ACTIVATION OF IMMUNE SYSTEM FUNCTION AGAINST CANCER BY HEAT SHOCK PROTEINS

by

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This dissertation is dedicated to those people whose lives have been affected by cancer whether they were the patient or their loving families. This includes close friends, Beverly Pearlman and Ed Goodman along with my grandfather who have all passed away from various forms of cancer. It is my hope that the cancer research taking place today will result in the development of better diagnostic indicators and improved treatments to ease the pain and suffering of both cancer patients and their families.

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ABSTRACT

Chaperone proteins such as heat-shock proteins 70, 90 and 110, glucose-related protein 94 and calreticulin have been reported to be effective anti-tumor vaccines when purified from a tumor source. We have developed a procedure utilizing a free-solution-isoelectric focusing technique to obtain vaccines from tumor or normal tissue sources that are rich in multiple immunogenic chaperone proteins, called Chaperone-Rich Cell Lysate (CRCL). Tumor-associated peptides are presumed to be the currency of T-cell mediated anti-cancer immunity, and tumor-derived chaperone vaccines are believed to be purveyors of such peptides. As a novel anti-cancer strategy, we have examined the extent to which the peptide repertoire of CRCL can be manipulated. Here, we explored the concept of creating a designer CRCL, utilizing the adjuvant properties and the carrying capacity of CRCL to deliver exogenous antigenic peptides for DC-based presentation and ultimately demonstrate the anti-tumor efficacy of the designer vaccine *in vivo*. Designer CRCL allows for the development of personalized vaccines to those afflicted with cancer expressing known antigens.

Growing evidence indicates that the stress response, specifically involving HSPs, has a profound impact on tumor immunogenicity. Enhancement of T-cell–mediated immunogenicity correlates with the expression of inducible heat shock protein 70 (iHSP70), the major heat-inducible member of the HSP70 family. In addition, studies have shown tumor-specific cell surface localization of iHSP70

correlates with an increased sensitivity to lysis mediated by human natural killer (NK) cells. Given these findings, investigating novel and effective means of modulating the heat shock response within tumor cells may bear great therapeutic potential and result in potent anti-tumor immune activity. Withaferin A (WA) is a compound isolated from the plant *Withania somnifera* that has been shown to induce a robust transcriptional heat shock response. In our studies, we found that WA treatment resulted in increased surface expression of iHSP70 in several tumor types leading to significant immunostimulatory effects. These findings indicated that WA-dependent modulation of the heat shock response may enhance tumor immunogenicity. Given the potent immunomodulatory and anti-tumor effects of WA as well as the adjuvanticity and specificity of peptide-complexed CRCL against tumors, these therapies individually have shown profound anti-cancer activity.

I. INTRODUCTION

History of Chronic Myelogenous Leukemia (CML)

The American Cancer Society estimates that 4,600 new cases of Chronic Myelogenous Leukemia (CML) will be diagnosed this year in the United States. CML is a clonal hematopoietic stem cell disorder characterized by the presence of the Philadelphia chromosome, a reciprocal translocation between chromosome 9 and 22, leading to the BCR-ABL chimeric oncoprotein (Figure 1). Enforced expression of the BCR-ABL chimeric protein in normal murine bone marrow cells is sufficient to cause a CML-like disease in mice. The BCR-ABL fusion protein is a tumor-specific antigen and represents a reasonable target for an immunologic approach to the treatment of CML. By screening large numbers of fusion peptides from the junctional sequences of BCR-ABL, several BCR-ABL derived fusion protein amino acid sequences with appropriate anchor motifs for binding to human leukocyte antigen (HLA) molecules have been identified. Examples of these peptides include: HSATGFKQSSK, KQSSKALQR, KQSSKALQRPV, QSSKALQRPV, SSKALQRPV, ATGFKQSSK and GFKQSSKAL (GFK), with the latter peptide sequence being utilized for the remainder of this report. Immunization with some of these fusion peptides has been shown to elicit specific CD4+ and CD8+ T cell responses against CML in humans (1).

Standard treatment options for patients with CML are allogeneic stem cell or bone marrow transplantation, hydroxyurea, busulfan, interferon-alpha (IFN-α) based regimens and more recently, imatinib mesylate, a potent and specific inhibitor of the BCR-ABL tyrosine kinase (2). Currently, allogeneic-matched sibling stem cell transplantation is favored for young patients in chronic phase who have higher "cure" rates (3). For older patients, the transplant-associated mortality rate is higher than the mortality rate in younger patients (4). Since few patients are eligible for stem cell transplantation, alternative treatments are desired. IFN-α had substantial activity in CML and was the non-transplant standard of care for chronic-phase disease until 1997 (5). IFN-α induced a state of tumor dormancy and delayed progression to advanced phase. Unfortunately, IFN-α was associated with substantial liver toxicity at therapeutic doses (6). Signal transduction inhibitor 571 (STI571; formerly CGP57148B), also known as imatinib mesylate or *Gleevec*^{TM}, is a rationally designed ABL-specific tyrosine kinase inhibitor that has been the front line treatment for CML since 1997 (7). Although STI571 has shown promise in clinical trials with responses in up to 90% of cases with advanced phase CML (8, 9), drug resistance and relapse often occur (10).

