MHC-UNRESTRICTED MUC1-SPECIFIC T CELL RECEPTOR FOR CANCER IMMUNOTHERAPY/GENE THERAPY

by

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MUC1 glycoprotein is overexpressed on the surface of a variety of epithelial tumors and has been under investigation as a target for immunotherapy. A number of cytotoxic lymphocyte clones were generated in our laboratory from breast and pancreatic cancer patients that recognized MUC1 on the surface of tumor cells in a TCR-mediated MHC-unrestricted manner. The purpose of this study was to test the feasibility, efficacy and safety of using MHC-unrestricted MUC1-specific T cell receptor (TCR) gene transfer as a tool for cancer immunotherapy. The TCR α and β chains were cloned from one MHC-unrestricted MUC1-specific CTL clone (MA). Various configurations of chimeric TCRs were constructed and were expressed on the surface of a variety of cell lines in vitro. The TCR-deficient T cell line, Jurkat JRT3.5, transfected with the TCR α and β chains from MA CTL clone fluxed calcium in response to stimulation by a MUC1+ pancreatic human tumor, HPAF. BWZ murine thymoma cells transfected with a single-chain TCR (scTCR) consisting of the TCR extracellular domain and the CD3 ζ signaling domain) were triggered to secrete IL-2 in response to stimulation with different MUC1+ tumor cells. The tumor recognition and rejection functions of this scTCR were tested in vivo when SCID mice were reconstituted with bone marrow (BM) cells transduced with scTCR-MFG retroviral supernatant and challenged with HPAF tumor cells. Tumor growth in mice
reconstituted with scTCR-transduced BM cells was significantly slower (P<0.05) than that seen in the control group. Tumor sections from TCR-reconstituted mice were infiltrated by neutrophils and macrophages, and to lesser extent, by NK cells. FACS analyses showed that BM cells transduced with scTCR-MFG could differentiate \textit{in vivo} into multiple immune lineages including T cells, B cells, granulocytes, monocytes and NK cells that express the scTCR. The scTCR was expressed on higher percentages of cells of the innate immune system when compared to T and B cells. Human MUC1 transgenic (Tg.) mice reconstituted with BM cells transduced with this MUC1-specific TCR did not show any signs of autoimmunity, abnormal cellular infiltration or destruction of MUC1-expressing tissues. Transduction of BM with tumor-specific TCR represents a potentially efficacious gene therapy/immunotherapy approach. MUC1-specific MHC-unrestricted TCR will make this treatment applicable to all cancer patients with MUC1+ tumors, regardless of their HLA type.
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Nehad M. Alajez
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<th>Abbreviation</th>
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<tr>
<td>ADCC</td>
<td>Antibody Dependant Cellular Cytotoxicity</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation Induced Cell Death</td>
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<td>APCs</td>
<td>Antigen Presenting Cells</td>
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<td>BM</td>
<td>Bone Marrow</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CDRs</td>
<td>Complementary Determinant Regions</td>
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<tr>
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<td>Carcinoembryonic Antigen</td>
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<td>GM-CSF</td>
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<td>Horseradish Peroxidase</td>
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<td>ICAM-1</td>
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<td>IFN</td>
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<td>RAGE</td>
<td>Renal Tumor Antigen</td>
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<td>RBL</td>
<td>Rat Basophilic Leukemia</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse-Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>SA</td>
<td>Streptavidin</td>
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<td>Sca-1</td>
<td>Stem Cell Antigen-1</td>
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<td>SCID</td>
<td>Severe Combined Immunodeficient</td>
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<tr>
<td>ScTCR</td>
<td>Single Chain T Cell Receptor</td>
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<td>SCF</td>
<td>Stem Cell Factor</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>SscFV</td>
<td>Soluble Single Chain Fraction Variable</td>
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<tr>
<td>SscTCR</td>
<td>Soluble Single Chain T Cell Receptor</td>
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<td>TAM</td>
<td>Tumor Associated Macrophages</td>
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<td>TCR</td>
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<td>Tg.</td>
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<td>Th</td>
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<td>TIL</td>
<td>Tumor Infiltrating Lymphocytes</td>
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<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>VLA</td>
<td>Very Late Antigen</td>
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1. INTRODUCTION

1.1. THE IMMUNE SYSTEM

1.1.1. Overview

The immune system is a collection of interdependent cell types that protect us from outside invaders, such as bacteria and viruses, and from abnormal cell growth from within, such as cancer cells, while at the same time tolerating normal tissues and organs. The balance between the capability of the immune system to recognize dangerous invaders and to induce vigorous immune responses against them, without any immune response against self, is what governs the proper function of the immune system. Improper activation of the immune system can result in autoimmunity, while inadequate activation can result in uncontrolled infection.

The discipline of immunology began as a branch of microbiology; it grew out of the study of infectious diseases and the body’s responses to them. Because microorganisms come in different forms, a wide variety of immune responses are required to deal with them. In general, immune responses fall into two main categories (i) adaptive and (ii) innate. T and B cells are the key players in adaptive immunity. Adaptive immunity is highly specific for a particular antigen and the response improves with each subsequent encounter with the same antigen. In contrast, innate immunity has limited specificity and does not improved upon second encounter with the same antigen. Innate immunity is
mediated by several cell types including macrophages, granulocytes, monocytes and NK cells. Innate immunity represents the first line of host defense against invading pathogens until specific immune response is generated.

Our understanding of how the immune system functions based on the tremendous knowledge of molecular biology and immunology acquired over the past decades, has led to various attempts by immunologists to manipulate the immune system; either to augment the immune response to protect the host from infections and cancers or to suppress pathologic immune response against self antigens to prevent autoimmune diseases.

1.1.2. Mechanisms of tolerance to self antigens

For most of the 20th century it was generally believed that the immune response could only be induced against foreign antigens and not against autologous tissue, based on the fact that self-reactive T lymphocytes are normally deleted during their development in the thymus. T lymphocytes capable of binding to self MHC are positively selected on thymic cortical epithelial cells. Positively selected T lymphocytes with high affinity receptors for self peptides in the context of self MHC are then deleted on bone marrow-derived antigen presenting cells (APCs) during the process of negative selection (Kisielow et al., 1988). However, some T lymphocytes with low affinity to self peptide-MHC complex (pMHC) may be spared (Teh et al., 1989). Since there are some self-antigens that are not expressed in the thymus (e.g., developmental stage antigens), potentially self-reactive T lymphocytes may survive the process of negative selection and migrate to the periphery. In fact, the presence of autoreactive T cells has been demonstrated in the repertoire of healthy individuals (Pette et al., 1990). The presence of efficient mechanisms
to regulate activation of peripheral T cells with autologous specificity is crucial to the prevention of autoimmune disease. Peripheral tolerance can be achieved by a variety of mechanisms, including deletion by apoptosis, anergy, T cell ignorance, and control by regulatory T cells (Stockinger, 1999). Prolonged exposure of high-affinity autoreactive T lymphocytes to self-antigens in the periphery can result in deletion via activation induced cell death (AICD) and apoptosis. For proper activation of naive T cells, signaling through costimulatory molecules is required in addition to signaling through the TCR. When self-reactive T cells encounter their specific antigen in the periphery, in the absence of costimulatory molecules, these cells enter a state of unresponsiveness termed anergy. A number of tissues (e.g. testes, brain, etc) are considered immunologically privileged sites, because they are not accessible to the immune system. In addition, most self-proteins are expressed at a level too low to be recognized by self-reactive T cells and, are thus usually ignored by the immune system. A subset of CD4+ T cells, referred to as regulatory T cells, was recently identified and been found to have the potential to actively suppress self-reactive T cells (Shevach, 2000). All these different mechanisms of peripheral tolerance ensure that self-reactive T cells are either deleted or remain inactive under normal circumstances. Since the majority of tumor antigens are normal antigens that are overexpressed on tumor cells, a fundamental goal of “tumor vaccinology” is to break down self-tolerance against these cryptic epitopes and to induce vigorous anti-tumor immune response. One of the major limitations for tumor vaccines is that the anti-tumor immune response could potentially result in the destruction of normal tissues expressing the same antigen (Ludewig et al., 2000).
1.1.3. MHC-restricted recognition of antigens by T cell

Although TCRs and antibodies are both assembled by genetic recombination of various V, D, J, and C gene segments, only antibodies undergo somatic hypermutation to increase their affinity for antigens. While antibodies can recognize a broad spectrum of different antigens including proteins, carbohydrates, and DNA (Wilson and Stanfield, 1994), TCRs, on the other hand, recognize small peptides presented by MHC molecules (Matsui et al., 1991). CD4+ T cells generally recognize exogenous peptides presented by MHC class II molecules, while CD8+ T cells generally recognize endogenous peptides presented by MHC class I molecules. The affinity of interaction between the TCR and peptide-MHC (pMHC) complex is very weak when compared to that of the interaction between the antibody and antigen. The pMHC binding site on the TCR is contributed by three complementary determinant regions (CDRs) in the variable domains of the TCRα and β chains (Hennecke and Wiley, 2001). The TCR/pMHC complex interaction is topologically constrained in such a way that the TCR Vα region interacts with the N-terminus of the peptide while the TCR Vβ region interacts with the C-terminus of the peptide. The affinity of interaction between the TCR αβ heterodimer and pMHC complex is mainly due to the direct interaction between the CDR loops of both chains and the peptide, with minimal contribution of the interaction between the TCR and MHC molecule itself (Wu et al., 2002). This has led to the hypothesis that TCR recognition of pMHC complex is a two-step event in which the interaction is initiated by the weak interaction between the TCR and MHC molecule, which then gets stabilized if the TCR interacts with the appropriate peptide in MHC groove. Theoretically, each TCR is capable of recognizing as many as $3 \times 10^5$ different pMHC in order to compensate for the large gap between the
available TCR repertoire within an individual and the much larger pool of pMHC complexes that could potentially be presented within the same individual (Mason, 1998). This had led to the concept that TCR recognition of pMHC is promiscuous; therefore the same TCR can bind to different pMHC complexes with variable affinities. This interaction is further stabilized by the engagement of other accessory molecules, such as CD8, CD4, CD2, CD28/CTLA-4, on T cells and their cognate ligands on target cells, such as MHC I, MHC II, CD48, and B7.1/B7.2, respectively. Whether T cell engagement with a target cell leads to a functional activation or not depends on the overall avidity and duration of the interaction between the T cell and its target.

1.1.4. MHC-unrestricted recognition of antigens by T cell

MHC-unrestricted recognition of antigens by T cells has also been described. TCR γδ+ T cells obtained from the synovial fluid of rheumatoid arthritis (RA) patients exhibited an MHC-unrestricted recognition of targets derived from RA synovium (Ohta and Sato, 1994) as well as mycobacterium (MT) antigens (Holoshitz et al., 1989). γδ T cells that recognized soluble MT antigens could be generated from mice immunized with MT (Janis et al., 1989). An idiootype-specific γδ T cells were established from a patient with Burkitt’s lymphoma (Wright et al., 1989). TCR γδ+ T cells that recognized herpes simplex virus type I transmembrane glycoprotein, gI, in an MHC-independent fashion, have been described as well (Sciammas et al., 1994). MHC-unrestricted recognition of non-peptide antigens by γδ T cells has previously been reported (Morita et al., 1995). MHC-unrestricted recognition of antigens by αβ T cells has been described as well. TCR αβ+ T cell lines specific for avidin or myelin basic protein (MBP) could respond to stimulation with native antigens in the
absence of APCs (Altmann et al., 1987). TCR αβ+ T cells that recognized the heme moiety of hemoglobin in an MHC-unrestricted fashion were generated by in vitro priming of mouse spleen cells with hemin (Sherman and Lara, 1989). Arsonate hapten-specific TCR αβ+ T cells could also recognize hapten-carrier proteins in the absence of APCs (Rao et al., 1984). TCR αβ+ T cell clones specific for the hapten fluorescein isothiocyanate (FITC) could also be activated with a multivalent form of the hapten in the absence of MHC presentation (Siliciano et al., 1986). Carbohydrate specific TCR αβ+ T cells have also been described (Abdel-Motal et al., 1996). These CTLs were specific for carbohydrate and were not restricted to particular MHC molecule.

1.2. TUMOR IMMUNOLOGY

1.2.1. History

It has been more than a century since the initial work done by William Coley in the early 1890s demonstrated that activation of the immune system with bacterial toxins resulted in tumor regression (Chamberlain and Kaufman, 2000). However, the first direct evidence that the immune system can recognize and reject tumor cells came from tumor rejection experiments in outbred animal strains and across species. These data clearly demonstrated that allogeneic and xenogeneic immune responses are capable of rejecting tumor cells; however these studies were not informative about the nature of tumor-specific immune responses. For a long time, it was generally believed that tumor cells are non-immunogenic in the host from which they arose. Then, elegant work by Klein in the early 1960s demonstrated that chemically or radiation-induced tumor cells can be rejected in
inbred animal strains that had previously been exposed to the same tumor cells (Klein at al. 1961). This was the first time that the concepts of specificity and memory of immune responses against cancers were introduced. Advances in molecular biology and in immunology over the past several decades have contributed tremendously to our understanding of the specific immune mechanisms underlying immune recognition of cancer cells.

1.2.2. Immunological surveillance theory

In the early 1970s, the concept of surveillance of tumor cells by the immune system emerged, following the demonstration that the immune system was capable of recognizing and rejecting transplantable tumors (Burnet, 1970). This theory proposed that if a tumor arose from a single cell that expressed an antigen that could be recognized by the immune system, then an intact immune system should be able to recognize and eliminate this tumor. Soon after it emerged, the immunological surveillance theory was challenged by the observation that nude mice, athymic mice that lack T cells, did not display higher rates of spontaneous tumors when compared to wild-type mice (Stutman, 1979). However, it is important to point out that nude mice do not completely lack the T-cell compartment, and that those mice still have an intact innate immune system. The immunological surveillance theory was supported by the observation that patients with various forms of immunodeficiencies have a higher incidence of malignancies than immunocompetent individuals (McClain, 1997). Another observation that supported the immunological surveillance theory came from the finding that mice deficient in IFN-γ have a higher incidence of spontaneous and carcinogen-induced tumors than wild-type mice (Kaplan et
A similar observation was made in mice lacking the rag-2 gene, which results in a lack of competent T and B cell compartments (Shankaran et al., 2001). Whether immunological surveillance exists in vivo or not; there is a compelling evidence that various cells of the immune system can recognize and control tumor growth in vivo.

1.2.3. Adaptive immune responses against cancer

The most direct evidence for the involvement of T cells in anti-tumor immunity came from the isolation of a tumor-specific, MHC-restricted CD8+ T cells infiltrating solid tumors (Topalian and Rosenberg, 1990). These tumor infiltrating lymphocytes (TILs) could be grown in culture and could lyse autologous tumor cells and tumors from HLA-matched individuals (Kawakami et al., 1992). These data, in addition to other observations by many groups over the last decade, led to the conclusion that T cells play a critical role in immune responses against tumor cells.

B cells are also considered an important component of anti-tumor immunity. Antibodies secreted by B cells can facilitate tumor lysis by antibody dependent cellular cytotoxicity (ADCC) or by activating the complement system (Naundorf et al., 2002). The importance of the humoral immune response in cancer immunity is further supported by the observation that some cancer patients do have antibody response against tumors (Disis et al., 1994). In the majority of breast cancer patients, no correlations between tumor regression and the presence of natural antibodies against tumors were found (Coronella-Wood and Hersh, 2003). However, an objective response was seen in 11.6% of breast cancer patients who received a humanized antibody against HER2/neu proto-oncogene (Herceptin) in a phase II clinical trial (Baselga et al., 1999).
1.2.4. **Innate immune responses against cancer**

Following their initial characterization as a unique subset of lymphocytes, the role of NK cells in tumor control was proposed (Herberman et al., 1975). Subsequently, it was demonstrated that NK cells can kill targets via ADCC (Koren and Williams, 1978). Later on, it was shown that nude mice inoculated with tumor cells had elevated levels of interferons (IFNs), which was followed by rapid increase in NK cells activity (Djeu et al., 1980). The role of NK cells in tumor immune surveillance was demonstrated recently when mice deficient in perforin, but not mice deficient in CD8+ T cells, had significantly higher rates of carcinogen-induced malignancies (van den Broek et al., 1996). NK cells have a unique antigen recognition system. The “missing self” theory of recognition by NK cells evolved following the demonstration that NK cells preferentially kill targets that have low levels of MHC class I molecules (Karre et al., 1986). This killing can be inhibited by transfecting MHC class I molecule into tumor cells (Hoglund et al., 1993). This recognition of “missing self” by NK cells is mediated by a family of inhibitory receptors on the surface of NK cells that can interact with MHC class I molecule on the surface of target cells. Normal cells express high levels of MHC class I molecules which interact with inhibitory receptors on NK cells, and thereby inhibit NK cell killing; while class I-deficient targets cannot do that and are generally more susceptible to NK-mediated lysis. Three families of inhibitory receptors have been identified on the surface of NK cells in mice and humans. The Ly49 family (in mouse), the KIR family (in humans) and the CD94/NKG2A family in both mice and humans (Karlhofer et al., 1992; Wagtmann et al., 1995; Brooks et al., 1997). Ly49 and KIR bind directly to classical MHC class I molecules, while the CD94/NKG2A heterodimer interacts with a peptide from the leader sequence of MHC class I molecules.
presented in the context of a non-classical MHC class I molecule (Qa-1 in mouse and HLA-E in human, Vance et al., 1998; Braud et al., 1998).

