi M, Sugita T. IL-6 and IL-6R in myeloma plasmacytoma. *Curr Top Microbiol Immunol* 1988; 166: 13-22

Tai Y-T, Teoh G, Shima Y, Chauhan D, Treon SP, Raje N, Hideshima T, Davies FE, Anderson KC. Isolation and characterization of human multiple myeloma cell enriched populations. *J Immunol Methods* 2000; 235: 11-19

Takahira H, Kozuru M, Hirata J, Obama K, Uike N, Iguchi H, Miyamura T, Yamashita S, Kono A, Umemura T. Establishment of a human myeloma cell line with growth-promoting activity for bone marrow-derived fibroblastoid colony-forming units. *Exp Hematol* 1994; 22: 261-266

Tarte K, Chuang Y, Klein B. Kaposi's sarcoma-associated herpesvirus and multiple myeloma: Lack of criteria for causality. *Blood* 1999; 93: 3159-3166

Tarte K, Zhang XG, Legouffe E, Hertog C, Mehtali M, Rossi J-F, Klein B. Induced expression of B7-1 on myeloma cells following retroviral gene transfer results in tumor-specific recognition by cytotoxic T cells. *J Immunol* 1999; 163: 514-524

Tefferi A, Schad CR, Pruthi RK, Ahmann GJ, Spurbeck JL, Dewald GW. Fluorescent in situ hybridization studies of lymphocytes and neutrophils in chronic granulocytic leukemia. *Cancer Genet Cytogenet* 1995; 83: 61-4

Teoh G, Chen L, Urashima M, Tai Y-T, Celi LA, Chen D, Chauhan D, Ogata A, Finberg RW, Webb IJ, Kufe DW, Anderson KC. Adenovirus vector-based purging of multiple myeloma cells. *Blood* 1998; 92: 4591-4601

Treon SP, Mollick JA, Urashima M, Teoh G, Chauhan D, Ogata A, Raje N, Hilgers JHM, Nadler L, Belch A, Pilarski LM, Anderson K. Muc-1 core protein is expressed on multiple myeloma cells and is induced by dexamethasone. *Blood* 1999; 93: 1287-1298

Troy AJ, Hart DNJ. Dendritic cells and cancer: progress toward a new cellular therapy. *J Hematother* 1997; 6: 523-533

Van Baren N, Brasseur F, Godelaine D, Hames G, Ferrant A, Lehmann F, Andre M, Ravoet C, Doyen C, Spagnoli GC, Bakkus M, Thielemans K, Boon T. Genes encoding tumor-specific antigens are expressed in human myeloma cells. *Blood* 1999; 94: 1156-1164

Verfaillie CM, Miller WJ, Boylan K, McGlave PB. Selection of benign primitive hematopoietic progenitors in chronic myelogenous leukemia on the basis of HLA-DR antigen expression. *Blood* 1992; 79: 1003-10

Vilpo J, Vilpo L, Hulkkonen J, Lankinen M, Kuusela P, Hurme M. Non-specific binding compromises the purification yields of leukemic B-cells in chronic lymphocytic leukemia: prevention by collagen coating. *Eur J Haematol* 1998; 60: 65-67

Wang Q, Finer M. Second-generation adenovirus vectors. *Nature Med* 1996; 2: 714-716

Wattel E, Vanrumbeke M, Abina MA, Cambier N, Preudhomme C, Haddada H, Fenaux P. Differential efficacy of adenoviral mediated gene transfer into cells from hematological cell lines and fresh hematological malignancies. *Leukemia* 1996; 10: 171-174

Weaver JC. Electroporation of cells and tissues. *IEEE Transac Plasma Sci* 2000; 28: 24-33

Wells DA, Sale GE, Shulman HM, Myerson D, Bryant EM, Gooley T, Loken MR. Multidimensional flow cytometry of marrow can differentiate leukemic from normal lymphoblasts and myeloblasts after chemotherapy and bone marrow transplantation. *Am J Clin Pathol* 1998; 110: 84-94

Wells AW. Cancer vaccines mades to order. *HMS Beagle: The BioMedNet Magazine* (http://www.biomednet.com/hmsbeagle/76/notes/profile) 2000; 76

Wendtner CM, Nolte A, Mangold E, Buhmann R, Maass G, Chiorini JA, Winnacker EL, Emmerich B, Kotin RM, Hallek M. Gene transfer of costimulatory molecules B7-1 and B7-2 into human multiple myeloma cells by recombinant adeno-associated virus enhances the cytolytic T cell response. *Gene Ther* 1997; 4: 726-735

Westendorf JJ, Ahmann GJ, Greipp PR, Witzig TE, Kyle RA, Lust JA, Jelinek DF. Establishment and characterization of three myeloma cell lines that demonstrate variable cytokine responses and abilites to produce autocrine interleukin-6. *Leukemia* 1996; 10: 866-876

Wickam TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* 1993; 73: 309-

Wijdenes J, Voojis WC, Clement C, Post J, Morard F, Vita N, Laurent P, Sun RX, Klein B, Dore JM. A plasmacyte selective monoclonal antibody (B-B4) recognizes syndecan-1. *Br J Haematol* 1996; 94: 318-323

Yeh P, Perricaudet M. Advances in adenoviral vectors: from genetic engineering to their biology. *FASEB J* 1997; 11: 615-623

Yi Q, Dabadghao S, Österborg A, Bergenbrant S, Holm G. Myeloma bone marrow plasma cells: Evidence for their capacity as antigen-presenting cells. *Blood* 1997; 90: 1960-1967

Zhang XG, Klein B, Bataille R. Interleukin-6 is a potent myeloma cell growth factor in patients with aggressive multiple myeloma. *Blood* 1989; 74: 11-

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