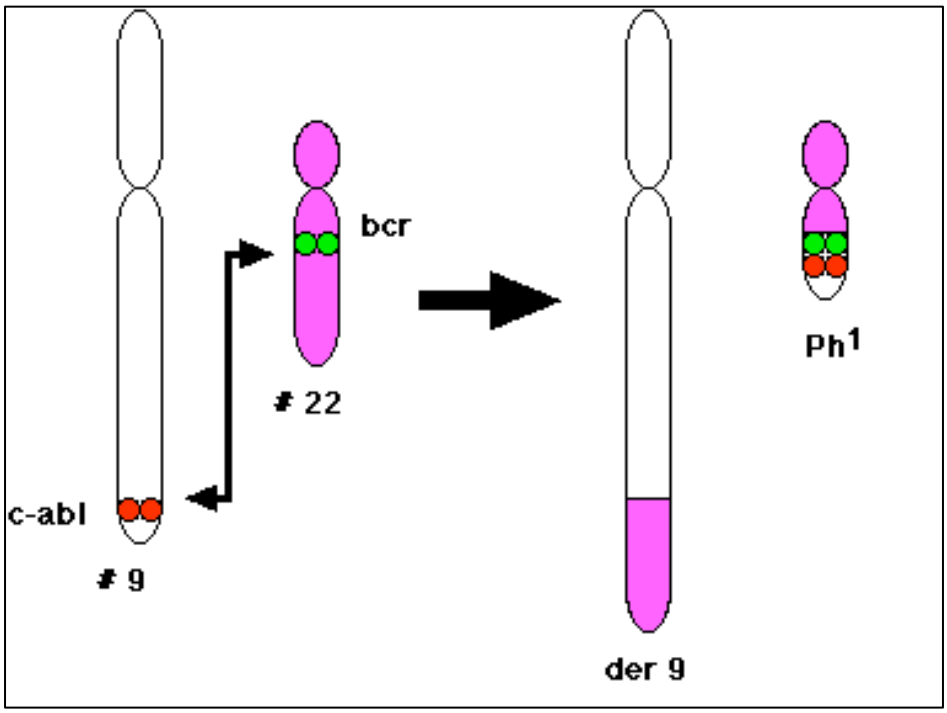


Figure 1: *Schematic view representing metaphase chromosomes.*

Reciprocal translocation between chromosome #9 and chromosome #22 forms an extra-long chromosome 9 (‘der 9”) and the Philadelphia chromosome (Ph^1) containing the fused BCR-ABL gene. Modified from (11).

Peptide-Based Vaccines

Tumor cells express antigens that can be recognized by the host's immune system. These tumor-associated antigens (TAAs) can be injected into cancer patients in an attempt to induce a systemic immune response that may result in the destruction of the cancer growing in different body tissues. This procedure is defined as active immunotherapy or vaccination since the host's immune system is either activated *de novo* or restimulated to mount an effective, tumor-specific immune reaction that may ultimately lead to tumor regression. However, until now, the vaccination approach for cancer has been carried out in the presence of the disease (i.e., in immunocompromised subjects) and not, as it occurs in prophylactic vaccination against infectious diseases, in healthy individuals. Moreover, although in infectious disease vaccination, the antibody response is of major importance, in anti-cancer vaccination, the focus is on the induction of T-lymphocyte responses. It is widely known that T-cells recognize antigens in the form of short peptides bound to major histocompatibility complex (MHC) molecules (12). In fact, a considerable amount of data from animal models and *in vitro* studies utilizing human cells indicates that T-cells are the major factor for the immunologic control of tumor growth when neoplastic cells express TAA.

Tumor antigens can be categorized into four groups: (a) antigens unique to an individual patient's tumor; (b) antigens common to a histologically similar group of tumors; (c) tissue-differentiation antigens; and (d) ubiquitous antigens expressed

by normal and malignant cells. To specifically target tumors, antigens must be over-expressed in tumor cells. Such unique tumor antigens (13) include mutated proteins, such as oncoprotein ras (14), tumor suppressor proteins p53 (13) and von Hippel Lindau. Fusion proteins created by chromosomal translocations, such as BCR-ABL in Chronic Myelogenous Leukemia (15), PAX-FHKR in alveolar rhabdomyosarcoma (13), EWS-FLI1 in Ewing sarcoma (16), and SYT-SSX in synovial sarcoma (17) are also included as specific tumor peptides. Another type of tumor antigen unique to cancer cells are antigens with tumor-specific post-translational modifications, exemplified by MUC1, which shows altered glycosylation in cancer cells, creating neoantigenic sites by exposing protein sequences normally masked by glycosylation (18, 19). Identification of TAAs expressed by different human tumors provides the basis for antigen-specific active immunotherapy or vaccination and facilitates the design of new vaccinations in clinical trials (20). Unfortunately, there are a number of barriers that may limit using peptides alone as potential immunotherapies. Some of these obstacles include poor bio-membrane permeability and enzymatic degradation early on after injection (21).

Immunotherapy with Peptide-Loaded Dendritic Cells

DCs are antigen-presenting cells that have the crucial function of presenting antigens, including TAAs, to naive T-cells in the lymph nodes. DCs are present in most tissues and can be recruited to the site of tumor cell growth by cytokines.

At the tumor site, DCs can internalize and process TAAs and then travel to the draining lymph nodes where they present, with high efficiency, peptide–MHC complexes to T-cells (22). In fact, the generation of tumor-specific T-cells against tumor peptides appears to require a phase of "antigen presentation" *in vivo* by antigen presenting cells, of which the most efficient appear to be DCs (23). This principle was suggested first by Bevan in 1976 (24) and termed "cross-priming." Essentially, cross-priming or cross-presentation is the ability of certain antigen-presenting cells to take up, process and present extracellular antigens with MHC class I molecules to $CD8^+$ T cells. This process is necessary for immunity against most tumors. It is also required for induction of cytotoxic immunity by tumor vaccination with protein antigens (e.g. CRCL). In 1989, Romani et al. (25) showed that murine DCs could take up proteins, process them, and present peptide–MHC complexes to T-cell clones. A similar observation was reported in a tumor model by Huang et al. (26). These findings (23-26) provided the rationale for using antigen-loaded DCs as potential activators of anti-tumor responses. An early study (27) showed that mice injected with antigen-loaded DCs were protected against subsequent challenge with the same tumor. Even in a therapeutic setting, such a vaccination was effective because tumors regressed in treated animals.