The other category of receptors on NK cells are the activating receptors. NKG2D is an activating receptor that is expressed by NK cells, activated and memory CD8+ αβ T cells, some γδ T cells in mice and humans, and by activated macrophages in mice (Jamieson et al., 2002; Bauer et al., 1999; Raulet, 2003). The ligands for NKG2D are molecules that are up-regulated on the cell surface of stressed cells, virally infected cells; and these molecules are also expressed on tumor cells. One group of ligands for NKG2D on human cells is the MHC class I-related protein A and B (MICA/B, Raulet, 2003). NKG2D receptors on NK cells function by interacting with DAP10 or DAP12 signaling adaptors and activation of the PI-3 kinase signaling pathway, which can result in activation of NK cells, release of granzymes, and eventually results in tumor cell lysis.

The role of tumor associated macrophages (TAM) in tumor immunity is still controversial. TAMs have high levels of MHC class I and MHC class II molecules that enable them to present tumor antigens to CD4+ and CD8+ T cells. In addition, TAMs have an intrinsic cytotoxic activity against tumor cells following their stimulation with IFN-γ (Mantovani et al., 1992). TAMs kill targets via release of TNF-α, IL-1, free radicals, proteases, and nitric oxide (Saio et al., 2001). TAM can also kill target cells via ADCC (te Velde and Figdor, 1992). Despite their intriguing role as anti-tumor effector cells, TAMs can also exert pro-tumor effects by secreting various cytokines that promote tumor growth (Leek and Harris, 2002). The role of other innate immune cells, such as mast cells, eosinophils, neutrophils, and NKT cells in tumor immunity is also well documented. Mast cells purified from human skin were cytotoxic toward a mouse sarcoma cell line (WEHI-164,
Mast cell cytotoxicity toward tumor cells was dependent on TNF-α and was further augmented by crosslinking IgE molecules on the surface of mast cells. Cytotoxic activity of mast cells also correlated with histamine release (Benyon et al., 1991). Eosinophils also have a critical role in immune responses against tumors. Eosinophils are considered a Type2 effector cells, and have been shown to infiltrate tumors in an IL5-dependent fashion (Hung et al., 1998). The cytotoxic activity of eosinophils is mediated by the production of reactive oxygen metabolites and nitric oxide and by degranulation at the tumor site (Reali et al., 2001). The role of polymorphonuclear cells (PMNs) in tumor immunity has been described as well (van Egmond et al., 2001). Activated PMNs co-express the FcγRI and the FcαRI. These receptors can bind to IgG and IgA antibodies, respectively and kill targets via ADCC. Activated PMNs produce several cytotoxic mediators, including reactive oxygen free radicals, proteases, membrane-perforating agents, and soluble mediators of cell killing, such as TNF-α, IL-1β, and IFNs (Di Carlo et al., 2001). Natural killer T (NKT) cells are a distinct T cell population that co-expresses CD3 and NK1.1 (Bendelac et al., 1994). Some NKT cells can recognize glycolipids presented in the context of the non-classical MHC class I-like molecule, CD1d, via an invariant TCR (Vα14Jα281 in the mouse and Vα24JαQ in human, Smyth and Godfrey, 2000). NKT cells are an intriguing population of cells because they can exert pro-tumor (Moodycliffe et al., 2000) or anti-tumor (Terabe et al., 2000) effects in vivo. NKT cells can suppress CD8+ CTLs in an IL4 and IL13 dependent fashion. On the other hand, NKT cells can exert anti-tumor function when stimulated to produce IFN-γ in response to proinflammatory cytokines, such as IL-12.
1.2.5. Immune evasion by tumor cells

Tumor cells utilize a variety of mechanisms to evade recognition by and to suppress functions of cells of the immune system. It is well documented that tumor cells can down-regulate the expression of MHC class I molecules (Bubenik, 2003; Zheng et al., 1999), which renders them invisible to T cells. Tumor cells normally lack expression of B7.1 and B7.2 co-stimulatory molecules that may result in partial activation of tumor-specific T cells and anergy (Banat et al., 2001). In fact, transfection of the B7 gene into tumor cells significantly enhanced their immunogenicity and resulted in complete regression when transplanted into syngeneic mice (Chen et al., 1994). Tumor cells can also lose the expression of a particular tumor antigen under the selective pressure of the immune system. In one study, the expression of melanoma antigen gp100 was significantly decreased (47% vs 34%) in melanoma patients who were vaccinated with gp100-derived peptide in combination with IL-2 (Riker et al., 1999). Tumor cells can express FasL that can induce apoptosis in tumor-reactive T cells (Strand et al., 1996). They also can down-regulate the expression of CD95 (Fas, (Landowski et al., 2001) and can evolve functional defects in the “death receptor” signaling pathways (Shin et al., 2002). In addition, tumor cells can produce a variety of cytokines that can negatively affect the function of immune cells. Vascular endothelial growth factor (VEGF) is one such cytokine produced by tumor cells that was shown to inhibit differentiation and maturation of dendritic cells (DCs, Oyama et al., 1998). Tumor cells can secrete IL-10 (Shurin et al., 2002), TGF-β (Beck et al., 2001), and prostaglandin E2 (Yang et al., 2003), all of which can down-modulate the immune response against tumors.
1.3. TUMOR ANTIGENS RECOGNIZED BY T CELLS

The identification of tumor antigens recognized by T cells began when it became possible to isolate tumor-specific T cell clones from cancer patients and to grow them in culture. Three approaches have been utilized to identify peptides presented by MHC class I molecules for CD8+ T cells. The “genetic” approach involves transfection of cDNA libraries from tumor cells into cell lines that express particular MHC class I molecule followed by screening transfected cell lines for their ability to stimulate CD8+ T cell clones from cancer patients (De Plaen et al., 1997). A second approach involves biochemical purification of peptides eluted from MHC class I molecules followed by testing the ability of these peptides to sensitize target cells for lysis by CD8+ T cell clones (Kao et al., 2001). Fractions containing peptides that have the potential to sensitize targets are then sequenced using mass spectrometry. A third approach is the “reverse immunology” approach which was used successfully to identify immunogenic peptides from various antigens over-expressed by tumor cells (Vonderheide et al., 2001). In this approach, tumor antigens are screened for peptides that have consensus anchor motifs for particular HLA allele, followed by testing the ability of these synthetic peptides to stimulate T cells in vitro. Tumor antigens recognized by T cells can be classified into four major categories (Boon and Old, 1997): (i) Unique tumor antigens which are expressed by a particular tumor as a result of mutation (e.g. cyclin-dependent kinase (CDK) 4 and β-Catenin). Because these antigens are tumor specific and are not expressed on normal tissues, targeting these antigens with immunotherapy normally does not result in autoimmune disease. However, because these antigens are unique for each tumor, these antigens cannot be used as generic targets for immunotherapy. (ii) Shared tumor-specific antigens that are expressed on a
number of different tumors but not on normal adult tissue (e.g. melanoma antigen encoding gene (MAGE-1), renal tumor antigen (RAGE), and MUC1). Some antigens in this category are expressed on melanoma cells; however, expression of RAGE tumor antigen has been detected in >30% of human renal cell carcinoma (Neumann et al., 1998). MAGE antigens are also expressed in testis tissue, but because testis tissue is MHC class I negative, these antigens are not presented by testis cells. MUC1 is a shared tumor antigen that is expressed on a wide variety of epithelial tumors and will be discussed in more depth in later sections. (iii) Tissue-specific differentiation antigens which are expressed on tumors and on normal tissue from which the tumor arose (Kirkin et al., 1998). Examples of this category of tumor antigens are the gp100 and tyrosinase antigens expressed by melanoma cells. Another member of this family is carcinoembryonic antigen (CEA) which is expressed on normal colon epithelia and on most gastrointestinal carcinomas (Hodge et al., 2003). (iv) Virus-associated tumor antigens that are expressed on some virally-induced cancers (e.g. human papilloma virus (HPV) E6 and E7 antigens). These antigens are expressed on the majority (>60%) of human squamous cervical carcinomas and could be recognized by CD4+ and CD8+ T cells (Bubenik, 2002; Santin et al., 1999). Shared tumor-specific antigens are the ideal candidates for cancer immunotherapy.

1.4. MUC1 TUMOR ANTIGEN

MUC1 was first identified in the milk fat globule membrane fraction and was described as a protein rich in serine, threonine, proline, glycine, and alanine (Shimizu and Yamauchi, 1982). It was found to contain a high percentage of O-linked carbohydrates that accounted for about 50% of its molecular weight. In 1987, Gendler and colleagues were
able to clone a fragment from this first human mucin gene by screening a mammary tumor cell line (MCF-7) cDNA library using antibodies raised against a chemically-deglycosylated form of milk mucin (Gendler et al., 1987). The cloned gene, located on chromosome 1q21 (Dekker et al., 2002), was sequenced and found to consist of numerous 60 base pair tandem repeats (Gendler et al., 1988; Siddiqui et al., 1988). Subsequently, cDNAs encoding splice variants of mucin were cloned from breast carcinoma cell lines (Ligtenberg et al., 1990), from human breast tumor tissue (Wreschner et al., 1990; Gendler et al., 1990), and from pancreatic tumors (Lan et al., 1990). This first human mucin gene was given the name MUC1 to replace pre-existing names that included epithelial membrane antigen (EMA, O'Brien et al., 1980), MAM-6 (Hilkens et al., 1984), DF3 antigen (Kufe et al., 1984), polymorphic epithelial mucin (PEM, Girling et al., 1989), polymorphic urinary mucin (PUM, Middleton-Price et al., 1989), and episcopal (Bork and Patthy, 1995).

Additional members of the mucin gene family were numbered in the order they were identified: MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6-9, MUC11-13, and MUC15-17. Mucins have certain structural features in common. They all consist of a peptide core with O-linked glycans attached to serine and threonine residues. The protein core consists of a variable number of repeated sequences (tandem repeats) distinct to each mucin. Mucins exists as secreted forms (gel-forming), membrane-bound forms or both. MUC1, MUC3-4 (Williams et al., 1999b; Moniaux et al., 2000), MUC12-13 (Williams et al., 1999a; Williams et al., 2001) and MUC15-17 (Pallesen et al., 2002; Yin et al., 2002; Gum et al., 2002) can be expressed as membrane-bound glycoproteins. These membrane-bound mucins have a transmembrane domain that facilitates their anchoring in the
membrane lipid bilayer. The rest of the mucins, MUC2, MUC5AC, MUC5B, MUC6-9, and MUC11, can only be expressed in soluble form.

1.4.1. MUC1 on normal and malignant tissue

MUC1 is normally present on the apical surface of most polarized epithelial tissues of the respiratory, genitourinary tract and digestive system (Zotter et al., 1988; Poland et al., 1997). It is also expressed in normal breast ducts. MUC1 is over-expressed on the majority of adenocarcinomas of the breast, lung, colon, pancreas, stomach, prostate, and ovary (Ho et al., 1993). MUC1 expressing cancers account for about 70% of new cancer cases expected in the year 2003 (Jemal et al., 2003).

The forms of MUC1 produced by tumor cells differ in many ways from normal MUC1. As an epithelial cell undergoes malignant transformation, it loses its normal apical-basolateral polarity and begins to express MUC1 on the entire cell surface. MUC1 expressed on tumors cells also lacks normal levels of glycosylation, which results in exposing the peptide backbone of the molecule to the immune system (Figure 1). The level of expression of aberrant MUC1 on tumor cells also increases, and a soluble form of MUC1 can be found in the serum of cancer patients (Beatty et al., 2001).

Similar to MUC1, other members of the mucin family also have an altered expression on different tumors. MUC4 is overexpressed in adenocarcinomas of the lung and expressed de novo in pancreatic and gastric cancers (Nguyen et al., 1996; Balague et al., 1994; Buisine et al., 2000). In one report, about 29% of lung cancer patients had high titers of anti-MUC4 IgG and IgM antibodies (Hanaoka et al., 2001). Over-expression of MUC6 and de novo expression of MUC2, MUC4, and MUC5AC have been demonstrated
on the surface of adenocarcinomas of the pancreas and on pancreatic tumor cell lines (Balague et al., 1994). However, there is limited information about the immunogenicity of these other mucins, and the prognostic significance of their altered expression is not known.
1.5. NATURALLY OCCURRING IMMUNE RESPONSES TO MUC1

1.5.1. In healthy humans

The presence of anti-MUC1 antibodies of IgM and IgG isotypes, as well as of circulating MUC1 antigen in sera from healthy women is well documented (Richards et al., 1998). Agrawal et al (Agrawal et al., 1996) have shown that MUC1-specific T cells can be primed during pregnancy, as T cells from biparous but not nulliparous women proliferated specifically in response to core MUC1 peptides. These findings could be explained by the fact that anatomical and physiological changes of MUC1-expressing organs (like the uterus and breast) during pregnancy and lactation can prompt changes in MUC1 production that eventually trigger priming of B, and possibly T cells to MUC1. Two recent studies (Croce et al., 2001a; Croce et al., 2001b) provided good analyses of antibody responses in healthy women in correlation with their current or previous pregnancy/lactation status. Plasma measurements of free circulating MUC1 as well as of MUC1 complexed with antibodies in immune complexes showed elevated levels in pregnant women, compared to non-pregnant women. During pregnancy, there was a dramatic increase in MUC1 during the second trimester up to puerperium. In contrast, although the levels of immune complexes were gradually increasing, there was a drop in the levels of free anti-MUC1 IgG and IgM antibodies, which reached their lowest value at puerperium and then gradually increased after delivery. Lactation may have also influenced anti-MUC1 antibody production, since the titer of IgG isotype was significantly higher in the lactating group when compared to non-lactating women.

Despite the fact that the studies discussed above provided a good description of the spectrum of anti-MUC1 immune responses arising spontaneously to a “self” molecule, the
significance of this natural immunization to MUC1 remains to be elucidated. Epidemiological studies performed to date suggest a correlation between pregnancy and lower risk of developing breast cancer (MacMahon et al., 1982; Kalache et al., 1993). In that regard, we have reported a case of a long-term breast cancer survivor whose pregnancy might have triggered MUC1-specific immune response that prevented recurrence of tumor (Jerome et al., 1997). The patient was diagnosed with a breast tumor that was successfully removed through a radical mastectomy. Five years later, she became pregnant and developed acute inflammatory cellulites in her remaining breast. Breast tissue from this patient expressed the same MUC1 immunodominant epitope as presented by the original tumor. The subject also had a high titer of circulating anti-MUC1 IgM and IgG antibodies, and a high frequency of MUC1-specific cytotoxic T lymphocytes (CTL) in the blood. She remained tumor-free for an additional 5 years of follow-up. It is possible that secondary immune responses against MUC1 were precipitated by pregnancy and prevented the recurrence of breast cancer. The importance of such natural immunity to MUC1 on the incidence of other cancers (like uterine and ovarian carcinomas) remains to be determined.

**1.5.2. Immune response to MUC1 in cancer patients**

In addition to the above findings that suggest immunization to a self antigen under physiologic conditions, many reports have shown that anti-MUC1 responses could also be triggered in cancer patients during the growth of MUC1+ tumors. In general, tumor cells are poorly recognized by the immune system as a result of tolerance to self-antigens. Moreover, tumor cells can utilize a variety of mechanisms to evade recognition and to suppress cells of the immune system (reviewed earlier in this chapter). Despite these
inhibitory mechanisms, given its characteristics that differentiate it from self, MUC1 made by tumor cells can still trigger humoral and cellular responses in cancer patients, although of low efficiency. Kotera and colleagues have reported anti-MUC1 IgM antibodies in sera from more than 10% of breast, colon, and pancreatic cancer patients (Kotera et al., 1994). The presence of only IgM isotype in these sera indicated a T helper independent anti-MUC1 immune response. Petrarca and colleagues were able to isolate in vivo-primed B cells from the draining lymph nodes of 6 out of 12 patients with epithelial tumors (Petrarca et al., 1999). These B cells secreted anti-MUC1 IgM and IgG antibodies when cultured in vitro. There was strong association between the ability to isolate B cells from these patients and the presence of anti-MUC1 IgM antibodies in their sera. A number of other reports demonstrated the presence of anti-MUC1 IgM and IgG antibodies in sera from patients with ovarian cancer (Snijdewint et al., 1999), pancreatic cancer (Hamanaka et al., 2003), and colorectal cancer (Nakamura et al., 1998).

A strong correlation between the presence of anti-MUC1 antibodies in sera from cancer patients and better prognosis and patient survival has been reported for patients with pancreatic tumors (Hamanaka et al., 2003) and breast tumors (von Mensdorff-Pouilly et al., 1996). Antibodies developed in cancer patients could bind to tumor antigens on the tumor cell surface and mediate complement-dependent cytotoxicity and/or ADCC (Maio et al., 2002). Such mechanisms are able to eliminate circulating tumor cells and micrometastases, as shown in preclinical and clinical studies by Zhang et al., (Zhang et al., 1998).