The efficacy of peptide-loaded DC vaccination in terms of CTL induction and anti-tumor activity depends on additional factors, such as the route of DC

administration and the origin of the DCs. Previous results with TAA peptide-loaded murine bone marrow-derived DCs indicated that subcutaneously injected DCs had greater anti-tumor activity than intravenously injected DCs and that subcutaneously injected DCs migrated to T-cell areas of the draining lymph nodes, whereas intravenously injected DCs migrated to the spleen (28). Although in mice the induced immunity was influenced by the route of administration of the vaccine, in cancer patients the induced immunity was independent of the route of administration (29). An early study revealed that the anti-tumor mechanisms activated by DCs requires cooperation between T-cell subsets and the expression of co-stimulatory molecules (such as B7–1), and Th1 cytokines (such as IL-2), interferon-γ (IFN- γ), and tumor necrosis factor-α (TNF-α) (30). Furthermore, the efficacy of DC-based vaccination against tumor growth can be substantially improved by combining the injection of peptide-loaded DCs with the administration of cytokines such as IL-12 (31). The mechanisms that allow DCs to promote effective anti-tumor immunity or to convert a tolerogenic peptide into a CTL priming peptide are currently being studied (32). Such mechanisms include the CD40L–CD40 T-cell co-stimulatory pathway, since the *in vivo* administration of an activating antibody to CD40 could convert a peptide vaccine from an inducer of CTL tolerance to an activator of tumor-specific immunity (33). Such activity is further increased by the *in vivo* administration of antibodies that block the cytotoxic T lymphocyte antigen 4 (CTLA-4) molecule, which is known to deliver an inhibitory signal to antigen activated T-cells (34).

Thus, DCs appear to be powerful adjuvants for peptide-based immunization strategies in animal models.

Overview and Functions of Heat Shock Proteins

Chaperone proteins, or heat shock proteins (HSPs), were primarily exposed in an array of species as a group of proteins that are strongly activated by heat shock and other chemical and physical stresses (35, 36). Later, these heat shock proteins were distinguished as molecular chaperones since they have the ability to modify the interactions and structures of other proteins (37-40). HSPs are recruited for action when other proteins are disorganized due to heat shock, oxidative stress or other events that damage proteins, because they are capable of regaining the order and function of denatured and aggregated protein conformations (35, 36, 39, 41). Following stress, there is an increase in the cellular levels of HSP27, 70, 90, and 110 (42-47). Cells also contain a large number of heat shock proteins that are constitutively expressed in addition to those induced by heat or other stresses (48). It has been shown that constitutive forms of heat shock proteins are found in multi-protein complexes containing both HSPs and other cofactors (49). These include HSP70- and HSP90-containing complexes involved in both specific association with key regulatory proteins within the cell and in protein-folding pathways. In addition HSP110 and HSP60 complexes are involved in protein folding as well (40, 50).

Vaccination with Heat Shock Proteins

Heat shock proteins (HSPs) are considered natural adjuvants that show promise in cancer vaccination because they can bind antigenic peptides within the tumor cell and chaperone peptides to antigen-presenting cells (such as DCs) in the lymph nodes (Figure 2), (51). Antigen presenting cells express a specific receptor (CD91) that can bind different families of HSPs (52). In the mouse system, HSPs of the 96, 70, and 110 kDa subfamilies have been shown to function as potent vaccines in both prophylactic and therapeutic settings, with the ability to induce strong, specific, anti-tumor T-cell immunity that results in tumor rejection (51). The ability of HSPs to bind tumor-associated peptides has been directly shown *in vitro* in mouse models of cancer and infectious diseases (51) as well as in human melanoma (53).

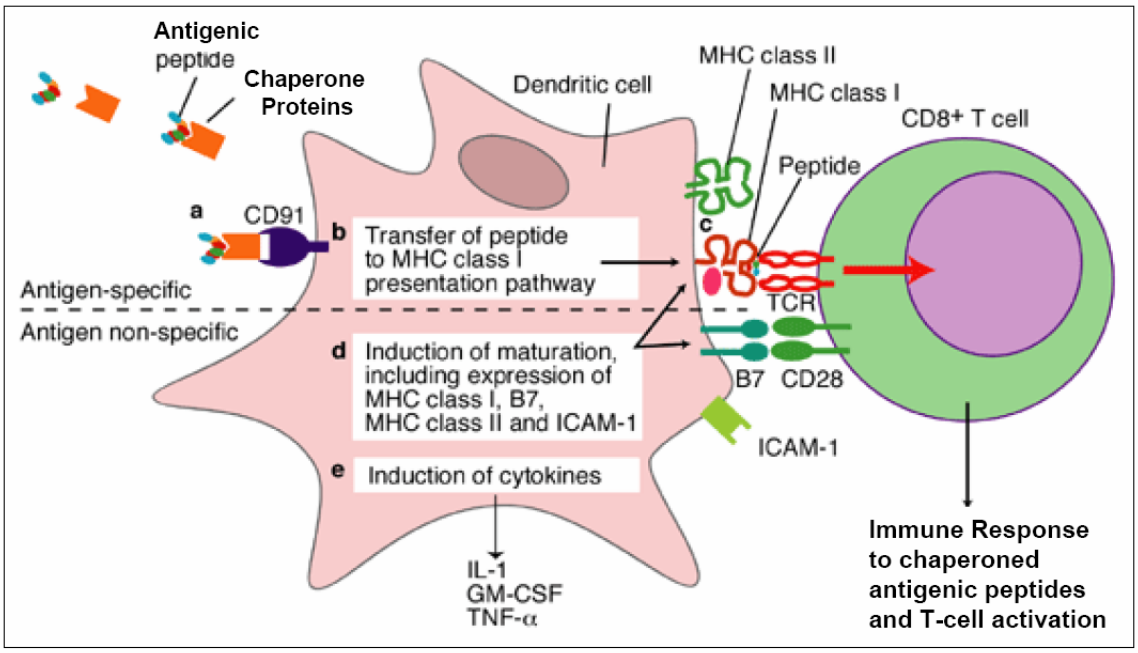


Figure 2: *Chaperone proteins deliver antigenic peptides and maturation signals to antigen-presenting cells, and induce release of cytokines.*

(a) Chaperone protein–peptide complexes bind to CD91 and are taken up by dendritic cells via receptor-mediated endocytosis. (b) Peptides carried on chaperone proteins are thus delivered to the major histocompatibility complex (MHC) class I presentation pathway and are (c) re-presented on the cell surface in association with MHC class I antigens for recognition by antigen-specific $CD8^+$ T-cells via the T-cell receptor (TCR) and associated molecules. (d) chaperone proteins also deliver maturation signals to the dendritic cells, and induces the expression of MHC antigens, co-stimulatory molecules such as B7 (which binds to CD28), and intercellular adhesion molecule 1 (ICAM-1). This, combined with (e) the induction of pro-inflammatory cytokines, promotes the generation of

immune responses to HSP-chaperoned peptides. Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-1, interleukin 1, TNF-a, tumor necrosis factor a. Figure adapted from (54)

Clinical studies have been initiated with the aim of immunizing cancer-bearing patients with autologous GRP94 (a.k.a. GRP 96 or HSP96), which is known to be immunogenic in mice (51). In a pilot study, Janetzki et al. (55) vaccinated 16 subjects, who already possessed different types of cancer, with autologous tumor-derived GRP94 preparations. No clinically significant toxicity was observed, and six of 12 patients that could be tested developed class I HLA-restricted, tumor-specific T-cells (55). A similar study was performed in 39 patients with metastatic melanoma (12). It was found that vaccination with peptide–GRP94 complexes obtained from autologous tumors led to an increase in specific T-cell responses against melanoma antigens in 48% of patients and to clinical responses in 18% of patients (12). Although this approach is interesting, it needs to be validated by showing that autologous tumor-derived HSPs do contain individual tumor peptide epitopes. Such peptide epitopes are thought to be more immunogenic than those derived from shared tumor peptides and are responsible for tumor regression in animal models (51).

Exogenous HSPs Deliver Antigenic Peptides to APCs

One of the major roles of heat shock proteins is their ability to act as chaperones for peptides and other proteins. HSPs bind an array of immunogenic (poly)peptides and different HSPs may carry different antigenic peptides. Evidence has continued to accumulate that HSPs are associated with peptides from a wide spectrum of antigens, including tumor antigens (56), viral antigens

(57) and model antigens (e.g. OVA, HIV gp120 and bacteriophage T4 Hsp10). The peptide-binding capacity appears to allow HSPs to acquire proteinaceous antigenic material within cells and when administered exogenously, to induce priming of $CD8^+$ T lymphocytes *in vivo*.

Exogenous HSP-peptide complexes or those released from dying cells have been shown to be efficiently internalized by APCs in a receptor-mediated way. CD91, which was initially identified as the receptor for GRP94, has been recently demonstrated to be the common receptor for HSP90, HSP70 and calreticulin (CRT) as well (52). Once internalized, HSPs traffic to a subcellular compartment(s) where peptide release can occur. Within the antigen presenting cells, HSP-derived peptides can be assembled onto MHC class I molecules for presentation on the APC surface. In the case of HSP70, presentation is via either a cytosolic (transporter associated with antigen processing [TAP] and proteasome-dependent) or an endosomal (TAP and proteasome-independent) route, with the preferred pathway determined by the sequence context of the optimal antigenic peptide within the HSP-associated material, thereby yielding the required and specific CD8+ T-cell responses that contribute to the process of tumor rejection (58).

Endogenous HSPs Assist Tumor Antigen Processing and Presentation

Several studies have shown that HSPs not only serve as chaperones to deliver antigenic peptides to APCs when released from dying cells, but endogenous HSPs might also enhance tumor immunogenicity by increasing the ability of APCs to process and present endogenous antigens directly to tumor specific T-cells (59-61). It is well known that most poorly immunogenic tumors cannot process and present endogenous tumor antigens because of the down regulation of MHC class I molecules and/or other components of the antigen-processing machinery, such as TAP molecules and proteasome subunits. Some studies have shown that poorly immunogenic melanoma cells, which normally express very low levels of MHC-I, exhibit increased levels of MHC-I antigens on their surface when stably transfected with either HSP72 or HSP65 (62, 63). The transfected HSPs were shown to complex with intracellular peptides, and unlike parental melanoma cells, HSP-expressing tumor cells processed and presented antigens directly to specific CTLs *in vitro*.

More evidence has been accumulated to indicate the important roles of HSPs in antigen processing and presentation. Cellular proteins undergo degradation in the cytosol, and the resulting peptides are transported into the endoplasmic reticulum (ER), generally through TAP. Within the ER, the peptides are loaded onto MHC I molecules (64). One of the key unresolved questions in this scheme pertains to the mechanism through which peptides are channeled to the TAP or

other transporters. It has been proposed that the peptides exist in association with peptide-binding proteins in the cytosol and the ER (59). Because HSPs are known to chaperone a wide array of molecules and because immunological and structural evidence suggests that HSPs chaperone antigenic peptides, it is believed that HSPs are the peptide-binding proteins that transport peptides (59). Supporting evidence includes reports which demonstrate that HSPs are required for the ubiquitination of certain proteins and are involved in the chaperoning of proteins to the cytosolic sites where the degradation of proteins occur, leading to the generation of antigenic peptides (65). Recently, more direct evidence indicates that HSPs play a necessary role in antigen processing and presentation. It was reported that free peptides in the cytosol are presented very inefficiently, while HSP70- or HSP90-chaperoned peptides are presented efficiently by MHC-I molecules (66).