MUC1 is also recognized by T cells from cancer patients. CTLs that recognized MUC1 on the surface of epithelial tumors were found in the draining lymph nodes of pancreatic cancer patients (Barnd et al., 1989). It was then demonstrated that these CTLs
recognized MUC1 on tumor cells in an MHC-unrestricted manner. These MUC1-specific T cells have α/β TCR and have a CD3+CD8+CD4− phenotype. This MHC-unrestricted recognition of MUC1 could be blocked using an antibody against the immunodominant epitope (APDTRP) in the tandem repeat of the extracellular domain of tumor MUC1 (Jerome et al., 1991). CTLs that recognize MUC1 in an MHC-unrestricted manner were also established from draining lymph nodes of breast cancer patients (Jerome et al., 1991) and from peripheral blood mononuclear cells (PBMCs) from patients with multiple myeloma (Takahashi et al., 1994). Extensive studies of these T cells showed that they have same intracellular signaling events as T cells that recognize conventional pMHC complexes (Magarian-Blander et al., 1998). In fact, these T cells showed a large calcium flux when stimulated with beads coated with a MUC1-derived synthetic 100mer peptide carrying five repeats from the VNTR region and thus carried five APDTRP epitopes. This phenomenon of MHC-unrestricted recognition of MUC1 may be explained by the fact that MUC1 has multiple repeated epitopes that can crosslink several TCRs on T cells (Figure 2). This hypothesis has gained support as the NMR structure of unglycosylated synthetic MUC1 peptide, determined by Fontenot and colleagues (Fontenot et al., 1993 ; Fontenot et al., 1995), revealed the presence of a knob-like structure protruding from the backbone of each MUC1 tandem repeat with the sequence APDTRP at the tip. MUC1-specific antibodies and T cells have increased accessibility to the immunogenic peptide backbone which exhibits shorter carbohydrate side chains on tumor cells, and which are otherwise masked by heavy glycosylation in normal epithelial tissues (Hinoda et al., 1998 ; Noto et al., 1997).
Figure 2. Underglycosylation of MUC1 on tumor cells results in the formation of a knob-like structure in each tandem repeat, which could bind and crosslink multiple TCRs on the surface of T cells, and could result in T cell activation. Featured are cytotoxic T cells (CTL).
DNA sequencing analyses of TCRs from a large number of MHC-unrestricted MUC1-specific CTL clones showed no association of a particular TCR α or β chain with MUC1 recognition (Kirii et al., 1998). However, TCR V\(\beta\)9, V\(\beta\)13.1 and V\(\beta\)17 gene segments were used by more than one clone. More interestingly, sequencing of the CDR3 region of these clones revealed the presence of a glutamine residue in the same position in all sequenced clones. It was then hypothesized that the negatively charged glutamine in the CDR3 region of the TCR interacts with the positively charged arginine in the APDTRP knob on MUC1. The affinity of TCR binding to the APDTRP knob is relatively low; however, the overall avidity of engagement of multiple TCRs to multiple APDTRP epitopes on MUC1 molecule is thought to be of sufficiently high avidity to trigger T cell activation.

T cells that recognized MUC1 via classical MHC class I presentation have also been detected in patients with epithelial tumors. A secondary CD8+ T cell response that recognized the STAPPAHGV peptide from the MUC1 tandem repeat presented by HLA-A11 was detected in the lymph node of an HLA-A11+ breast cancer patient (Domenech et al., 1995). Recently, the LLLLTVLTV peptide derived from MUC1 leader sequence was shown to have high binding affinity for HLA-A*0201 (Brossart et al., 1999). In a study of 84 breast cancer patients and using HLA-A2*0201/LLLLTVLTV tetramer staining technology, the frequency of CD8+ T cells specific for the LLLLTVLTV peptide presented by HLA-A*0201 was as high as 0.9% in BM and PBMCs from these patients (Feuerer et al., 2001).
1.6. MUC1-BASED IMMUNOTHERAPY

The observation that mice, vaccinated with different types of MUC1 vaccines, were able to reject MUC1+ tumor challenges led to the design of a number of MUC1 vaccine trials for patients with various malignancies. Karanikas and colleges conducted a mannan-MUC1 fusion protein vaccine trial in patients with advanced carcinomas of the breast, colon, and stomach (Karanikas et al., 1997). This clinical trial demonstrated that a high titer of MUC1-specific IgG antibody could be generated in these patients, and to a lesser extent, some MUC1-specific CTL responses were also observed. These results were surprising, because mice that were vaccinated with the same regimen were able to mount strong CTL response (Apostolopoulos et al., 1995). Whether this vaccine and the presence of a high titer of MUC1-specific IgG antibody has clinical significance remains an open question. In another study, the same group tested the efficacy of using oxidized mannan fused to MUC1 as a vaccine for patients with various advanced adenocarcinomas (Karanikas et al., 2001). This vaccine regimen was proven effective in eliciting a strong CTL response to MUC1 in vaccinated mice (Apostolopoulos et al., 1997). Patients received the vaccine either intramuscularly or intraperitoneally. About 60% of the vaccinated patients demonstrated high titer IgG antibody; while 28% of them showed MUC1-specific cellular immune responses. However, there was no objective clinical response in any of the vaccinated subjects. The outcome of a recent clinical trial using a MUC1-KLH vaccine plus Qs-21 adjuvant in high-risk breast cancer patients has been reported (Gilewski et al., 2000). Vaccinated individuals developed high titers of IgM and IgG antibodies; however, there was no evidence of MUC1-specific cellular immune responses. Another group has tested the possibility of using live Vaccinia virus encoding MUC1 to vaccinate advanced breast
cancer patients (Scholl et al., 2000). Vaccinated patients mounted strong antibody and CTL responses against Vaccinia virus; however, the response directed against MUC1 was minimal. Another interesting clinical trial has recently been conducted in which DCs loaded with peptides derived from Her-2/neu or from MUC1 were used to vaccinate patients with advanced breast and ovarian cancers (Brossart et al., 2000). Vaccinated subjects demonstrated detectable CTL responses against Her-2/neu and MUC1 peptides. More interestingly, one patient who was immunized with DC-MUC1 showed CEA and MAGE-3 specific CTL responses. Another patient who received the DC-Her-2/neu vaccine showed a MUC1-specific T cell response. These results demonstrated that patients vaccinated with DCs loaded with defined epitopes can develop immune responses against other epitopes presented by tumor cells as a result of “epitope spreading”. The significance of “epitope spreading” was demonstrated in one study in which “epitope spreading” was seen only in a melanoma patient who achieved complete response following immunization with DCs pulsed with MART-1-derived peptide (Butterfield et al., 2003). Recently, Pecher and colleges used DCs transfected with a MUC1 cDNA to vaccinate patients with advanced breast, pancreatic or papillary cancers (Pecher et al., 2002). Four of 10 patients in this clinical trial showed an increase in the frequency of MUC1-specific IFN-γ secreting CD8+ T cells.

The data obtained from various MUC1 vaccine clinical trials demonstrate that it is safe to vaccinate patients against MUC1 and it is feasible to generate immune responses to MUC1 in vaccinated patients. These responses included high titers of IgG antibodies and variable degrees of CTL responses. Unfortunately, most of the MUC1 vaccine clinical trials conducted to date did not result in objective clinical responses in vaccinated subjects.
These data were disappointing because they were in contrast to the preclinical vaccination experiments done in mice that showed clearly that mice could be successfully immunized against MUC1, could mount a strong CTL response and could control and reject MUC1+ tumor challenges. It is important to point out that most of these preclinical trials were conducted in wild type mice, which are not tolerant to MUC1. This may explain why the same vaccines were not as effective in cancer patients who are tolerant to the self antigen MUC1. In addition, most of MUC1 clinical trials were conducted in advanced late stage cancer patients. These patients have a weakened immune system as a result of previous conventional treatments (radiation or chemotherapy) and as a result of the systemic immune suppression by tumors. A better outcome using MUC1 vaccines could be achieved if the vaccine trials could be conducted in young patients with early stage disease.

1.7. STATEMENT OF AIMS

The purpose of this study was to explore the unique potential of an MHC-unrestricted TCR, specific for the tumor antigen MUC1, as a broadly applicable therapeutic tool for the treatment of over 83% of human solid tumors which over-express underglycosylated MUC1 antigen. We hypothesized that bone marrow cells engineered to express this MHC-unrestricted MUC1-specific TCR would differentiate in vivo into multiple immune lineages among which a high percentage of cells would express that TCR. These cells would be expected to be activated by the binding of the TCR to MUC1 on tumor cells and to destroy tumor cells via several different effector mechanisms.
Rationale

The role of different cells of the adaptive immune system in tumor immunity is well documented. However, tumor cells evolve in such a way that allows evasion of recognition by T cells. An elegant strategy to direct the specificity of cells of the innate immune system toward particular tumor antigens has been described using chimeric immunoreceptors consisting of the antigen binding domain of an antibody or human CD4 and the signaling domain of the FcγR or CD3ζ chain (Hege et al., 1996; Wang et al., 1998). The expression of such receptors is not dependent on the presence of endogenous CD3 components in the host cells; therefore all innate immune cells did express these receptors. To date, there are no reports in the literature describing the use of single chain T cell receptors (TCR) to modify BM cells and redirect the specificity of various cells of the innate immune system toward a particular tumor antigen in vivo to confer tumor immunity. One of the reasons is that, unlike antibodies, TCRs are MHC-restricted and thus of limited use only in patients with specific MHC haplotype. MHC-unrestricted TCR has the universal advantage use of an antibody; and in addition, the optimal binding affinity of a TCR. Modifying BM cells with such a TCR would provide an unremitting source of genetically-modified immune cells that can target tumors.

The overall goal of this project was: (i) to clone the TCR from an MHC-unrestricted MUC1-specific CTL clone (MA), and to design different configurations of chimeric TCRs that could potentially be used for therapeutic purposes; and (ii) to test the efficacy and safety of using MUC1-specific MHC-unrestricted TCR transduced BM cells for cancer immunotherapy in severe combine immunodeficient (SCID) and MUC1 Tg. mouse models.
Specific Aim 1: To clone, express and analyze in vitro the MHC-unrestricted MUC1-specific TCRs.

Previous work done by our group demonstrated that recognition of MUC1 by MHC-unrestricted T cells can be blocked with monoclonal antibodies against the immunodominant epitope APDTRP or with antibody against the TCR/CD3 complex. These data showed indirectly that MHC-unrestricted CTLs recognized this epitope on the native MUC1 molecule via their $\alpha\beta$ TCR. However, no one had shown directly that this specificity was conferred by the TCR $\alpha\beta$ heterodimer. TCR reconstitution experiments in TCR-deficient T cells have been widely used to test the specificity of TCR for a particular pMHC complex. We used the same approach to test the specificity of the TCR expressed by the MA CTL clone. In addition, we wanted to design various chimeric molecules consisting of the extracellular domain of the TCR and the signaling domain of CD3$\zeta$, and to compare their levels of expression and functions using different in vitro systems.

Specific Aim 2: To analyze in vivo the expression and anti-tumor function of the MHC-unrestricted MUC1-specific scTCR

Cells of the innate immune system have been targeted towards particular tumors using chimeric antibody-Fc$\gamma$R or CD4-$\zeta$ immune receptors. These cells were able to control the growth of tumor cells bearing the target antigens in vivo. However, to date no one has addressed the possibility of using chimeric TCR$\zeta$ receptor to modify BM cells as a measure of conferring tumor immunity. In this aim, we tested the potential of SCID mice reconstituted with BM cells, engineered to express an MHC-unrestricted TCR specific for the tumor antigen MUC1, to control the growth of MUC1+ tumors. In addition, we determined what population(s) of immune cells could be targeted with this receptor in
reconstituted mice. We also addressed the hypothesis that grafting a large percentage of immune cells with MUC1-specific TCR could result in autoimmunity in MUC1 Tg. mice.
2. CLONING, EXPRESSION AND IN VITRO ANALYSES OF MHC-UNRESTRICTED MUC1-SPECIFIC TCRS

2.1. INTRODUCTION

We have previously reported the generation of MUC1-specific MHC-unrestricted CTL clones from the draining lymph nodes of breast and pancreatic cancer patients (Jerome et al., 1991). Two types of MHC-unrestricted T cell clones could be established in vitro. One type of CTL clone recognized MUC1 on the surface of tumor cells in an MHC-unrestricted manner, independent of other accessory molecules on the surface of T cell and tumor cells. This was attributed to a high affinity of their TCRs. We were unable to grow these T cell clones in vitro to sufficient numbers to permit functional and TCR usage analyses. The other type of CTL clone also recognized MUC1 on tumor cells in an MHC-unrestricted manner. However, this recognition was dependent on an interaction between other accessory molecules on the surface of T cells and ligands on target cells (Magarian-Blander et al., 1998), presumably due to a low affinity of their TCRs. These accessory molecules included CD8/MHC I, ICAM-1/LFA-1 and LFA-3/CD2. One such CTL clone, MA clone, was successfully grown and expanded in vitro to large numbers to permit extensive analyses. The MA CTL clone recognized a synthetic MUC1 100mer, which mimics the tumor form of MUC1, on microbeads and fluxed calcium as a result of this recognition (Magarian-Blander et al., 1998). This observation suggested that even though the interaction between MA CTL and MUC1 on tumor cells was dependent on the
engagement of other molecules, the interaction between the TCR on the MA CTL clone and MUC1, in the absence of these accessory molecules, could still result in downstream signaling, as evidenced by the calcium flux. The presence of a high density of MUC1 on tumor cells more likely increased the overall avidity of interaction between the TCR and MUC1. Semi-quantitative RT-PCR analysis of TCR usage by MA CTL clone (Alter, 1998) showed that the MA CTL clone had a single TCR α (Vα23.1) and single TCR β (Vβ8.3) transcripts. The ability of the MA CTL clone to recognize synthetic MUC1 on beads, in addition to its clonality, made it a good candidate for use for MUC1 immunotherapy. What is presented in this chapter: (i) shows that the MUC1 specificity of the MA CTL clone is conferred by the TCR Vα23.1/Vβ8.3 heterodimer; (ii) different configurations of chimeric TCRs can be designed for use in immunotherapy; and (iii) comparisons are made between the expression and function of these chimeric receptors using various in vitro systems. Additional work is presented on the generation of a soluble form of this TCR that will eventually enable us to better understand the interaction between the TCR and MUC1 using biophysical analyses. The first section in this chapter presents cloning of the full-length TCR α and β chains from the MA CTL clone followed by studies on its expression and function in JRT3.5, a TCR deficient Jurkat line. The second part of this chapter involves the construction of different configurations of chimeric TCRs and the comparison of their level of expression and function in different cell lines in vitro. Finally, we constructed and expressed a soluble form of the TCR using a mammalian expression system. The in vivo function of the TCR will be discussed in chapter 3 in this thesis.
2.2. MATERIALS AND METHODS

2.2.1. Cloning of Full-Length TCR α and β Chains from MA CTL Clone

The MA CTL clone was cultured in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml Penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), and recombinant human IL-2 (rhIL-2) at 20 U/ml (DuPont, Williamngton, NC). Twenty million cells were lysed and total RNA was extracted using a Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacture’s instructions. One µg of RNA was used for reverse-transcriptase polymerase chain reaction (RT-PCR) using GeneAmp RT-PCR kit (Applied Biosystems, Foster City, CA). The sequences for primers used in this chapter are listed in Appendix 1. Three µl of cDNA were then used for PCR amplification using Vα (P1) and Vβ (P2) leader sequence specific forward primers and Cα (P3) and Cβ (P4) specific reverse primers. PCR amplification was done using AmpliTaq Gold DNA polymerase (Applied Biosystems). For some experiments, we also used the pfu turbo DNA polymerase (Stratagene, La Jolla, CA). PCR was done using a Perkin Elmer thermocycler. The TCR α and β chains were cloned into pcDNA3.1 and pEF6 TA vectors (Invitrogen), respectively. The TCR α chain was then cloned into the Xho I and EcoR I sites in multiple cloning site (MCS) A; while the TCR β chain was subsequently cloned into the Sal I and Not I sites in MCS B in the pIRES vector (Clontech Laboratories, Palo Alto, CA). Eventually the TCRα-IRES-TCRβ cassette was cloned into the Xho I and Not I sites in the pEF4 mammalian expression vector (Invitrogen, Figure 3B). Expression from this plasmid is driven by the human Elongation factor 1-alpha (EF-1α) promoter. Human CD8α chain
was cloned into pEF6 TA mammalian expression vector (Invitrogen) using CD8α Forward (P5) and reverse (P6) primers (Figure 3A). The sequence, orientation and presence of open reading frames in all cloned genes were confirmed by DNA sequencing at the Department of Molecular Genetics and Biochemistry (MGB) DNA Sequencing Facility, University of Pittsburgh.

### 2.2.2. Construction of a Two Chain TCR (tcTCR) and a Single Chain TCR (scTCR)

The human CD3 ζ chain was cloned using a forward primer beginning at nucleotide number 80 (P7) in the extracellular domain and a reverse primer termination at the stop codon in the cytoplasmic domain of CD3 ζ (P8). This cloning strategy maintained an endogenous BamH I site at nucleotide number 80 in the extracellular domain of human CD3 ζ. The PCR product was cloned into the pCDNA3.1 TA vector (Invitrogen). The extracellular domains of the TCR α and β chains were cloned using leader sequence-specific primers (P1, P2) and reverse primers specific for the extracellular domain of the human TCR α (P9) or β (P10) chains, respectively. Both TCR α and β chains were terminated after the last cystine in the TCR constant region. A BamH I site was introduced into both forward and reverse primers. The CD3 ζ/pCDNA 3.1 vector was digested with BamH I restriction enzyme (New England BioLabs, Beverly, MA) and the TCR α or β chains were cloned in-frame with the CD3 ζ chain at the BamH I site (Figure 6A & 6B). The sequence and orientation of the chimeric TCR αζ and TCR βζ was confirmed by DNA sequencing. The TCR αζ and βζ were then cloned into the pIRES vector and eventually the αζ-IRES-βζ cassette was cloned into the pLNCX2 expression vector (Figure 6C). The initial work on construction of the scTCR was done by Mark Alter (Alter, 1998). The TCR
VαJα was cloned and joined to the TCR VβDβJβCβ region using a 15 amino acid flexible linker encoding 3 repeats of the sequence GGGGS. The TCR VαJα-VβDβJβCβ was then ligated in-frame with the murine CD3 ζ chain. A short amino acid linker (GDLVPRGSSRLD) encoding a thrombin cleavage site was also introduced between the TCR β chain constant and the CD3 ζ transmembrane domain. The construct was then cloned into the MFG (Ohashi et al., 1992) retroviral vector at the Neo I and BamH I sites. For in vitro expression experiments, the scTCR was cloned into the pEF6 mammalian expression vector.

2.2.3. Construction of scTCR-CD4TM-hζ Mammalian Expression Vectors

The scTCR VαJα-VβDβJβCβ extracellular domain, terminating before the last cystine in the TCR β chain constant region, was amplified by PCR using Vα (P1) forward and Cβ reverse (P11); and was cloned into the pEF6 vector. An Asc I site was introduced at the C-terminus in the TCR Cβ region. The human CD4 transmembrane (TM) domain fused to the human CD3 ζ cytoplasmic domain was amplified from the hCD4ζ vector (kindly provided by Dr. Margo R. Roberts, University of Virginia) using forward (P12) and reverse (P8) primers. Asc I and Sac II sites were introduced into the forward and reverse primers, respectively that allowed in-frame ligation to the scTCR extracellular domain. A modified version of this vector was created by inserting 3 amino acid (AGD) linker between the TCR Cβ region and the CD4 TM domain to provide flexibility to this molecule. In the latter, PCR was carried out using P8 and P13.
2.2.4. Surface Biotin Labeling and Thrombin Cleavage of scTCR

Twenty millions and half rat basophilic leukemia (RBL) cells transfected with the scTCR were washed 2 X in phosphate buffered saline (PBS) and were resuspended in 1 ml of 0.5 mg/ml sulfo-NHS-biotin (Pierce Biotechnology Inc., Rockford, IL) in PBS, pH 8.0 for 30 minutes at RT. Cells were washed 3X in PBS, pH 8.0 and were then incubated with 0.5 units of thrombin (Sigma, St. Louis, MO) in 100 µl of thrombin cleavage buffer (50 mM Tris-HCL, PH 8.0, 100 mM Na Cl2, 2.5 mM Ca Cl2, 0.1% 2-ME) and incubated at RT for 1 hour. The soluble fraction was then collected and was incubated with protein G sepharose beads coated with anti-TCR BF1 antibody (Endogen, Rockford, IL) for 2 hours at 4°C. Beads were then washed 3X in PBS + 0.05% tween 20 and boiled in 50 µl of 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2X loading buffer: 1.0 ml glycerol, 0.5 ml 2-ME, 3.0 ml of 10% SDS, 1.25 ml of 1.0 M Tris-HCl, PH 6.7 and 1.0 mg of bromophenol blue). Beads were then briefly centrifuged and 20 µl of supernatant was used for PAGE analysis. Samples were run using electrophoresis buffer (0.96 M Glycine, 0.125 M Trizma base, 0.5% SDS) for 1.5 hours at 100 V, and were then transferred to nitrocellulose membrane for 2 hours at 80 V using (25 mM Tris, 192 mM Glycine, 10.0 % Methanol) transfer buffer. The membrane was then blocked for 1 hour with PBS + 5 % BSA + 0.05 % tween 20 followed by adding streptavidin-horse radish peroxidase (SA-HRP, Sigma) at 1: 500 dilution in blocking buffer, and incubation for 1 hour at RT. The membrane was then washed 3X in PBS + 0.05 % tween 20, and given a final wash in PBS. The membrane was then developed using an ECL chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Film was exposed for 1-10 minutes and was developed using a Kodak film processor.
2.2.5. **Construction of Soluble scTCR**

A soluble, single chain fraction variable domain (sscFV) encoding the TCR Vα-Jα-Vβ-Dβ-Jβ or soluble scTCR (sscTCR) domain consisting of the TCR Vα-Jα-Vβ-Dβ-Jβ-Cβ was cloned by RT-PCR amplification using a combination of P1 and P14, or P2 and P15 primers, respectively into pCDNA3.1 mammalian expression vector. C-myc and hemaglutinin (HA) tags were inserted at the C-terminus of the sscFV construct, while a 6-histidine (6-His) and Flag tags were inserted at the C-terminus of the sscTCR. Modified versions of the sscTCR vector were created by fusing the sscTCR to the granulocyte-macrophage colony-stimulating factor (GM-CSF, PCR was done using P16 and P15), or Ig-κ light chain leader sequences (PCR was done using P17 and P15). Different tags were inserted to facilitate detection and purification of recombinant proteins.

2.2.6. **Transfection of 293H Cells With Expression Plasmids**

Human embryonic kidney cells, 293H, were grown in DMEM-10 (DMEM + 10% FBS, 2 mM L-glutamine, 100 U/ml Penicillin, 100 U/ml streptomycin). Cells were harvested using 0.05% trypsin and 2X10⁵ cells were seeded in 500 µl of DMEM-10 (containing no antibiotics) in 24 well plates. Twenty-four hours later, 1.0 µg of DNA was diluted in 50 µl of DMEM and 3 µl of lipofectamine 2000 (Invitrogen) was diluted in 50 µl of DMEM medium. The diluted DNA was mixed with the diluted lipofectamine 2000 and the mixture was incubated at RT for an additional 20 minutes. After that, the mixture was added to 293H cells, drop wise, and cells were agitated to uniformly distribute the transfection mixture. Cells were analyzed for protein expression 48-72 hours post transfection.
2.2.7. Electroporation of BWZ and Jurkat Cells

BWZ murine T cells were kindly provided by Dr. Nilabh Shastri at the University of California, Berkeley. BWZ cells were grown in cRPMI-10 (RPMI + 10% FBS, 2 mM L-glutamine, 100 U/ml Penicillin, 100 U/ml streptomycin, 10 µM 2-ME, 1X non-essential amino acids, and 1X sodium pyruvate). Jurkat cells were grown in RPMI-10 medium. Five million cells were harvested, washed once in PBS, and resuspended in 400 µl of normal growth medium (cRPMI-10). Fifty µg of DNA were then added and mixed with the cells. Cells were electroporated using 960 µF and 200 V settings using a BioRad Gene Pulser II (Bio-Rad Laboratories, Hercules, CA). Cells were mixed gently and kept at RT for an additional 10 minutes before seeding into T25 Flask in cRPMI-10. Forty-eight hrs later, cells were harvested, and 1X10^4 cells were plated in 200 µl of cRPMI-10 medium supplemented with the appropriate selection antibiotic (G418 at 1 mg/ml, Zeocin at 200 µg/ml, or Blasticidin S at 3 µg/ml) in 96 well plate. Plates were then monitored for clone growth 10-20 days post selection. Clones that grew were then expanded in 24 well plates and tested for surface expression of TCRs using FACS analyses.

2.2.8. Flow Cytometric Analyses for TCR Expression

Five-hundred thousand cells were washed in FACS buffer (PBS + 5% FBS + 0.01% Sodium Azide) and were resuspended in 100 µl of FACS buffer containing 1 µg of the appropriate antibody, on ice for 30 minutes. For some experiments, cells were first “blocked” using 1 µg of anti-CD16/CD32 antibody (BD Pharmingen, San Diego, CA) /10^6 cells for 20 minutes, on ice. Thirty minutes later, cells were washed 3 times in FACS
buffer, and 100 µl of 1:100 diluted Alexa flour 488-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR) was added and incubated for additional 30 minutes, on ice. Cells were then washed 3 times and resuspended in FACS buffer containing 1% paraformaldehyde (PFA) and were analyzed using BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

2.2.9. Purification of sscTCR and Western Blotting

293H cells were either transiently transfected with plasmid encoding the appropriate construct or were selected for stable expression of the transfected gene. Cells were grown in DMEM-10. In some experiments, cells were grown until they became confluent and then medium was replaced with DMEM-10 containing no FBS. In other experiments, cells were grown entirely in 293CD medium (Invitrogen). Seventy-two hours later, culture medium was harvested and was either stored at -80°C or used immediately for protein purification. Anti-HA, c-Myc, and 6-His antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-Flag M2 antibody was purchased from Sigma. For constructs encoding c-Myc or HA tagged proteins, supernatants were incubated with Protein G Sepharose beads (Amersham Biosciences) coated with the appropriate anti-tag antibody for 2 hours at 4°C. Beads were washed in PBS + 0.05% tween 20 3X and were then boiled for 5 minutes in 1X SDS-PAGE sample loading buffer. Beads were then briefly centrifuged and 20 µl of supernatant was used for SDS-PAGE analyses. For vectors encoding 6-His tagged proteins, culture supernatant was run on a Nickel-Agarose column (Qiagen) at 1 ml/min flow rate. The column was then washed using buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0). Proteins were then eluted using
different concentration of imidazole in wash buffer. Eluted fractions were mixed with equal
volumes of 2X SDS-PAGE sample loading buffer, boiled for 5 minutes, centrifuged briefly
and 20 µl of supernatant were then loaded on a 10% Tris SDS-PAGE gel. Samples were
run for 1.5 hours at 100 V and were then transferred to a nitrocellulose membrane for 2
hours at 80 V using transfer buffer. The membrane was “blocked” for 1 hour using PBS +
5% non-fat milk (NFM) + 0.05% tween 20 followed by adding the appropriate antibody at
1 µg/ml in blocking buffer and incubation for 1 hour at RT. The membrane was washed 3X
in PBS + 0.05% tween 20 then goat anti-mouse IgG was added at 1:5000 dilution
(Amersham Biosciences, Piscataway, NJ) in blocking buffer and incubated for 45 min at
RT. The membrane was then washed 3X in PBS + 0.05% tween 20 and given one final
wash in PBS. The membrane was then developed using an ECL chemiluminescence
detection system (Amersham Biosciences). Film was exposed for 1-10 minutes and was
developed using a Kodak film processor.

2.2.10. Live imaging microscopy for calcium flux

Fura-2 AM was purchased from Molecular Probes (Eugene, OR) and was prepared
according to the manufacture’s recommendations. Briefly, 50 µg of Fura-2 Am was
dissolved in 50 µl of DMSO, 20 µl of that was added to 1X10⁷ cells in 10 ml of RPMI
medium containing no serum, and cells were then incubated at 37⁰ C for 30 minutes. Cells
were spun down for 5 minutes and the pellet was resuspended in 750 µl of live imaging
medium (DMEM without sodium bicarbonate + 10% FBS, 10mM HEPES, 2 mM L-
glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, pH 7.1-7.2) and incubated at 37⁰ C
for 15 minutes. HPAF tumor cells were seeded at 1X10⁵/ml in a 2-chamber slide (Fisher
Scientific, Pittsburgh, PA) and grown over night at 37° C. Cells were then rinsed gently with PBS, and 500 µl of warm imaging medium was added. Fifty µl of Fura-2 Am labeled cells was then added, and images were taken at 340 and 380 nm excitation λ every 10 seconds for a total of 10 minutes using a Zeiss live imaging microscope (Thornwood, NY) at the Center for Biologic Imaging, University of Pittsburgh. Data were presented as the overlay of the emission at 510 nm for cells exited at 340 and 380 nm.

2.2.11. mIL-2 Detection in Culture Supernatant by ELISA

BWZ or BWZ-scTCR cells were plated in U-bottom 96 well plates at 1X10⁵ /100 µl of cRPMI-10 medium, and 2X10⁴ tumor cells (irradiated 6000 rad) were added as stimulators in 100 µl of cRPMI-10 medium. Thirty six hours later, the amount of mIL-2 in culture supernatants was measured using a mouse IL-2 OptEIA kit (BD Pharmingen) according to the manufacture’s recommendations. Briefly, Immulon 4 flat bottom 96-well plates (Dynex Technologies, Chantilly, VA) were coated overnight with anti-mouse IL-2 capture antibody in coating buffer (0.1 M Carbonate, pH 9.5). Plates were then washed 3X in PBS + 0.05% tween 20. Plates were blocked using 200 µl of cRPMI-10 and were incubated at RT for 1 hour. Plates were washed 3X in PBS + 0.05% tween 20 and 100 µl of culture supernatant or mIL-2 standard was added and incubated for 2 hours at RT. Plates were washed for 5X in PBS + 0.05% tween 20, and 100 µl of working detection complex (anti-mouse IL-2/Avidin-HRP) was added and incubated for 1 hour at RT. Plates were then washed 7X in PBS + 0.05% tween 20, and 100 µl of TetraMethyBenzidine (TMB, BD Pharmingen) substrate was added for 30 minutes at RT. Reactions were then stopped using 2.5 M H₂SO₄, and absorbance was taken at 450 nm.
2.3. RESULTS

2.3.1. Reconstitution of the TCR/CD3 Complex on the Surface of JRT3.5 Jurkat Line Transfected with the TCR α and β Chains from MA CTL Clone.

Our first experiments involved cloning the TCR α and β chains into mammalian expression vectors. Initially, we cloned the full-length TCR α and β chains from the MA CTL clone by RT-PCR into pCDNA3.1 and pEF6 vectors, respectively. To test the specificity of the TCR α and β chains from the MA CTL clone, we decided to take advantage of a TCR-deficient Jurkat line (Ohashi et al., 1985). This Jurkat line (JRT3.5) lacks the TCR β transcript and has low levels of TCR α chain message. This made this Jurkat line an ideal model for TCR reconstitution and function analyses. The binding of MUC1-specific MHC-unrestricted TCR to MUC1 is thought to be of low affinity. In this regard, it was previously shown that MA CTL lysis activity is dependent on CD8/MHC I interactions, as well as on the engagement of other accessory molecules on the surface of T cells and tumor cells (Magarian-Blander et al., 1998). Therefore, we thought necessary to transfect JRT3.5 line with the human CD8, in addition to the TCR α and β chains, in order to be able to detect specific function of this TCR in vitro. CD8 normally exist on T cell surface as a heterodimer of the CD8α and the CD8β chains; however, many reports had shown that CD8αα homodimers can bind to MHC class I molecules with similar kinetics to those of CD8αβ heterodimers (Kern et al., 1999). The human CD8α chain was cloned by
Figure 3. Mammalian expression vectors for human CD8 α and TCR α/β chains from MA CTL clone. hCD8 α was cloned by RT-PCR into pEF6 expression vector (A) while the TCR α-IRES-β cassette was cloned into the pEF4 expression vector (B). IRES stands for internal ribosomal entry site. Blasticidin and Zeocin are antibiotic resistance genes.
RT-PCR into the pEF6 mammalian expression vector (Figure 3A). Following stable transfection of the cloned hCD8α into the JRT3.5 cell line, high levels of surface expression of the hCD8α was observed (figures 4A-B). Transfection of only the TCR β chain resulted in very low levels of expression of TCR/CD3 complex on the surface of the JRT3.5 line (Figure 4C). This was expected because JRT3.5 has a very low level of TCR α chain transcript which is the limiting factor for TCR/CD3 expression, even though the cells were transfected with the TCR β chain gene. The TCR α and TCR β chain genes were then cloned into the MCS A and MCS B in the pIRES vector. Subsequently, the TCR α-IRES-β cassette was cloned into the pEF4 mammalian expression vector (Figure 3B). We chose to use the pEF6 and the pEF4 mammalian expression vectors to clone the hCD8α and the TCR α and β chains because expression from these vectors is driven by the human elongation factor-1 alpha (EF-1α) promoter, which is expected to be more transcriptionally active and stable than viral promoters. We also decided to clone the TCR α and β chains into a vector that has an IRES sequence that permits expression of the TCR α and TCR β chain genes from the same message. This was expected to result in similar levels of expression of both genes, in contrast to vectors that have different promoters. As shown in Figure 4D, stable transfection of the TCRα-IRES-β pEF4 vector into JRT3.5 cells resulted in high levels of TCR/CD3 complex surface expression. These cells also maintained CD8α expression at the end of this process (data not shown).
Figure 4. Expression of the hCD8α and TCR α/β from MA CTL clone on the surface of a TCR-deficient Jurkat line (JRT3.5). JRT3.5 (A) was transfected with human CD8 α pEF6 (B), TCRβ pEF6 (C), or hCD8α pEF6/TCR α-IRES-β pEF4 (D) expression vectors. Cells were stained with anti-CD8 α (A & B filled blue line) or anti-CD3ε (C & D), green line. Blue lines in C and D indicate staining with isotype controls.
2.3.2. JRT3.5 Cells Reconstituted with the TCR α/β Chains, and hCD8 α Fluxed Ca++ in Response to Stimulation with MUC1+ Tumor.