Tumor-derived chaperone proteins have proven to be effective immunogens in animal models, and their advantages as anti-cancer vaccines have been documented (51, 60, 67-69). Chaperone proteins carry peptides as part of their escort duties and are components of the antigen presentation machinery of a cell. The chaperoned peptides, associated with the various chaperone protein family members, represent perhaps the entire protein repertoire of the cell. In a normal cell, the peptides should not be antigenic; however, in a tumor cell, mutated or ill-expressed chaperoned peptides may serve as antigens. The

chaperoned peptides are diverse in size, sequence and protein of origin, increasing the possibility that some of them are ligands for MHC class I and class II molecules. Such peptide diversity also lends itself to a polyclonal T-cell response. In an encounter between immune system cells and exogenous chaperone proteins, the association of the peptides with chaperones results in ‘‘privileged access’’ to the antigen presentation pathways of specialized antigen presenting cells (APCs), most notably, dendritic cells (DC). Recent reports of receptors on APCs for chaperone proteins indicate that uptake of exogenous chaperones is a specific, receptor-mediated process for APCs (52, 58, 70-74). An additional advantage of using chaperone proteins as anti-cancer agents is their cytokine-like activity on APCs, which appears to also enhance the abilities of APCs to process and present antigen and to stimulate T-cell responses (52, 58, 72, 73).

Individual chaperone proteins (e.g., GRP94/gp96, HSP90, HSP70 and calreticulin) purified from a given tumor are effective vaccines against that tumor, even in pre-existing models (75-79). After years of such pre-clinical studies, clinical trials utilizing tumor-derived GRP94 have begun (55, 60, 67, 80, 81). Our laboratory has focused its efforts on studying the potential benefits of multiple tumor-derived chaperone protein vaccines.

Multiple Chaperone Protein Vaccines

Despite multiple approaches to therapy and prevention, cancer remains a major cause of death worldwide. Most non-surgical approaches targeting rapidly dividing cells, using radiotherapy or chemotherapy, also affect normal cells and result in side effects that limit treatment. In principle, the exquisite specificity of the immune system could be harnessed to precisely target cancer cells without harming normal cells. To take advantage of the immune system's specificity, one must find antigens that clearly mark the cancer cells as different from host cells (18, 82), limiting the number of antigens available.

Developing more effective anti-cancer vaccines has been one of the major goals of cancer immunotherapy. Purified tumor-derived chaperone proteins such as heat-shock protein 70 and 90 (HSP70 and 90), GRP94, and calreticulin (CRT) have shown promise as vaccines, capable of generating tumor-specific T-cell responses and protective anti-tumor immunity in numerous animal models (56, 61, 76, 79, 83, 84). These studies have indicated that it is not the chaperone proteins, per se, but rather the tumor antigen repertoire (ie, peptides) carried by the chaperones that is immunogenic. In the normal cellular environment, chaperone proteins perform their intracellular functions as multi-protein complexes consisting of chaperones, co-chaperones, substrate molecules, and others (85). Vaccination studies have demonstrated that when purified away from their normal cellular environment, individual chaperone proteins retain

effective anti-tumor activity. However, a remaining question has been whether multi-chaperone/co-chaperone vaccines would be more effective than single-component HSP vaccines. Our lab has developed a relatively simple, rapid, and efficient procedure utilizing a free-solution-isoelectric focusing (FS-IEF) technique to obtain fractions rich in multiple immunogenic chaperone proteins derived from clarified tumor lysates. This final product is referred to as Chaperone-Rich Cell Lysate (CRCL). We are able to generate approximately 1-2 mg of CRCL vaccine per gram of tumor (85-87). By comparison, we are only able to obtain 20-100 μg per gram of tumor for any individual chaperone protein in a targeted, single chaperone purification (76, 86, 87). Thus, in our hands, even if one sums the maximum quantities of GRP94, HSP90, HSP70, and calreticulin that we are able to obtain by standard purifications, we still acquire 2 to 5 times more vaccine material via our FS-IEF method, and in far less time than is necessary for more typical chromatographic protein purifications. This makes the FS-IEF method of multiple chaperone enrichment desirable from a clinical standpoint in terms of high yield from a potentially limited tumor source, and with a rapid turn-around time from tumor harvest to treatment of the patient.

Heat Stress Response in Relation to Immunotherapy

The stress response on a cellular level has been a useful tool for determining the mechanisms of inducible gene expression in eukaryotes (88, 89). Through the molecular analysis of heat shock protein (HSP) genes the heat shock element

(HSE) was identified. The HSE is a stress-responsive promoter element essential for heat shock inducibility. They are located at various distances upstream of the site of transcription initiation. Specifically, in vertebrates inducible transcription requires the de novo binding of heat shock transcription factors (HSFs) transiently to the HSEs (90, 91). In human cells, there have been three HSFs characterized (HSF1, HSF2, and HSF4) (90, 91). HSF1 is ubiquitously expressed and plays a primary role in the stress-induced expression of HSPs.

At the molecular level, the cellular response to stress is represented by the induction of HSP synthesis. HSPs have been categorized into six major groups according to their molecular weight: HSP100, HSP90, HSP70, HSP60, HSP40, and small heat shock proteins. Members within each gene family are found to be inducibly regulated, constitutively expressed, and/or targeted to different compartments of the cell (92). For example, HSP90 functions in both the cytosolic and nuclear compartments, whereas GRP94 performs a similar function in the endoplasmic reticulum. Similarly, members of the HSP70 family display intricate patterns of growth-regulated and stress-induced gene expression and are targeted to different sub-cellular compartments.