One problem that we encountered when using IL-2 production as a readout to test the specificity of the TCR α/β chains transfected into JRT3.5 was that JRT3.5 TCR α/β hCD8α was unable to secrete IL-2 even when the TCR/CD3 complex was crosslinked with anti-CD3 antibody or when the cells were stimulated with ionomycin and PMA (data not shown). As an alternative, we chose to use earlier events in T cell activation as a readout, rather than cytokine production, which is a late event in T cell activation. We decided to test whether JRT3.5 TCR α/β hCD8α cells can recognize and respond to MUC1+ targets by measuring intracellular changes in Ca++ levels. Cells were labeled with fura-2 AM, a calcium sensitive dye. Fura-2 complexed with Ca++ can be excited at 340 nm (represented as blue color in Figure 5), while free fura-2 is excitable at 380 nm (represented by green color in Figure 5). Both free fura-2 and fura-2 complexed with Ca++ will emit at 510 nm. The change in the emission spectra from 380 nm to 340 nm represents an increase in intracellular calcium levels, which indicates T cell activation. The data are presented in Figure 5 as the overlay in the emission spectra for cells exited at 340 nm/380 nm. Panel 5A-D clearly shows that JRT3.5 TCR α/β hCD8α T cells can flux calcium when stimulated with HPAF MUC1+ tumor; however, no calcium flux was seen when the parental JRT3.5 cells were stimulated with the same tumor (Figures 5E-H).
Figure 5. Live imaging microscopy to measure changes in intracellular calcium levels in JRT3.5 or JRT3.5 hCD8α/ TCRα/β cells. JRT3.5 cells stably transfected with hCD8α and TCR α/β (A-D) from MA CTL clone or untransfected JRT3.5 (E-F) were incubated with HPAF, a human MUC1+ pancreatic tumor, and intracellular calcium levels were monitored as described in materials and methods. Blue color represents increased intracellular calcium level above background level. Panel A-D and E-H represent sequential images that were taken at different time points.
2.3.3. **Chimeric MUC1-specific Two Chain TCRs (tcTCR) Were Constructed and Expressed on the Surface of 293H Cells In Vitro.**

The ultimate goal of this project is to use the TCR from MA CTL clone for cancer immunotherapy. We thought it is more appropriate to construct chimeric TCRs rather than to use the native full-length TCR α and β chains. The expression of these chimeric TCRs is no longer dependent on the presence of the CD3 molecules in the host cells; hence these chimeric αζ and βζ TCRs can be expressed on the surface of both T cells and non T cells (Engel et al., 1992). This is very important when considering an immunotherapeutic approach that will result in the expression of MUC1-specific TCR on the surface of various immune lineages that are expected to target and purge MUC1+ tumors. This would also eliminate any possibility that the transferred TCRs could pair with endogenous TCR α or β chains to form a potentially autoreactive TCR (Willemsen et al., 2000). Construction of the TCR αζ and βζ was done as detailed in the Materials and Methods section in this chapter. The extracellular domain of the TCR α chain was cloned in-frame to the human CD3 ζ transmembrane and cytoplasmic domains (Figure 6A). This construct also contained most of the extracellular domain of the hCD3 ζ chain. The TCR βζ was constructed in the same configuration as described for the TCR αζ (Figure 6B). The TCR αζ and βζ were then cloned into the pIRES vector and subsequently the TCR αζ-IRES-βζ cassette was cloned into the pLNCX2 vector (Figure 6C). As shown in Figure 7A, transfection of TCR βζ alone into 293H cells resulted in no surface expression. However, co-transfection of the TCR αζ and TCR βζ constructs into 293H cells resulted in surface expression of the heterodimer (Figure 7B). A similar level of surface expression was also obtained when 293H cells were transfected with the TCR αζ-IRES-βζ pLNCX2 vector (Figure 7C).
Figure 6. Mammalian expression vectors for TCR αζ and βζ chimeric receptors. The TCR α and β chains were cloned inframe with the human CD3 ζ chain as depicted in (A) and (B). αζ-IRES-βζ cassette was then cloned into the pLNCX2 expression vector (C). IRES stands for internal ribosomal entry site, Neomycin and Blasticidin are antibiotic resistance genes.
Figure 7. Transient expression of $\alpha\zeta/\beta\zeta$ heterodimer on 293H cells. No surface expression was achieved when 293H cells were transfected with the TCR $\beta\zeta$ alone (A); however, co-transfection of the TCR $\alpha\zeta$ and $\beta\zeta$ resulted in surface expression of the $\alpha\zeta/\beta\zeta$ heterodimer (B). Similar level of surface expression was also achieved by transfecting 293H cells with the TCR $\alpha\zeta$-IRES-$\beta\zeta$ pLNCX2 vector (C). Filled blue curves indicate staining with isotype control, while green lines represent staining with anti-TCR BF1 antibody.

2.3.4. Different Configurations of Single Chain TCRs (scTCRs) Were Expressed at Various Levels on the Surface of 293H Cells.

The data presented in Figure 7 showed that co-transfection of the TCR $\alpha\zeta$ and $\beta\zeta$ is required for surface expression of the heterodimer on the cell surface. Therefore, we thought it is more advantageous to construct a single chain TCR (scTCR). We also thought that construction of a scTCR-retroviral vector is easier and more practical than constructing a retroviral vector that expresses the TCR $\alpha\zeta$ and TCR $\beta\zeta$ chains. Other groups have previously reported successful construction and expression of scTCR and single chain antibodies (Novotny et al., 1991; Eshhar et al., 1996). The scTCR was constructed as
described in the Materials and Methods section in this chapter. We also constructed different configurations of the scTCR to compare their levels of surface expression. Figure 8 shows the configuration of the scTCR (A), scTCR-CD4TM-hζ (B) and the scTCR-AGD-CD4TM-hζ (C). These scTCRs consists of the TCR antigen binding domain and a signaling component from the CD3 ζ chain. All constructs were cloned into the pEF6 mammalian expression vector. As shown in Figure 9B, transfection of the scTCR into 293H cells resulted in substantial levels of surface expression. However, transfection of the scTCR-CD4TM-hζ construct into 293H cells resulted in very low levels of surface expression (Figure 9B). Surprisingly, inserting a 3 amino acids (AGD) linker between the TCR Cβ chain and the CD4 TM domain restored high levels of surface expression of the scTCR (Figure 9D). It is hypothysized that these 3 amino acids provided some form of flexibility to the scTCR construct that resulted in proper folding of the molecule and normal level of surface expression. Figure 10 shows quantitative analyses of surface expression of different scTCRs transiently transfected into 293H cells. Even though the level of surface expression of the scTCR-AGD-CD4TM-hζ was not statistically significant when compared to that of the scTCR, the difference in surface expression was statistically significant when compared to that of the scTCR-CD4TM-hζ. The rest of the work in this chapter was done using the scTCR-pEF6 construct.
Figure 8. Different configurations of scTCR mammalian expression vectors. The scTCR-pEF6 was constructed as described in Materials and Methods and as depicted in (A). The scTCR-CD4TM-hζ and scTCR-AGD-CD4TM-hζ were constructed as depicted in (B) and (C), respectively. Different configurations of scTCRs were cloned into pEF6 mammalian expression vector.
Figure 9. Expression of different scTCRs on the surface of 293H cells. 293H cells were transiently transfected with control plasmid (A), scTCR-pEF6 (B), scTCR-CD4TM-hζ pEF6 (C) or scTCR-AGD-CD4TM-hζ pEF6 (D) expression vectors. Cells were stained with anti-TCRβ (BF1) antibody (green line) or with isotype control antibody (filled blue curves).
2.3.5. **Functional MHC-Unrestricted MUC1-Specific scTCR Was Expressed on the Surface of T and Non-T immune Cells In Vitro.**

We had demonstrated that the scTCR can be successfully expressed on the surface of 293H cells (Figure 9). To show that the scTCR can also be expressed on the surface of different immune cell types, Rat Basophilic Leukemia (RBL) and BWZ mouse T cell lines were stably transfected with the scTCR-pEF6 expression vector. Figure 11 shows that the scTCR could be expressed on the surface of RBL (Figure 11A) and BWZ cell lines (Figure

![Figure 10. Comparison of surface expression of different configurations of scTCRs transiently transfected into 293H cells. Y-axis shows fold increase in mean fluorescent intensity (MFI) of specific staining with anti-TCR BF1 antibody over isotype control staining. X-axis shows different constructs that were transfected into 293H cells.](image-url)
11B). These data further confirmed the hypothesis that the scTCR can be expressed in T cells, as well as in non-T cells. The next task was to show that we expressed a functional scTCR. BWZ cells transfected with the scTCR were incubated with DM6 (MUC1-), HPAF (MUC1+), or T3M4 (MUC1+) tumors for 36 hours and mIL-2 in supernatant of these cultures was measured by ELISA as described in the Materials and Methods. No significant IL-2 production was detected when BWZ or BWZ-scTCR cells were incubated with DM6 tumor (Figure 12). However, substantial level of mIL-2 production was seen when BWZ-scTCR cells were incubated with HPAF tumor (Figure 12). No IL-2 production was seen

Figure 11. Surface expression of scTCR on non-lymphoid and lymphoid cells. ScTCR-pEF6 vector was stably transfected into rat basophilic leukemia cell line RBL (A) or BWZ mouse T cell line (B). Cells were stained with anti TCR BF1 antibody (green and orange lines) or with isotype control (filled curves).
Figure 12. mIL-2 ELISA for mouse T cell line (BWZ) or BWZ cells stably transfected with the scTCR-pEF6 vector following stimulation with different targets. Five hundred thousand BWZ or BWZ-scTCR cells were cultured with DM6 (MUC1− tumor), HPAF or T3M4 (MUC+ tumors) at 5:1 effectors to target ratio for 36 hours and mIL-2 in culture supernatant was measured by ELISA and values were plotted on the y-axis as pg/ml. Cells were also stimulated with anti-TCR BF1 antibody or with ionomycin + PMA (I/P) as indicated.
when untransfected BWZ cells were cultured with the same HPAF tumor. Low levels of IL-2 production, though statistically significant, were seen when BWZ-scTCR cells were stimulated with T3M4. HPAF and T3M4 share no HLA alleles. These data further confirmed the fact that the cloned scTCR still has the same specificity of recognizing MUC1+ targets, regardless of the HLA allele present on tumor cells. The difference in IL-2 production by BWZ-scTCR in response to HPAF compared to T3M4 can be attributed to a difference in the levels of expression of the epitope recognized by the scTCR on the surface of these MUC1+ tumors. BWZ-scTCR cells produced a substantial amount of IL-2 when the scTCR was cross-linked with plate-bound anti-TCR BF1 antibody. Taken together, these data confirmed that we had constructed and expressed a functional MUC1-specific scTCR.

2.3.6. Soluble scTCR Was Expressed Using a 293H Mammalian Expression System.

To date, the interaction between the MHC-unrestricted MUC1-specific TCR and MUC1 molecule has not been studied at the molecular level. This interaction can be studied using Biacore analyses, which has been used successfully to measure the affinity of interactions between the TCR and the pMHC complex (Baker et al., 2001). Once the affinity and kinetics of interaction between MHC-unrestricted MUC1-specific TCR and MUC1 is determined, we can then use site-directed mutagenesis to alter the amino acid residues in the TCR CDR2 and CDR3 region that contributes to binding to the MUC1 immunodominant knob and then to correlate the affinity of mutated TCRs and their ability to function in vitro and in vivo. In order to carryout these studies, it was necessary to generate a soluble form of the MUC1-specific scTCR (sscTCR). This sscTCR could
potentially be used as a vehicle to deliver and target therapeutic drugs to MUC1+ tumors, as well. The first attempt that we made to generate sscTCR was to introduce a thrombin cleavage site between the scTCR Cβ region and the CD3 ζ transmembrane domain. Theoretically, this scTCR could be cleaved from the surface of cells transfected to express the protein following treatment with thrombin. Figure 13A shows that the scTCR containing the thrombin cleavage site can be cleaved from the surface of RBL cells transfected to express the protein following treatment with thrombin. We also showed that the sscTCR can be purified from the soluble fraction following thrombin cleavage (Figure 13B) using an anti-TCR BF1 affinity column. As expected, the sscTCR has a lower molecular weight than the membrane bound scTCR (Figure 13B, lanes 2 and 4). The sscTCR can only be eluted from the affinity column under high pH elution conditions (Figure 13B, lane 8). Even though this approach was successful in generating sscTCR, the amount that was obtained was extremely low. Therefore, we attempted other approaches to generate soluble scTCR. Figure 14 shows the configuration of different constructs that were created to generate secreted forms of the scTCR. Other groups have reported expression of a soluble, single chain fraction variable (sscFV) domain of both antibody and T cell receptor (Pavlinkova et al., 2000; Gregoire et al., 1996). Figure 14A shows the design of the sscFV. Basically, this construct encoded the TCR VαJα-linker-VβDβJβ. The sscFV was terminated just right after the Jβ domain in the scTCR. Figure 14B shows the configuration of the sscTCR which encodes the VαJα-linker-VβDβJβCβ. The construct was terminated just before the proximal cystine in the TCR β chain constant region. Two other configurations of the sscTCR were constructed as described in Figure 14B, with the
exception of replacing the Vα leader sequence in the sscTCR with either a GM-CSF (Figure 14C) or Ig-κ light chain (Figure 14D) signal peptide. As shown in Figure 14,
different constructs have different epitope tags that were fused at the C-terminus to facilitate detection and purification of recombinant proteins. All constructs were cloned into the pcDNA3.1 mammalian expression vector (Invitrogen). When the sscFV construct

![Diagram A](image1)

**Figure 14.** Design of different secreted scTCR Expression vectors. Various constructs were made as described in Materials and Methods. The single chain fraction variable (scFV) domain was cloned and fused to a C-terminus HA and c-myc epitope tags (A). B shows the configuration of soluble scTCR (sscTCR) that was cloned and was fused to a C-terminus Flag and His epitope tags. C and D show the design of the sscTCR as described in B, with the exception that the sscTCR was fused to the leader sequence from GM-CSF (C) or Ig-kappa light chain (D). All constructs were cloned into the pcDNA3.1 mammalian expression vector.
was transfected into 293H cells, no recombinant sscFV protein could be detected in culture supernatant (Figure 15, lanes 1 and 2). However, transfection of the sscTCR construct into 293H cells resulted in significant amounts of recombinant protein that were secreted in culture supernatants (Figure 15, lane 3). Transfection of the sscTCR that was fused to the Ig-κ light chain leader sequence resulted in a lower level of protein expression than was seen with the sscTCR construct (Figure 15, lane 5), while transfection of the sscTCR construct that was fused to the GM-CSF leader sequence resulted in no recombinant protein secretion in culture supernatant (Figure 15, lane 4). In our expression system, it is evident that the presence of the TCR β chain constant region is absolutely required for expression.

Figure 15. Expression of sscFV or sscTCR recombinant proteins in 293H cells. Culture supernatants from 293H cells transiently transfected with a control plasmid (1) or with Vα-Jα-Vβ-Dβ-Jβ-HA-myc were immunoprecipitated with anti-HA antibody and were blotted with anti-c-myc antibody. Culture supernatant from 293H cells transfected with Vα-Jα-Vβ-Dβ-Jβ-Cβ-Flag-His (3), GM-CSF Vα-Jα-Vβ-Dβ-Jβ-Cβ-Flag-His (4) or Ig-k Vα-Jα-Vβ-Dβ-Jβ-Cβ-Flag-His (5) constructs was affinity purified using nickel agarose beads and were blotted with anti-Flag antibody. Lane 6 is control culture supernatant.
of the sscTCR. We hypothesized that the TCR β chain constant region is important for proper folding of the protein or maybe that the TCR β chain constant region does interact and mask other hydrophobic amino acid residues in the TCR β chain VDJ region, otherwise the scFV is rendered insoluble. To test the effects of culture medium and conditions on sscTCR production, 293H cells transfected with the sscTCR construct were cultured under different culture conditions (Figure 16). The data presented in figure 16 show that maximum amount of recombinant protein is produced when transfected 293H cells were grown until confluence and then the medium was replaced with fresh DMEM containing no FBS (Figure 16A, lane 3). A very low amount of protein was secreted when cells were grown entirely in 293CD medium (Figure 16A, lane 2). Figure 16B & 16C, lanes 5-8 illustrate that the recombinant sscTCR can be purified from culture supernatant using a nickel column. In conclusion, we were able to express a soluble form of the scTCR in mammalian cells in sufficient quantities that can be used for biacore analysis as well as for other studies. Continuation of these studies was beyond the scope of this thesis.

2.4. Discussion

MUC1 is a unique tumor antigen in that it is able to trigger CTL activation in the absence of processing and presentation in the context of MHC class I. This uniqueness of MUC1 is attributed to the fact that the extracellular domain of the MUC1 molecule is composed of a large number of repeated sequences. Each repeated sequence of MUC1 expressed on tumor cells has a knob like structure with the immunodominant epitope (APDTRP) exposed on the MUC1 backbone. Engagement of multiple epitopes of MUC1 molecule on tumor cells
Figure 16. Expression and purification of recombinant sscCTR in 293H cells. Panel A shows the effect of different medium and culture conditions on the amount of sscCTR secreted in culture supernatant. Stably transfected 293H cells were cultured in DMEM medium + 10% FBS until confluence then medium was replaced with 293CD medium (1), DMEM + no FBS (3), DMEM + 1% FBS (4) or with DMEM + 10% FBS (5). Cells were also grown entirely in 293CD medium (2). Lane 6 is MW marker. Panel B shows commassie blue staining of fractions from culture supernatant A3 purified using nickel column. Lane B1 is MW marker, B2 is culture supernatant before purification, B3 is flow through, B4 is wash, and B5-9 are different eluted fractions. C is western blot of panel B using anti-Flag antibody. For details refer to text.
with multiple TCRs on the surface of a T cell can result in T cell activation. MHC unrestricted recognition of antigens by TCR $\alpha\beta^+$ T cells has also been described for other molecules (Siliciano et al., 1986; Altmann et al., 1987; Abdel-Motal et al., 1996). These molecules share a common feature with MUC1, in that they all consist of multiple repeated sequences. Therefore, they are all able to crosslink the TCR and activate specific T cells.

Previous work done by our group demonstrated that recognition of MUC1 by MHC-unrestricted T cells can be blocked with monoclonal antibodies against the immunodominant epitope APDTRP or with antibody against the TCR/CD3 complex. These data showed indirectly that MHC-unrestricted CTLs recognized this epitope on the native MUC1 molecule via their $\alpha\beta$ TCR. However, no one had shown directly that this specificity was conferred by the TCR $\alpha\beta$ heterodimer. TCR reconstitution experiments have been widely used to test the specificity of TCR for a particular pMHC complex. We used the same approach to directly show the specificity of the TCR from MA CTL clone for MUC1. We showed here that the full length TCR $\alpha$ and $\beta$ chains from MA CTL clone reconstituted the TCR/CD3 complex on the surface of a TCR deficient Jurkat line (JRT3.5) and that the TCR-reconstituted JRT3.5, but not the parental JRT3.5, was able to recognize MUC1+ targets (Figure 5).

Additionally, we showed that, even though expression of the TCR $\alpha\zeta$ and $\beta\zeta$ constructs was independent on the presence of endogenous CD3 molecules in host cells, surface expression of the TCR $\beta\zeta$ was still dependent on the co-expression of the TCR $\alpha\zeta$. We hypothesized that pairing of the TCR $\alpha\zeta$ to the TCR $\beta\zeta$ was crucial for proper folding and transport of the heterodimer through the endoplasmic reticulum (ER) and Golgi and eventually to the cell surface.
The single chain approach was initially used to create single chain antibodies and was later applied to TCRs. In contrast to the expression of two chain TCRs, functional scTCRs can be expressed on the cell surface from a single mRNA transcript. Here we constructed a single chain TCR specific for the tumor antigen MUC1, and this scTCR was expressed on the surface of different immune cell types \textit{in vitro}. More interestingly, BWZ murine T cells transfected with the scTCR were able to recognize MUC1+ targets and secrete IL-2 as a result of this recognition. Even though this scTCR contained a single CD3ζ chain which encoded 3 immunoreceptor tyrosine kinase activation motifs (ITAMs), this was sufficient to mediate T cell activation. This can be explained by the simultaneous cross-linking of multiple TCRs on the surface of T cells, which compensates for the lower number of ITAM motifs in the CD3 ζ in the scTCR. The expression of the scTCR on the surface of transfected BWZ or RBL cells was lower than the level of expression of normal TCR on the surface of T cells (data not shown). In an attempt to increase the level of surface expression of the scTCR, we replaced the TM domain of CD3ζ in the scTCR with the TM domain of human CD4. However, this new construct was expressed at very low levels when transfected into 293H cells. It was previously reported that the presence of a short linker between the TCR constant region and the CD3ζ TM region was critical for surface expression of chimeric TCRs (Engel et al., 1992). Therefore, we inserted a 3 amino acid (AGD) linker between the scTCR constant region and the CD4 TM domain and tested surface expression. Surface expression of the scTCR was restored in this new construct. The short linker provided either flexibility or sufficient spacing between the TCR constant and CD4 TM to allow normal surface expression. In the end, however, expression of the
scTCR with the human CD4 TM domain was not much different than that with the CD3ζ TM domain (Figure 10), therefore the later being used in the continuation of our studies.

Most previous attempts to generate soluble scTCRs were made using prokaryotic expression systems. However, proteins expressed in prokaryotic cells lack post-translational modifications, and may be improperly folded. In order to avoid these potential problems, we chose to express soluble MUC1-specific scTCR using mammalian expression systems. First, we showed that it was feasible to generate a soluble scTCR by inserting a thrombin cleavage site in the extracellular domain of the scTCR, followed by thrombin treatment. The yield of soluble scTCR using this approach was extremely low and not sufficient for other analyses. In another attempt to generate secreted scTCR, we terminated the construct just before the TM domain of the CD3ζ chain. Following transfection into 293H cells, large amount of soluble scTCR was detected in culture supernatants. We also replaced the leader sequence of the TCR α chain in the scTCR with that of the Ig-κ light chain, which lowered the amount of secreted protein. Replacing the scTCR leader sequence with that of GM-CSF resulted in no protein secretion. It is possible that the GM-CSF sequence lacked amino acids required for proper cleavage of the leader sequence in the ER. The scTCR expressed using the construct that has the native TCR α leader sequence will eventually be used for Biacore analyses to measure the affinity of interaction between the TCR and MUC1 molecule. For optimal function of TCR, it is important that its affinity for antigen falls within a certain range. It is possible that increasing the affinity of the scTCR may enhance the in vivo function of this receptor and anti-tumor effect. Based on computer modeling, we could predict which amino acids are involved in the interaction between the scTCR and MUC1 molecule (Alter, 1998). We propose that mutating these amino acids
(either in MUC1 or in scTCR) will increase the affinity of interaction between the scTCR and MUC1, which may serve better anti-tumor function. Measuring the kinetic of interaction between the scTCR and MUC1 is beyond the scope of this thesis.
3. **IN VIVO ANALYSES OF MHC-UNRESTRICTED MUC1-SPECIFIC scTCR**

3.1. **INTRODUCTION**

The inability to induce vigorous anti-tumor immune responses in cancer patients through vaccination is attributed to their weakened immune systems as a result of tumor growth and immunosuppressive treatments such as radiotherapy and/or chemotherapy. One way to overcome this problem is to adoptively transfer *ex vivo* expanded tumor-reactive T cell clones or tumor-infiltrating lymphocytes (TIL). Recently, Dudley and colleagues (Dudley et al., 2002) reported that adoptive transfer of *in vitro* expanded melanoma-specific TILs administered in conjunction with high dose IL-2 to advanced melanoma patients resulted in objective clinical responses, including regression of metastasized tumors in about half of the treated patients. This study clearly demonstrated that tumor specific T cells can recognize and eliminate tumor cells *in vivo*. Yee et al have also shown that adoptively transferred melanoma-specific CD8+ T cell clones administered with low dose IL-2 could traffic to the tumor site, effect tumor regression and persist *in vivo* for at least 21 days post T cell infusion. (Yee et al., 2002). Even though these studies demonstrated that adoptive transfer of tumor-specific T cells is feasible, relatively safe and effective in eliminating tumor cells, there are limitations to using this approach. These limitations range from the difficulty of obtaining sufficient numbers of TILs from most cancer patients to the presumably limited *in vivo* survival of adoptively transferred T cells.
In fact, Dreno and colleagues reported that the overall survival of stage III melanoma patients who received melanoma-specific TILs with IL-2 was not different than that of patients receiving IL-2 alone (Dreno et al., 2002). This is hypothesized to be due to the fact that infused T cell clones do not survive *in vivo* long enough to prevent recurrence and growth of residual tumor cells.

To overcome the limitation of generating sufficient numbers of T cells for adoptive transfer, a number of investigators have reported successful transfer of the TCR α and β chains from tumor-reactive T cell clones to *in vitro* activated primary T lymphocytes (Rubinstein et al., 2003; Morgan et al., 2003). These T cells retrovirally transduced with the TCR α and β chains from Tumor-specific T cell clones were able to lyse tumor cells that express the antigen of interest. This approach was proven successful in eliminating tumor cells that express the antigen recognized by the TCR in animal models (Stanislawski et al., 2001). The limitations of using full-length TCR α and β chain gene transfer approach for cancer immunotherapy is that the expression of the TCR is restricted only to T cells. In contrast, TCRs fused to other signaling molecules, such as the FCγR or the CD3ζ chain can be expressed in T and non-T cells. This can broaden the spectrum of immune cells that can be endowed with particular TCR specificity and targeted to the tumor site, thus resulting in a more robust anti-tumor immune response. Willemsen and colleagues reported that primary human T lymphocytes transduced with melanoma MAGE-A1-specific tcTCR or scTCR can recognize MAGE-A1 positive tumors *in vitro*. Other groups were able to transduce BM cells with a retroviral vector encoding a single chain antibody fused to the CD3ζ signaling component (T body) or with a CD4-ζ chimeric universal receptor to target HIV-infected cells (Wang et al., 1998; Hege et al., 1996). Transduced BM cells could
differentiate *in vivo* into multiple immune lineages that expressed the chimeric receptors and could reject tumor cells that expressed the target antigen or HIV gp120 protein.

The role of the innate and adaptive arms of the immune system in controlling tumor growth is well documented. For the past few decades, many groups have focused their efforts on inducing tumor-specific T cell responses in an attempt to control tumor growth in animal models and in human clinical trials. We believe that an effective immune response against cancers that could potentially result in tumor regression and clinical responses has to involve both innate and adaptive mechanisms of the immune system. An intriguing strategy to redirect the specificity of cells of the innate immune system towards a particular tumor antigen has been described recently using either chimeric CD4-ζ universal receptors or using single chain antibodies (T-bodies) (Hege et al., 1996; Wang et al., 1998). The specificity of the CD4-ζ is limited to HIV gp120 expressing targets. On the other hand, the affinity of T-bodies to antigens is relatively high (10^{-7}–10^{-9} M, (Hudson and Souriau, 2003) which could result in apoptosis of cells that express the T-body following interaction with tumor cells via activation induce cell death. T cell receptors have lower binding affinity (10^{-4}-10^{-5} M; Davis and Chien, 1993) for their target cells; therefore a single T cell can engage and kill multiple targets. The only limitation of using scTCRs for cancer immunotherapy is that recognition of tumor cells via conventional TCR requires MHC class I presentation by tumor cells. However, tumor cells can down-regulate MHC class I processing and presentation (Bubenik, 2003; Cabrera et al., 2003). In addition, scTCRs specific for particular pMHC complex can only be used to treat patients who express that particular HLA allele. MUC1 is a unique tumor antigen as it can be recognized by CTL in the absence of MHC processing and presentation. This phenomenon of MHC-unrestricted
recognition of MUC1 has been extensively studied by our group (Barnd et al., 1989; Jerome et al., 1991; Magarian-Blander et al., 1998). Tumor cells have defects in their glycosylation machinery that results in expression of new form of MUC1 on the surface of tumor cells (Sotiropoulou et al., 2002). Underglycosylation of MUC1 on tumor cells results in exposing the immunodominant epitope of MUC1 backbone on tumor cells (Fontenot et al., 1993). This epitope is able to bind directly to the TCR on the surface of CD8+ T cells. MUC1 is a large molecule that consists of more than 100-200 repeats of a 20 amino acid, each presenting one immunodominant epitope. Therefore, this molecule is able to bind and crosslink multiple TCRs on the surface of CD8+ T cells and hence is able to activate T cells. This unique structure of MUC1 expressed by tumor cells, in addition to our ability to establish MUC1-specific MHC-unrestricted CTL clones in our laboratory, gave us the opportunity to explore the potential of using such TCRs for cancer immunotherapy.

Because MUC1 is expressed on the surface of more than 83% of human cancers, we proposed to use the MHC-unrestricted MUC1-specific scTCR as a universal tumor-specific receptor for the treatment of MUC1+ epithelial tumors. To date, there are no reports in the literature describing scTCR as a therapeutic tool to modify BM cells and to confer tumor immunity. In this chapter we describe the efficacy and safety of the MHC-unrestricted MUC1-specific scTCR for cancer immunotherapy in SCID and MUC1 Tg. mouse models.

We showed that SCID mice reconstituted with BM cells transduced with the scTCR retroviral vector controlled the growth of MUC1+ tumor cells. Construction of a scTCR-EGFP retroviral reporter vector enabled us to track the scTCR-expressing cells in vivo. BM cells transduced with the scTCR-EGFP retroviral vector could differentiate in vivo into T, B, NK, monocytes, and granulocytes that expressed the scTCR. ScTCR-expressing cells
were detected in both primary and secondary lymphoid organs in reconstituted mice. Expression of this scTCR on large percentage of immune cells was safe as evident by no signs of autoimmunity, tissue destruction, or infiltration of MUC1-expressing organs in MUC1 transgenic mice.

3.2. MATERIALS AND METHODS

3.2.1. Construction of scTCR-EGFP Retroviral Vector

The IRES-EGFP cassette was cloned by PCR from the pIRES2-EGFP vector (Clontech Laboratories, Palo Alto, CA) using IRES forward (P18) and EGFP reverse (P19) primers. Two Bgl II sites were introduced into the forward and reverse primers. The PCR product was first cloned into the pcDNA3.1 TA vector and was then digested with the Bgl II restriction enzyme (New England BioLabs, Beverly, MA). The digested fragment was then cloned into the BamH I site in the scTCR-MFG retroviral vector. The orientation and sequence of the final vector was confirmed by DNA sequencing at the department of MGB DNA sequencing facility, University of Pittsburgh.

3.2.2. Production of Retroviral Supernatant

ScTCR-Neo MFG or scTCR-EGFP MFG retroviral vectors were transfected into the Phoenix amphotropic retroviral packaging cell line using the calcium phosphate method and according to the manufactures recommendations (Invitrogen). Forty eight hours post-transfection, retroviral supernatant was harvested and was used to transduce the GP-E86 ecotropic retroviral packaging cell line (ATCC, Manassas, VA) in the presence of 8 µg/ml
polybrene (Sigma, St. Louis, MO). GP-E86 cells were maintained in DMEM-10 (DMED + 10% FBS, 2 mM L-glutamine, 100 U/ml Penicillin and 100 U/ml streptomycin). Cells transduced with the scTCR-Neo MFG vector were selected in DMEM-10 medium supplemented with 1 mg/ml G418. GP-E86 cells transduced with the scTCR-EGFP MFG vector were cultured for 5 days followed by sorting the EGFP<sup>high</sup> population at the UPCI Flow Cytometry facility. Sorted cells were then cultured in DMEM-10 until confluent and then the medium was replaced with fresh DMEM-15 (DMEM + 15% FBS, 2 mM L-glutamine, 100 U/ml Penicillin, 100 U/ml streptomycin). Thirty six hours later, retroviral supernatant was harvested and was frozen at -80°C in 1.5 ml aliquots until use.

### 3.2.3. Fluorescence Microscopy

NIH 3T3 cells transduced with the scTCR-EGFP MFG retroviral vector were cultured in 2-chamber slides at 2X10<sup>5</sup>/ml overnight at 37°C. Slides were then washed 3X in PBS and fixed for 15 minutes at RT in PBS + 2% PFA. Slide were then washed once with PBS followed by 3 washes with BSA buffer (PBS + 0.5% BSA + 0.15% glycine). Slides were then blocked with PBS + 5% normal goat serum for 30 minutes at RT followed by 3 washes with BSA buffer. The anti-TCR BF1 antibody (Endogen) was added at 2 µg/ml in BSA buffer and was incubated at RT for 1 hour. Slides were washed 3X in BSA buffer, and Alexa flour 546-conjugated goat anti-mouse IgG was added at 2 µg/ml in BSA buffer and slides were incubated for 1 hour at RT. Slides were washed 3X in PBS and cover slips were mounted on the slide using Gel Mount (Sigma). Cells on slides were visualized under Olympus fluorescence microscope at the Center for Biologic Imaging, University of Pittsburgh.
3.2.4. Retroviral Transduction of BM Cells

Six to eight week old (SCID, Balb/c or C57BL/6) donor mice were injected intraperitoneally with 150 mg/kg 5-FU (Invivogen, San Diego, CA); and five days later, mice were sacrificed and BM cells were isolated. Residual red blood cells (RBCs) in BM preparations were lysed using RBC lysing buffer (Sigma). BM cells were cultured at 1X10^6/ml in retroviral supernatant diluted 1:1 with fresh DMEM-15 medium supplemented with 8µg/ml polybrene. Retroviral supernatant was hemi-depleted after 2 hours, and equal volume of fresh retroviral supernatant with polybrene was added. For some experiments, BM cells were pre-stimulated for 72 hours in the presence of 50 ng/ml SCF, 10 ng/ml mIL-3 and 10 ng/ml mIL-6 (PeproTech Inc., Rocky Hill, NJ) in DMEM-15. BM cells were then harvested and resuspended in retroviral supernatant supplemented with 50 ng/ml SCF, 10 ng/ml mIL-3, 10 ng/ml mIL-6 and polybrene at 8µg/ml. In all BM transduction experiments, cells were plated in 24-well plates pre-coated with recombinant fibronectin CH-296 fragment (Takara Bio Inc., Madison, WN). In some experiments, retroviral supernatant was first adsorbed to plates coated with CH-296 for 2 hours at 37°C prior to transduction of BM cells. Cells were then centrifuged for 30 minutes at RT at 800 X g in a Sorvall T6000B centrifuge and were put back in culture at 37°C. Twelve or 24 hours post transduction, retroviral supernatant was hemi-depleted and fresh retroviral supernatant with cytokine and polybrene was added. Plates were centrifuged for 30 minutes at 800 X g at RT. The transduction process was repeated every 12 or 24 hours for a total transduction period of 48 hours.
3.2.5. Flow Cytometric Analyses of Transduced BM

All antibodies used for flow cytometry were obtained from BD Pharmingen, unless stated otherwise. Transduced BM cells were harvested on day 7, and 5X10^5 cells were blocked with 1 µg of anti-CD16/CD32 antibody in 100 µl FACS buffer for 20 minutes on ice. One µg of PE-conjugated anti-c-kit or anti-CD34 antibody was added to cells and incubated for an additional 30 minutes on ice. Cells were washed 3X in FACS buffer (1X PBS + 5% FBS + 0.01% sodium azide) and were resuspended in 1% PFA before analyses using BD FACSCalibur. In some experiments, BM cells were blocked for 20 minutes with anti-CD16/CD32 antibody, and then 1 µg of PE-conjugated anti-c-Kit, APC-conjugated anti-Sca1, and Biotinylated anti-lineage cocktail (Stem Cell Technologies, Vancouver, BC) antibodies were added and incubated for an additional 30 minutes on ice. Cells were then washed 3X in FACS buffer and 10 ng of SA-PE-Cy7 was added in 100 µl FACS buffer and incubated for additional 30 min on ice. Cells were washed 3 X in FACS buffer and fixed in 1% PFA before analyses.