Molecular chaperones, including HSP70, are becoming very useful in tumor immunotherapy. HSP70 has been used as an adjuvant when combined with other therapies to activate APCs and break tolerance to tumor-associated

antigens (93). It is also employed as a carrier protein to shuttle tumor antigens to MHC class I molecules on APCs (93, 94). In a wide variety of cancers, HSP70 levels are elevated. Even under non-stress conditions, HSP70 upregulation protects emerging cancer cells from apoptosis that is associated with many steps of transformation. However, this HSP70 increase also creates an opportunity for immune attack (48). HSP70 can be seen on the plasma membrane when induced by heat or chemical stress in tumor cells but not in normal untransformed cells (95). HSP70 was first described as an extra-cellular protein when it was found to be released into the blood stream and stimulated production of anti-HSP70 antibodies (96). Circulating HSP70 is a result of dying cells and/or from HSP70 actively released from intact cells (83, 96-100). The final stages of necrosis of tumor cells seems to favor antigen uptake by HSP70 (101, 102). Over-expression of HSP70 in the presence of slow necrotic death is a very powerful approach to breaking tolerance and inducing specific immune destruction of tumors (103). HSP70, when released from the tumor, is complexed with peptide and constitutes a ‘danger signal’ attracting and activating APCs (94, 104). Such HSP70 complexes are, however, not merely danger signals but carry tumor peptides which mediate the adaptive immune response. Injection of HSP70-peptide complexes or multi-chaperone based vaccines into tumor-bearing hosts may show similar adaptive immune responses (93). HSP70 released from tumor cells become diluted upon entering the interstitial fluid. Therefore, APCs response to low levels of HSP70 from tumor implies the

presence of high-affinity receptors. HSP70 has at least two activating effects on APCs: the first being the induction of innate immunity (105) and second, the induction of the adaptive immune response through the transport of peptide antigens into APCs to further deliver to MHC class I and activate cytotoxic T-cells (93). Four main candidate receptors have been suggested, including: the CD14/TLR 2/4 complex, the CD91 receptor, CD40 and scavenger receptors-especially lectin-like receptor for oxidizing low-density lipoprotein (LOX-1) (106).

Hyperthermia in the fever-range (~42-45°C), or heat shock, also serves as a biological adjuvant. It has the ability to activate APCs and tumor immunity through various mechanisms which appear to include both HSP-dependent and –independent effects (107). At higher temperatures, heat shock may function to enhance HSP expression and release HSPs outside of the cell as well as inducing independent effects on immune cell activation. Indeed, the combination of fever range heat shock with chaperone-based vaccines seems a highly promising approach.

Withania somnifera and Withaferin A

Ayurveda is one of the traditional systems of medicine practiced in India and Sri Lanka and can be dated back over 3000 years ago (Samhita, 1949). As in other medical systems practiced in the world, Ayurveda originated from folk medicine and now holds a powerful position in the countries mentioned. Ayurvedic

treatments are based largely upon herbal and herbomineral preparations and have specific diagnostic and therapeutic principles (108). *Withania somnifera*, (commonly known as Ashwagandha) belonging to the family of Solanaceae, is used in many indigenous drug preparations. *Withania* extract was found to possess anti-tumor (109-111), anti-oxidant (112) and immunomodulatory activities (113). In particular, studies have shown that *Withania* treatment could increase the total white blood cell count, bone marrow cell count as well as the stimulation of CTL production in mice (113). *Withania somnifera* has also been determined to augment cell mediated immune responses including natural killer (NK) activity (113). This reported NK activity was attributed to interferon induction. Likewise, *Withania* treatment produced an increase in antibody-dependent cellular cytotoxicity (ADCC), which is the interaction of humoral and cell mediated immune effectors (113).

Withaferin A (WA), a steroidal lactone, isolated from the plant *Withania somnifera*, has been reported to inhibit the growth of Ehrlich Ascites Carcinoma in mice, and went on to show that WA rendered cured mice refractory to rechallenge with the same tumor due to induction of a marked anti-tumor immune response (111, 114). Specifically, immunity was characterized by the presence of circulating antibodies with cytotoxic effects toward Ehrlich Ascites cells (111). Furthermore, this study found that levels of engulfed tumor cells in macrophages usually reached its peak values by the 4th day of WA treatment (111). During

this time rosette and giant-cell formations were observed and macrophages loaded with cell debris were seen. Moreover, when WA-cured mice were re-challenged with Ehrlich Ascites tumor cells, the macrophage response was almost immediate and reached peak values as soon as the first day (111). Although these studies were observational, they indicated that when WA was administered to tumor-bearing mice, it had the ability to eradicate the tumor while acquiring immunity toward a subsequent tumor re-challenge.

Finally, WA was identified as a compound of interest by our laboratory due to the massive heat shock response induced by this small molecule natural product. It was determined that WA induces an extremely robust cellular heat shock response (up to 25 fold above vehicle treated cells) at low micromolar concentrations (R. Falsey et. al. manuscript submitted). Given the strong heat shock response induced by WA and the previously described immunomodulatory effects of *Withania somnifera* extracts and WA, investigating a plausible link between these properties merits development. For instance, when such a massive shock response occurs as a result of WA treatment in tumors, heat shock proteins may be overexpressed on the tumor cell surface thereby activating an immune response against the otherwise unnoticed tumor. Indeed, numerous studies propose that the immune system may be highly sensitive to factors that increase the expression of inducible HSPs on the tumor cell surface, suggesting the WA can have profound effects on tumor based immunity.

Statement of the Problem

For most advanced stage cancers, chemotherapy continues to be the treatment option of choice. In light of many set backs seen with conventional cancer therapies, novel approaches to treating and eliminating cancer are greatly needed. In particular, due to long-term drug resistance for many cancers, it is critical to find other means of treatment (115). Cancer immunotherapy represents a unique therapeutic approach that may be able to specifically target tumor cells while sparing normal, healthy tissue.

Purified tumor-derived chaperone proteins such as heat-shock protein 70 and 90 (HSP70 and 90), GRP94, and calreticulin have been well documented as effective anti-tumor vaccines, generating tumor-specific cytotoxic T-lymphocyte (CTL) responses and anti-tumor immunity in numerous animal models (51, 116). CRCL vaccine has a more pronounced immunologic effect per unit material of protein than most of the individual chaperone proteins used as a vaccine alone (85-87). Moreover, we have demonstrated that the antigenicity of CRCL can be augmented by loading it onto dendritic cells (DCs). In addition, CRCL has been shown to mature and activate dendritic cells (85), thus enhancing antigen presentation.