3.2.6. Reconstitution of Sub-Lethally Irradiated Mice With Transduced BM Cells

Six to eight week old SCID, Balb/c and C57BL/6 mice were obtained from Jackson Laboratories (Jackson Laboratory, Bar Harbor, ME). MUC1 Tg. mice were obtained from the Mayo Clinic (Scottsdale, AZ). Transduced BM cells were resuspended in PBS at 1X10^7/ml, and 200 µl of cell suspension was injected via the tail vein into sub-lethally irradiated recipient mice (SCID 3500 rad, Balb/c 900 rad, C57BL/6 1000 rad). With the exception of SCID mice, all mice were given a split dose of radiation (50/50) 3 hours apart.
Reconstituted mice were maintained in a germ-free environment and were put on acidified water (pH 2.5) for 3 weeks post-reconstitution.

3.2.7. Isolation of Immune Cells From Reconstituted Mice and Flow Cytometric Analyses

At different time points post-reconstitution, mice were bled from the tail artery and 200 µl of blood was collected in 0.5 ml PBS containing 3 µg/ml EDTA, 20 U/ml heparin (Sigma). Heparinized blood was then mixed with 0.5 ml of 20 mg/ml dextran T-500 (Amersham Biosciences, Piscataway, NJ) and incubated at 37° C for 30 min. The upper layer containing leukocytes was then collected and washed once in PBS. Residual RBC cells were then lysed using RBC lysing buffer. Cells were then stained with the appropriate anti-surface marker antibody (PE-conjugated anti-CD3, -B220, -GR-1, -Mac-3 and -DX5) or APC-conjugated anti-F4/80 (eBiosciences, San Diego, CA) antibodies. Cells isolated from blood, spleen, lymph nodes (LN), thymus or BM were stained as described above.

3.2.8. Tumor Challenge

Mice reconstituted with BM Cells transduced with scTCR-Neo MFG or with a control retroviral supernatant were challenged with HPAF tumor cells 5 weeks post-reconstitution. Mice were first anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL) followed by subcutaneous injection of 2X10^6 HPAF tumor cells in 100 µl of PBS in the upper hind flank region. Tumor size was measured every 2-3 days using caliber. Tumors from control or treated mice were fixed in 10% formalin, paraffin embedded, and sections were stained with H&E, anti-Myeloperoxidase (Labvision, Fremont, CA), anti-
3.2.9. Fluorescent Microscopic Analyses of Tissue Sections From Reconstituted mice

Six weeks post-reconstitution, C57BL/6 mice or MUC1 Tg. mice reconstituted with scTCR-EGFP transduced BM cells were sacrificed and spleen, lung, and pancreas were harvested and fixed in 2% PFA in PBS. Tissues were then frozen and sectioned and visualized for the infiltration with EGFP+ immune cells at the Center for Biologic Imaging, University of Pittsburgh.

3.3. RESULTS

3.3.1. Tumor Rejection in Mice Reconstituted With BM Cells Transduced With The MHC-Unrestricted MUC1-Specific scTCR Retroviral Vector

Donor SCID mice were treated with 5-FU 5 days before BM isolation. It was previously reported that BM cells from 5-FU treated mice have a more primitive phenotype and have more long-term reconstitution potential compared to BM cells from untreated mice (Randall and Weissman, 1997; Harrison and Lerner, 1991; Bodine et al., 1991). BM cells were transduced with an ecotropic retroviral supernatant prepared using the GP-E86 retroviral packaging line as described in Materials and Methods. The retroviral titer from GP-E86 cells varied between 1-4 X 10^6 pfu/ml. Figure 17A shows RT-PCR analysis of the scTCR expression in transduced BM cells 72 hours post-transduction. We did not have a directly-labeled antibody against the scTCR that we could use for flow cytometry;
therefore we decided to do RT-PCR analyses to confirm the expression of the scTCR gene in transduced cells. Transduced BM cells expressed high levels of the scTCR transcript compared to control BM cells. Recipient SCID mice received a sub-lethal dose of total

Figure 17. RT-PCR analyses of scTCR expression in transduced BM cells and in splenocytes and LN cells from reconstituted SCID mice. Panel A shows the expression of the scTCR mRNA in transduced BM cells 72 hours post-transduction. Expression of the scTCR mRNA was also detected in splenocytes and LN cells from reconstituted mice 60 days post-reconstitution (B). M is 1 Kb DNA molecular weight marker. β-actin is RT-PCR control.
body irradiation (350 rad) just before BM transplant. Irradiation of recipient mice is critical for successful engraftment of donor hematopoietic stem cells (Zhong et al., 2002). It was hypothesized that irradiation of recipient mice will create room for the transplanted stem cells, hence enhances engraftment. Recipient mice received an average of $2 \times 10^6$ transduced BM cells via tail vein injection. Expression of the scTCR mRNA was detected by RT-PCR in splenocytes and LN cells 8 weeks post-reconstitution (Figure 17B). Mice reconstituted with BM cells transduced with the scTCR, or with control retroviral supernatant, were challenged 5 weeks post-reconstitution with $2 \times 10^6$ HPAF tumor cells injected subcutaneously. Tumor size was measured every 2-3 days using Caliber. Figure 18 shows that mice reconstituted with the scTCR-transduced BM cells were able to control tumor growth compared to control mice. The difference in tumor size between the two groups was statistically significant ($P< 0.05$). p-Values were calculated by running two-tails t-test using Excel software. H&E staining of tissue sections of regressed tumors from mice reconstituted with scTCR-transduced BM cells showed massive destruction of the tumors (Figure 19A). There was no destruction of tumors in sections from the control mice (Figure 19B). The majority of the immune cells that infiltrated tumors in the scTCR-MFG-reconstituted mice were neutrophils that stained positive for myeloperoxidase (Figure 19C). Tumor sections from these mice were also infiltrated by macrophages (F4/80+, Figure 19D) and NK cells (Granzyme B+, Figure 19E).

3.3.2. Simultaneous Expression of scTCR and EGFP in Cells Transduced With The scTCR-EGFP Retroviral Vector

EGFP has been widely used as a marker to track differentiation and migration of genetically engineered hematopoietic cells *in vivo* (Kume et al., 2000; Yang et al., 2002).
Figure 18. Tumor challenge of SCID mice reconstituted with BM cells transduced with scTCR-MFG or with control retroviral supernatant. SCID mice were challenged after 5 weeks with HPAF (MUC1+) pancreatic tumors. Tumor size is expressed on the y-axis while days post-tumor challenge are plotted on the x-axis. Blue line (triangle) represents control mice, while yellow line (circle) represents mice reconstituted with scTCR-MFG transduced BM cells. P-Values were calculated by running the t-test using Excel software.
Figure 19. Destruction and infiltration of tumor sections from SCID mice reconstituted with scTCR-MFG-transduced BM cells. Tumor sections from control mice (A) or scTCR-MFG-reconstituted mice (B) were stained with H & E. Tumor sections from scTCR-MFG-reconstituted mice were also stained for myeloperoxidase (neutrophil marker, C), F4/80 (monocytes/macrophage marker, D), or Granzyme B (NK cells marker, E). Pictures were taken under 20X magnification. Pictures in lower right squares in C, D, and E were taken under 100X magnification.
The advantage of using the EGFP retroviral system is that it eliminates the necessity for tedious and often difficult multi-color immunofluorescence staining of immune cells. This system is also useful when directly-conjugated antibodies against the protein of interest are not available. Figure 20A depicts the design of the scTCR-EGFP MFG vector. The IRES-EGFP cassette was cloned downstream of the scTCR gene in the MFG retroviral vector. The presence of the IRES sequence mediates expression of the scTCR and EGFP proteins from the same mRNA transcript. Figure 20B (left panel) shows that NIH 3T3 cells transduced with the scTCR-EGFP MFG vector express EGFP. To confirm that both the scTCR and EGFP are expressed simultaneously, NIH 3T3 cells transduced with the scTCR-EGFP MFG were also stained for surface expression of the scTCR (Figure 20B middle panel). It is evident that cells that expressed EGFP also expressed the scTCR. Figure 20B (right panel) shows the overlay of EGFP (green), scTCR staining (red) and nucleus staining (blue).

3.3.3. Comparison of BM Transduction Efficiencies Using Different Transduction Protocols

Different protocols for retroviral transduction of murine BM cells have been described in the literature. To compare the efficiencies of BM transduction under different conditions, BM cells from 5-FU treated mice were isolated and transduced for 4 hours on fibronectin-coated plates, in the presence of 8 µg/ml polybrene (Figure 21B). Figure 21A is control untransduced BM cells. Plates were coated with fibronectin fragment because it was reported that recombinant fibronectin fragment (CH-296) can bind to VLA-4 and VLA-5, expressed on many cell types, and to the envelope proteins on retroviral particles,
Figure 20. Construction of a scTCR-EGFP MFG retroviral vector and expression in NIH 3T3 cells. The configuration of the scTCR-EGFP MFG retroviral vector is depicted in A. B shows fluorescent microscopy of 3T3 cells transduced with the scTCR-EGFP retroviral vector. Transduced cells expressed EGFP (green-left panel). Transduced cells were stained for scTCR-expression using BFI antibody and Alexa547-conjugated goat-anti-mouse antibody (red-middle panel). Overlay of EGFP, TCR and nucleus staining (Hoechst, blue color) is shown in B (right panel).
which results in higher efficiency of transduction (Hanenberg et al., 1996; Abonour et al., 2000). Under these transduction conditions, about 8.0% of BM cells were transduced with the retroviral vector (Figure 21B). To address the effect of prior activation of BM cells (Luskey et al., 1992) and the effect of centrifugation on the efficiency of transduction (Bunnell et al., 1995), BM cells were first cultured in DMEM-15 medium containing 50 ng/ml SCF, 10 ng/ml mIL-3 and 10 ng/ml mIL-6 for 72 hours followed by 2 cycles of transduction. Each cycle consisted of plating activated BM cells in the retroviral supernatant in the presence of cytokines and polybrene and centrifugation for 30 min at 1000 X g. This procedure was repeated after 24 hours. Under these conditions, about 30% of the cells in the culture were transduced with the retroviral vector (Figure 21C). When BM cells were subjected to 4 cycles of transduction each done every 12 hours, the efficiency of transduction was increased to 50% (Figure 21D). In order to further increase the efficiency of transduction, retroviral supernatant was first adsorbed to fibronectin-coated plates for 2 hours at 37°C and then transduction was done as described in Figure 21D. Under these conditions, about 60% of the cells in the culture were transduced with the retroviral vector (Figure 21E). The efficiency of transduction using the latter transduction protocol varied from 50-75% among different experiments.

3.3.4. Phenotype of Transduced BM Cells in Culture

BM cells were transduced as described in Figure 21E and were kept in culture in the presence of cytokines (50 ng/ml SCF, 10 ng/ml mIL-3 and 10 ng/ml mIL-6) for additional 3 days. On day 7, cells were harvested and stained for different surface markers. The overall efficiency of BM transduction in the experiment presented in Figure 22 was 72.0%
Figure 21. BM transduction efficiencies with scTCR-EGFP retroviral supernatant under different transduction protocols (B-E) and control (A). For details, refer to text section 3.3.3. Staining for CD34 (a hematopoietic progenitor cells surface marker) is plotted on the y-axis while EGFP expression is blotted on the x-axis.
(data not shown). As shown in Figure 22B, about 50% of total cells were positive for both EGFP and c-kit. C-Kit (CD117) is the receptor for stem cell factor (SCF) and is a marker of early hematopoietic progenitor and stem cells (Ogawa et al., 1991). Hematopoietic stem cells also express high levels of Sca-1 (Okada et al., 1992). Figure 22D shows that about 60% of the Sca-1+ cells were transduced with the scTCR-EGFP vector. Hematopoietic stem cells that have the capacity for self-renew and that have long-term reconstitution potential are contained within the Thy1.1−Lin−c-Kit+ Sca-1+ population (Kondo et al., 2003). To show that under these transduction conditions, the long-term reconstituting hematopoietic stem cells were transduced with the scTCR-EGFP retroviral vector, cells were stained with PE-conjugated anti-c-kit, APC-conjugated anti-Sca-1 and with PE-Cy7-conjugated anti-Lin cocktail antibodies. We gated on the c-kithigh Sca-1high Lin− population and plotted cells within those gates (R2 & R3) against EGFP. As shown in Figure 22H, about 39% of these cells were transduced with the scTCR-EGFP vector. More than 99.0% of the cells under these culture conditions were negative for thy1.1 (data not shown). Taken together, it is apparent that, under these culture conditions, the long-term reconstituting hematopoietic stem cells were transduced with the scTCR-EGFP retroviral vector. This needs to be further confirmed by long-term follow up of reconstituted mice and by doing secondary BM reconstitution experiments.

3.3.5. In Vitro Differentiation of Transduced BM Cells

In order to test the potential of BM cells transduced with the scTCR-EGFP MFG vector to differentiate in vitro into different immune lineages that express the scTCR, transduced BM cells were cultured for a total of 12 days in DMEM-15 medium containing
Figure 22. Phenotype of transduced BM cells on day 7 in culture. BM cells were transduced with scTCR-EGFP retroviral supernatant as described in Figure 21E. On day 7, cells were stained for hematopoietic stem cells surface markers (c-Kit and Sca-1), and for lineage markers (Lin). Expression of these surface markers was plotted on the y-axis as function of EGFP expression (x-axis). For details, refer to text section 3.3.4.
50 ng/ml SCF, 10 ng/ml mIL-3 and 10 ng/ml mIL-6. Cells were then stained for GR-1 (granulocyte lineage differentiation marker) and for F4/80 (monocyte/macrophage lineage differentiation marker). Figures 23A and 23B show that transduced BM cells can differentiate \textit{in vitro} into F4/80+ and GR-1+ cells that express the scTCR. Similar results were obtained when cells were cultured for an additional 8-12 days in AIM-V medium containing 1000 U/ml mGM-CSF and 1000 U/ml mIL-4 (Figures 23C & 23D). Under the latter culture conditions, transduced BM cells also differentiated into CD11c+ cells that expressed the scTCR (Figure 23E). Data presented in Figures 23C, 23D, and 23E were gated on F4/80+, GR-1+, and CD11c+ cells, respectively.

3.3.6. \textit{In Vivo} Differentiation of Transduced BM Cells

To test the \textit{in vivo} engraftment and differentiation potential of BM cells transduced with the scTCR-EGFP MFG retroviral vector, 1-2 X 10^6 BM cells that were transduced for 4 hours or that were transduced for 48 hours after activation with cytokines, were transplanted into a group of sub-lethally irradiated recipient syngeneic Balb/c mice. Mice were sacrificed 5 weeks post-reconstitution, and the percentage of scTCR-expressing immune cells in the blood, spleen, LN, thymus and BM were evaluated using FACS analyses. Figures 24 (B, E, & H) shows that at 5 week post-reconstitution with 4 hours transduced BM cells, an average of 4% of immune cells in the blood, spleen, and LN expressed the scTCR. However, a lower percentage of cells in the thymus (1.5%) and bone marrow (0.2%) expressed the scTCR (Figures 24 K, M, & O). On the other hand, 1.5%-2.8% of immune cells in the blood, spleen, and LN of mice reconstituted with day 6
Figure 23. *In vitro* differentiation of BM cell transduced with scTCR-EGFP MFG vector. BM cells were transduced with the scTCR-EGFP MFG and were cultured for an additional 8 days in DMEM-15 supplemented with mSCF, mIL-3 and mIL-6 (A & B) or in AIM-V medium supplemented with mGM-CSF & mIL-4 (C-E). Cells were stained for F4/80 (A & C), GR-1 (B & D) or CD11c (E) and were plotted against EGFP. Data presented in C, D, and E were gated on cells positive for F4/80, GR-1, or CD11c, respectively.
Figure 24. *In vivo* differentiation of BM cells transduced with scTCR-EGFP MFG using different transduction protocols into immune cells that express the scTCR. Five weeks post-reconstitution, cells from blood (A-C), LN (D-F), spleen (G-I), thymus (J-L), and BM (M-P) of reconstituted mice were stained with different surface markers and were plotted on the y-axis against EGFP expression (x-axis). A, D, G, and J are cells from control mice. For details, refer to text section 3.3.6.
transduced BM cells expressed the scTCR (Figures 24 C, F, and I) while a higher percentage was seen in the thymus (9.5%) and BM (9.4%-10.0%). These data suggested that when transducing BM cells for 4 hours, mainly mature and committed progenitor cells get transduced resulting in higher percentages of scTCR-expressing cells in secondary lymphoid organs. On the other hand, reconstituting mice with day 6 transduced BM cells resulted in a low percentage of scTCR-expressing cells in the secondary lymphoid organs after 5 weeks and a higher percentage in primary lymphoid organs. This suggested that activating BM cells for 3 days prior to transduction allows transduction of BM cells with a more primitive phenotype.

3.3.7. Kinetics of Immune Reconstitution With scTCR-expressing Cells in Irradiated Recipient Mice

Irradiated Balb/c mice receiving 2 X 10^6 day 6-transduced BM cells, were bled at 3, 6, and 11 weeks post-reconstitution; and percentages of scTCR-expressing cells in immune lineages were evaluated. The efficiency of transduction of BM cells in this experiment was about 50% (data not shown). As shown in Figure 25B, at 3 weeks post-reconstitution 5.6% of GR-1+ cells expressed the scTCR. This percentage didn’t change much at 6 weeks (Figure 25C); however 16.5% of GR-1+ cells expressed the scTCR at 11 weeks post-reconstitution (Figure 25D). Reconstitution of the monocyte/macrophage lineage followed a similar kinetics where 5.7% of these cells expressed the scTCR at 3 weeks (Figure 25F), 9.9% at 6 week (Figure 24G) and 32.0% at 11 weeks (Figure 25H). Similarly, in the NK lineage, DX5+ cells that expressed the scTCR were first detected at 3 weeks and reached a maximum of 27.2% at 11 weeks post-reconstitution (Figure 25L). When looking at T and B
Figure 25. Kinetics of \textit{in vivo} differentiation of transduced BM cells into multiple immune lineages that express the scTCR. Balb/c mice were reconstituted with BM cells transduced with the scTCR-EGFP MFG and at different time points post-reconstitution, mice were bled and immune cells were stained for the appropriate cell surface marker (GR-1 for granulocyte, DX5 for NK cells, mac-3/F4/80 for monocyte/macrophages, CD3 for T cells, B220 for B cells.) and were plotted on the y-axis. The percentage of EGFP positive cells in each lineage is plotted on the x-axis.
cells, at 3 weeks post-reconstitution there was 0.1% scTCR expression on CD3+ cells in the periphery. However, 5.4% of CD3+ cells were positive for scTCR at 6 weeks post-reconstitution (Figures 25N & 25O). This number dropped to 3.4% at 11 weeks post-reconstitution (Figure 25P). It has been reported that the first wave of T cells that appears in the periphery following BM and stem cell transplantation has a memory phenotype (CD45RO+) and may represent the recipient’s T cells that survived irradiation and expanded in the secondary lymphoid organs (Dreger et al., 1999). The first wave of donor-derived T cells that have gone through thymic selection is usually seen after about 5 weeks after BM transplantation (Kume et al., 2000). About 2.0% of B220+ cells expressed the scTCR at 3 weeks post-reconstitution (Figure 25R). This number reached a maximum of 3.1% at week 6 (Figure 25S) and eventually dropped down to 1.9% at 11 weeks (Figure 25T). Taken together, these data demonstrated that cells of the innate immune system get reconstituted faster than T and B cells and that the percentage of scTCR-expressing cells in the myeloid lineage and NK cells is much higher than that seen in T and B cells. To test the possibility that there was selection in the thymus against T cells that express this scTCR, we stained cells from the thymus of reconstituted mice with CD4 and CD8 and looked at scTCR expression at different stages of T cell maturation. Figure 26 shows that similar percentages of T cells expressed the scTCR at the CD4+CD8+ (Figure 26B), CD4+CD- (Figure 26C), CD4-CD8+ (Figure 26D), and the CD4-CD8- (Figure 26E) stages. These data ruled out the possibility that expression of the scTCR in immature thymocytes can block their differentiation and maturation. Even though the percentages of T and B cells that expressed the scTCR were low relative to the myeloid and NK cells, the percentage is as high as would be seen during a viral infection (Blattman et al., 2002) and is thought to be
sufficient to initiate and drive an effective immune response against targets that express MUC1.

Figure 26. ScTCR expression on T cell at different stages of maturation in the thymus of Balb/c mice reconstituted with scTCR-transduced BM cells. Five weeks post-reconstitution, mice were sacrificed and thymocytes were stained with PE-conjugated anti-CD4 and APC-conjugated anti-CD8. (A) shows normal staining of CD8 and CD4 on thymocytes. CD4+CD8+ (B), CD4-CD8+ (C), CD4+CD8-(D), and CD4-CD8-(E) were plotted against EGFP expression.
3.3.8. Immune Reconstitution With scTCR-Expressing Cells in Irradiated MUC1 Tg. Mice

Data presented above (Figure 25) demonstrated that it was feasible to reconstitute the innate and adaptive immune compartments of Balb/c mice with scTCR-expressing immune cells. However, we were interested in testing whether MUC1 Tg. mice could be reconstituted with scTCR-expressing immune cells as well. MUC1 Tg mice express human MUC1 as “self” molecule. MUC1 expression in these mice is driven by the MUC1 endogenous promoter; hence, these mice have the same pattern of MUC1 expression as in humans (Peat et al., 1992). Because MUC1 Tg. mice express MUC1 as “self” molecule, it was possible that T cells transduced to express the MUC1-specific scTCR could be deleted during thymic selection. To address this question, C57BL/6 or MUC1 Tg. mice received split doses (50/50) of 1000 rad total body irradiation and were reconstituted with 2 X 10^6 BM cells transduced with the scTCR-EGFP MFG retroviral vector. The efficiency of BM transduction in this experiment was 72.0% (Data not shown). Six weeks post-reconstitution, mice were bled and the percentages of scTCR-expressing immune cells in the blood were evaluated. As shown in Table 1, about 9.0% of CD3+ cells expressed the scTCR in MUC1 Tg. mice. This number was slightly higher than that seen in C57BL/6 mice (6.4%). Similar percentages of scTCR expressing B220+ cells (9.5%) were seen in C57BL/6 and MUC1 Tg. mice. When looking at the innate immune compartment, about 30% of GR-1+ cells in both C57BL/6 and MUC1 Tg. mice expressed the scTCR. About 18.5% and 14.6% of DX5+ and F4/80+ cells expressed the scTCR in MUC1 Tg. mice. These numbers were slightly lower than those seen in C57BL/6 mice (28.5 % and 20.4 %).
These data demonstrated that T cells expressing the MUC1-specific scTCR survived thymic selection in MUC1 Tg. mice and ended up in the periphery.

Table 1. ScTCR expression in immune cells from C57BL/6 and MUC1 Tg. mice 6 weeks post-reconstitution. Data are presented as mean% EGFP+ cells +/- s.d.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>C57BL/6</th>
<th>MUC1 Tg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>0.2 ± 0</td>
<td>6.4 ± 1.3</td>
<td>9.0 ± 5.7</td>
</tr>
<tr>
<td>B220</td>
<td>0.2 ± 0</td>
<td>9.7 ± 0.3</td>
<td>9.5 ± 1.6</td>
</tr>
<tr>
<td>GR-1</td>
<td>0.2 ± 0.7</td>
<td>30.8 ± 21.5</td>
<td>27.4 ± 9.2</td>
</tr>
<tr>
<td>DX5</td>
<td>0.1 ± 0</td>
<td>28.5 ± 4.3</td>
<td>18.5 ± 3.7</td>
</tr>
<tr>
<td>F4/80</td>
<td>0.1 ± 0.2</td>
<td>20.4 ± 5.0</td>
<td>14.6 ± 2.7</td>
</tr>
</tbody>
</table>

3.3.9. Absence of Tissue Destruction or Immune Infiltration of MUC1 Tissues in MUC1 Tg. Mice.

Six weeks post-reconstitution with scTCR-transduced BM cells, C57BL6 and MUC1 Tg. mice were sacrificed and spleen, lung, and pancreas tissues were harvested. Figures 27B and 27C show that scTCR-expressing cells (green) could be detected in the spleen of C57BL6 and MUC1 Tg. mice, respectively. There was no large infiltration of the lung (Figure 27E), or pancreas (Figure 27H) from reconstituted C57BL6 mice. Similarly,
Figure 27. Immunofluorescent visualization of tissue sections from control C57BL/6 mice (A, D, G), and from C57BL6 mice (B, E, H) or from MUC1 Tg. mice (C, F, I) that were reconstituted with BM cells transduced with the scTCR-EGFP MFG retroviral vector. Spleen (A-C), lung (D-F), and pancreas (G-I) tissue from indicated mice were harvested 6 weeks post-reconstitution and were microscopically visualized for infiltration with EGFP+ cells.
there was no massive infiltration of the lung (Figure 27F) or pancreas (Figure 27I) from reconstituted MUC1 Tg. mice. Lung and pancreatic tissue from MUC1 Tg. mice normally express human MUC1 as self antigen. H&E staining of lung and pancreas tissue sections from reconstituted C57BL6 mice (Figures 28G & 28M) or from MUC1 Tg. mice (Figures 28I & 28O) showed no signs of tissue destruction. These results were expected because the MUC1 epitope recognized by the scTCR is expressed mainly by tumor cells and not by normal tissues.

Figure 28. H & E staining of tissue sections from control C57BL/6 mice (A, D), and from C57BL6 mice (B, E) or from MUC1 Tg. mice (C, F) that were reconstituted with BM cells transduced with the scTCR-EGFP MFG retroviral vector. Lung (A-C) or Pancreas (D-F) tissues were harvested from reconstituted mice 6 weeks post-reconstitution and were stained with H & E and visualized microscopically under 20X magnification.
3.4. DISCUSSION

To date, there are no reports in the literature showing that single chain TCR can be used to modify BM cells and confer tumor immunity. Herein, we reported successful construction and expression of a functional MUC1-specific MHC-unrestricted scTCR on multiple immune cell types \textit{in vitro}. We found that BM cells transduced with the scTCR could differentiate \textit{in vivo} into multiple immune lineages that expressed the scTCR. Cells of the innate immune system that expressed scTCR were first seen at 3 weeks post-reconstitution. These numbers increased gradually and reached its maximum at 11 weeks. We report here successful immunotherapy in SCID mice reconstituted with BM cells transduced with MUC1-specific scTCR. Tumor growth in these mice was significantly slower than in mice reconstituted with BM cells transduced with control retroviral supernatant. Neutrophils and macrophages were the predominant immune cells that infiltrated tumors from the TCR-reconstituted mice. In addition, these tumors were also infiltrated by NK cells. These data are very interesting because they show that it is feasible to direct the specificity of the innate immune cells with particular TCR which can then target and prevent tumor growth \textit{in vivo}. Because this scTCR was introduced into BM/stem cells, the expression of the scTCR on immune cells persisted for several months in reconstituted mice. We think this therapeutic approach can be applied for breast cancer patients who receive BM transplantation as an adjuvant therapy.

Two-color staining of T cells in the thymus of reconstituted Balb/c mice showed a normal distribution of CD4+ and CD8+ T cells that expressed the scTCR (Figure 25). These data also demonstrated that T cells expressing the scTCR are not deleted in the thymus. The same pattern of scTCR expression was also seen in the thymus of MUC1 Tg.
mice (data not shown). These results are expected because the form of MUC1 that is recognized by this scTCR is expressed solely by tumor cells and is not expressed in the thymus of wild type or MUC1 Tg. mice (Magarian-Blander et al., 1993; Peat et al., 1992). Surprisingly, the number of T and B cells that expressed the scTCR in the periphery was relatively low when compared to the percentages of innate immune cells that expressed the scTCR in the periphery. It has been reported earlier that lymphoid cells transduced with retroviral vectors have the tendency to silence expression of genes driven by the retroviral LTR promoter (Klug et al., 2000). This may explain the finding that fewer T and B cells than innate immune cells expressed the scTCR in the periphery. It is possible that T and B cells are more resistant to irradiation; therefore most of the cells that appear in the periphery post-reconstitution represent recipient’s cells that survived the radiation dose.

From an immunological point of view, the presence of 1.9-3.4% of antigen specific T and B cells in the periphery is considered sufficient to drive a vigorous and effective immune response. For instance, the frequency of naïve T cells specific for a single LCMV epitope was estimated to be 0.0005% in the repertoire of un-immunized C57Bl/6 mice (Blattman et al., 2002). This number expanded to 25% of the T cell pool during acute infection, and eventually more than 1.0% of antigen specific memory CD8+ T cells were retained. It will be interesting to see if the 1.9-3.4% of T cells expressing the scTCR could expand and persist as memory cells upon challenge with MUC1+ tumors.

To address the possibility that reconstituting MUC1 Tg. mice with BM cells engineered to express MUC1-specific MHC-unrestricted scTCR could result in destruction of MUC1-expressing organs, reconstituted MUC1 Tg. mice were sacrificed six weeks post-reconstitution and lung, pancreas and breast tissues were examined microscopically for
infiltration and potential destruction. There were no signs of infiltration with scTCR-expressing immune cells. These results were not surprising considering that the epitope that is recognized by this particular scTCR is expressed mainly by tumor cells. Previous data from our laboratory have shown that the APDTRP epitope presented by tumor MUC1 can also be expressed on the ducts of lactating human breast. It will be interesting to see if scTCR-expressing immune cells can infiltrate breast tissues of lactating MUC1 Tg. mice. Another important issue that we would like to address is the contribution of T and B cells expressing the scTCR to tumor immunity. We reconstituted a group of MUC1 Tg. mice with BM cells transduced with the scTCR. These mice will be challenged with RMA or RMA-MUC1 and tumor growth in these mice will be monitored. There is not much information available about the capability of scTCRs bearing a single CD3ζ chain to activate naïve T and to induce T cell proliferation. It will be interesting to see if scTCR-expressing T and B cells can expand in vivo upon challenge with MUC1-expressing tumor cells.

In summary we described herein a safe and effective immunotherapy approach for the treatment of subjects with MUC+ tumors. Further work is needed to investigate the efficacy of this approach in preventing spontaneous tumors. Long-term follow-up of reconstituted MUC1 Tg. is warranted before this therapeutic approach can be considered for clinical application.
APPENDIX A

Primers List

P1    Vα FWD
  5’ CGG GAT CCT CGA GAT GGA GAC CCT CTT GGG CCT GCT TA 3’

P2    V β FWD
  5’ CGG GAT CCG TCG ACA TGG CCA CCA GGC TCC TCT GCT G 3’

P3    BamHI EcoRI TCR α FL REV
  5’ CGG GAT CCG GAA TTC TCA GCT GGA CCA CAG CCG CAG CGT 3’

P4    NotI-BamHI TCR β FL REV
  5’ ATA GTT TAG CGG CCG CCT CGG ATC CTC AGA AAT CCT TCT TCT TCT TGA CCA 3’

P5    BamHI CD8 α FWD
  5’ CGG GAT CCA TGG CCT TAC CAG TGA CCG C 3’

P6    Not I CD8 α REV
  5’ ATA GTT TAG CGG CCG CTT AGA CTG ATC TCG CCG AAA GG 3’

P7    BamHI CD3 ζ FWD
  5’ CGG GAT CCC AAA CTC TGC TAC CTG CTG C 3’

P8    Sac II Not I EcoR I h ζ REV
  5’ TCC CCG CGG CGG CGG CGA ATT CTT AGC GAG GGG GCA GGG CCT GCA TG 3’

P9    BamHI C α REV (Cystine)
  5’ CGG GAT CCA GAT CCC CAC AGG AAC TTT CTG GGC TGG GGA AG 3’

P10   C β REV (Cystine)
  5’ CGG GAT CCA GAT CCC CAC AGT CTG CTC TAC CCC AGG CCT CG 3’

P11   AscI scTCR REV
  5’ AGG CGC GCC CCC AGG CCT CGG CGC TGA CGA TC 3’

12    AscI CD4-TM FWD
  5’ AGG CGC GCC GAC ATG GCC CTG ATT GTG CTG CTG GGG GGC 3’
P13  AscI AGD-CD4TM FWD
5` AGG CGC GCC GAC GCT GGG GAT ATG GCC CTG ATT GTG CTG GG 3`

P14  scFV REV
5` CTA AGC GTA GTC TGG GAC GTC GTA TGG GTA CAG ATC CTC TTC TGA GAT
GAG TTT TTG TTC TAC AAC GGT TAA CCT GGT C 3`

P15  Flag-His TCR β REV
5` CCT GCA GGT CAA TGG TGA TGG TGA TGC TTG TCA TCG TCA TCC TTG
TAG TCA GCG TCT GCT CTA CCC CAG G 3`

P16  GM-CSF Vα FWD
5` ATG TGG CTG CAG AGC CTG CTG CTC TTG GGC ACT GTG GCC TGC AGC ATC
TCT GCA CCC CAG GAG GTG ACG CAG ATT C 3`

P17  Ig-κ Vα FWD
5` CCA TGG AGA CAG ACA CAC TCC TGC TAT GGG TAC TGC TGC TCT GGT TTC
CAG GTT CCA CTG GTG ACG CGG CCC AGG AGG TGA CGC AGA TTC 3`

P18  Bgl II IRES FWD
5` GAA GAT CTG CCC CTC TCC CTC CCC CCC C 3`

P19  Bgl II EGFP REV
5` GAA GAT CTT TAC TTG TAC AGC TCG TCC ATG CC 3'


are present in ovarian cancer patients and healthy women. Cancer Immunol Immunother 46, 245-252.


tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. Nat Immunol 2, 962-970.