Tumor-associated peptides are presumed to be the currency of T-cell mediated anti-cancer immunity. As a novel anti-cancer strategy, we examined the extent

to which the peptide repertoire of CRCL could be manipulated. The concept of creating a “designer” CRCL offers the ability to enhance the already personalized vaccine that is tumor-derived CRCL to those afflicted with cancer containing known antigens, such as the BCR-ABL fusion peptide. In other words, it utilizes the adjuvant properties and the extraordinary carrying capacity of CRCL to deliver desired antigenic peptides that are taken up by APCs and presented by those cells to T-cells in order to augment responses against specific tumor types. Our findings show that exogenous tumor-associated peptides can be incorporated into CRCL, thus enhancing the vaccine’s efficacy against a variety of cancers, i.e. BCR-ABL peptide incorporation into 12B1 (leukemia)-derived CRCL a.k.a. peptide-embedded 12B1 CRCL.

Typically, immunotherapy and chemotherapy have been considered to elicit opposite effects on the body, therefore there are limited reports investigating the relationship between these therapies. Contributing factors to this assumption include an abnormally low number of lymphocytes in the blood, which is a common side effect of many anti-cancer agents and the fact that most chemotherapeutic agents tend to destroy tumor cells via apoptosis. This form of cell death has been considered non-immunostimulatory. Apoptosis is also able to induce immune tolerance, which is a condition where T-cells can no longer induce an immune response after being presented with an antigen. However, when a chemotherapeutic agent induces a strong heat shock response prior to

cell death, such as the case with WA, heat shock proteins are either secreted or highly expressed on the tumor cell surfaces and are known to activate immune function. Our laboratory has previously shown that heat shock proteins, including HSP70, that are induced by stressed apoptotic tumor cells, increase their ability to activate dendritic cells. Heat-stressed tumor cells induce changes in DCs, including an upregulation of co-stimulatory molecules (CD40 and CD80) along with an increase in MHC II expression. In this study we found that WA may contribute to several processes that prime the immune system against tumors including: enhanced lymphocyte proliferation, the generation of activated/mature DCs by non-adherent WA-treated tumor cells and also by directly killing tumor cells whose antigens can be cross-presented to APCs.

II. EXPERIMENTAL PROCEDURES

Cell Culture/Tumor Generation/Murine Bone Marrow-Derived Dendritic Cell Generation

Chapter III

12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the human BCR-ABL fusion gene, and these cells express the p210 BCR-ABL protein. The 12B1 cell line was kindly provided by Dr Wei Chen (Cleveland Clinic, Cleveland, OH) (85).

All tissue/cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). 12B1 cells were cultured as described previously (117). In brief, cells were prepared for injection by washing and resuspending in 1x phosphate buffered saline solution. The cells were counted and adjusted to a concentration of 50 x 10^6 cells/mL. Female BALB/c mice were injected with 100µl (5 x 10^6 cells) subcutaneously in both flanks and were monitored for tumor development. Tumors larger than 1 cm in diameter were harvested from killed mice and processed into a cell suspension which was then filtered through a cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to remove debris and then centrifuged. The cell pellet was resuspended, washed, counted, and injected into new experimental mice.

Dendritic cells (DC) were harvested and cultured from syngeneic mouse bone marrow as described previously in detail (118, 119).

The B3Z cell line is a murine (H2Kb) T-cell hybridoma containing a gene construct of Escherichia coli LacZ reporter gene linked to the nuclear factor of activated T-cells. The B3Z cells have specific reactivity against the ovalbumin peptide OVA257-264 in the context of MHC I by the T-cell receptor (TCR). It has been engineered to express beta-galactosidase upon triggering of the TCR with peptide ligand that can be measured by absorbance spectrophotometry (120, 121). It was cultured at 37°C (10% CO2) in DMEM supplemented with 10% heat-inactivated fetal calf serum and supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin sulfate, 0.05mM MEM nonessential amino acids and 1mM sodium pyruvate (DMEM Complete media).

Chapter IV

EMT6 and 4T1 are mouse mammary carcinoma cell lines that were kindly provided by Dr. Emmanuel Akporiaye (University of Arizona, Tucson, AZ). The mouse renal carcinoma cell line, RENCA, was generously obtained from Dr. Thomas Sayers (National Cancer Institute, Frederick, MD). The mouse colorectal carcinoma cell line, CT26 was obtained from American Type Tissue Culture Collection (Rockville, MD).

The EMT6, 4T1, RENCA and CT26 cell lines were cultured at 37°C (6% CO2) in RPMI supplemented with 10% heat-inactivated fetal calf serum and supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin sulfate, 0.05mM MEM nonessential amino acids and 1mM sodium pyruvate (RPMI Complete media).

Peptides

Antigenic peptide derived from ovalbumin protein (AnaSpec, Inc., San Jose, CA) was reconstituted in water and is abbreviated from their amino acid sequence as the following: OVA257-264 peptide – SIINFEKL.

Antigenic peptide derived from the chimeric protein of BCR/ABL (AnaSpec, Inc., San Jose, CA) was reconstituted in water and is abbreviated from its amino acid sequence as the following: GFKQSSKAL – GFK. In addition, this peptide was also synthesized with a FITC conjugate at the N-terminus.

Chaperone Protein Enrichment and Purification

12B1 tumors were used in the making of tumor-derived Chaperone-Rich Cell Lysate (CRCL). Normal livers were harvested from BALB/c and C57BL/6 mice for the preparation of (normal tissue-derived) liver CRCL. Free solution-isoelectric focusing (FS-IEF) enrichment of tumor and normal tissue-derived

CRCL was performed as described previously (87). Vaccines were tested for endotoxin with the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD.) and were found to contain less than 0.01EU/μg protein (85).

For FS-IEF peptide incorporation studies, 5mg SIINFEKL peptide were added to the liver homogenate isofocusing mixture prior to the loading of the Rotofor device and application of power. Following isofocusing, each of the 20 fractions was handled individually for dialysis, chaperone protein characterization, vaccine preparation, and analysis for peptide content (see below).

Preparation of Vaccines

Following the dialysis steps described previously (87), proteins were prepared for use as vaccines by concentration in Centricon-10 devices (Millipore, Bedford, Mass.) and passage over Extracti-gel D columns (Pierce Endogen, Rockford, IL.) to remove residual detergents. Protein concentrations were determined using BCA assays with bovine serum albumin as standard, and proteins were diluted to 50μg/ml in sterile saline for pulsing onto dendritic cells.

Peptide Incorporation into Chaperone-Rich Cell Lysate by Simple Mixing

Peptides were added to CRCL vaccine at a 1:1 microgram ratio and incubated at room temperature for 40 minutes. The peptide/CRCL solution was centrifuged at 9.5 x 103 rpm via a 10kDa cut-off membrane filter (Vivaspin 500, ISCBioexpress,

Kaysville, UT). The retentate was then washed three times with 1x PBS by centrifugation through the filter and then collected for use.

Native Agarose Gel Electrophoresis and Sample Extractions

Sixty micrograms of CRCL containing FITC-peptide was loaded onto a 1% agarose gel (Amresco, Solon, OH) run under native conditions at 100V for 4 hours at 4°C. The high molecular weight region of the gel where the FITC label migrated was excised. To remove the protein sample from the agarose, a 1.7mL tube with a small hole punctured in the bottom was packed with glass wool and the agarose band was set on top of the glass wool. The entire 1.7mL tube was placed inside a larger tube to collect the liquid sample. This apparatus was centrifuged at 1000 rpm for 10-15 minutes until the liquid sample dissociated from the agarose and was collected in the bottom of the larger tube. The sample was now concentrated using a 10kDa cut-off membrane filter (Vivaspin 500, ISCBioexpress, Kaysville, UT). The sample was then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and further stained with Sypro Ruby (Molecular Probes) for band detection. The bands detected were excised and submitted to the Arizona Proteomics Consortium Core Facility at the University of Arizona for mass spectrometry analysis.

LC-MS/MS Analysis

Analysis of excised, in-gel digested bands was carried out using a quadrupole ion trap LCQ Classic mass spectrometer from ThermoFinnigan (122). The LCQ Classic was equipped with a Michrom MAGIC 2002 HPLC and a nanoelectrospray ionization source (University of Washington, Seattle, WA). Peptides were eluted from a 15cm pulled-tip capillary column (100µm I.D. x 360µm O.D; 3–5µm tip opening) packed with 8–9 cm Vydac C18 material (5µm, 300 Å pore size), using a gradient of 0–65% solvent B (98% methanol/2% water/0.5% formic acid/0.01% trifluoroacetic acid) over a 60 minute period at a flow rate of 200–300nl min-1. The sequences of individual peptides were identified using the Turbo SEQUEST algorithm to search and correlate the MS/MS spectra with amino acid sequences in the nonredundant protein database (123).

Western Blotting

Chapter III

The protein identified by mass spectrometry as a peptide binding target was confirmed by Western blotting. CRCL derived from liver was used as a positive control. Following SDS-PAGE, the gels were electroblotted to nitrocellulose using an Idea Scientific electroblotter (Minneapolis, MN). Gels were transferred in 25mM Tris, 200mM glycine, 20% methanol for 1 hour and 15 minutes at

500mAmps, stained with Ponceau Red, and destained in TBST (50mM Tris-Cl, 150mM NaCl, 0.1% Tween 20, pH 7.4). Blots were blocked in 3% nonfat dried milk in TBST for 30 minutes, followed by three 5-minute rinses in TBST. The protein of interest was identified using a rat monoclonal antibody to HSC70 (Stressgen, Vancouver, British Columbia). Primary antibody solutions were prepared in 0.3% milk solution from above, and blots were incubated in primary antibody overnight at 4°C, followed by three 5-minute rinses in TBST. Alkaline phosphatase-conjugated goat anti-rat secondary antibody was applied for 1 hour at room temperature followed by detection with substrate (BCIP/NBT; Roche Diagnostics, Indianapolis IN).

Chapter IV

Tumor cells that were treated with increasing amounts of WA were lysed and Western Blots were performed to identify levels of inducible HSP70. In addition, tumor cells were treated with WA followed by harvesting of proteins expressed on the cell surfaces. Western Blot analysis was subsequently performed to identify levels of inducible HSP70 on the cells' surface. Following SDS-PAGE, the gels were electroblotted to nitrocellulose using an Idea Scientific electroblotter (Minneapolis, MN). Gels were transferred in 25mM Tris, 200mM glycine, 20% methanol for 1 hour and 15 minutes at 500mAmps, stained with Ponceau Red, and destained in TBST (50mM Tris-Cl, 150mM NaCl, 0.1% Tween

20, pH 7.4). Blots were blocked in 3% nonfat dried milk in TBST for 30 minutes, followed by three 5-minute rinses in TBST. The protein of interest was identified using a mouse monoclonal antibody to HSC70 (Stressgen, Vancouver, British Columbia), a mouse monoclonal antibody to actin (Sigma, St. Louis, MO) and a rabbit polyclonal antibody to IGF1R antibody (Oncogene, San Diego, CA). Primary antibody solutions were prepared in 0.3% milk solution from above, and blots were incubated in primary antibody overnight at 4°C, followed by three 5-minute rinses in TBST. Alkaline phosphatase-conjugated goat anti-rat secondary antibody was applied for 1 hour at room temperature followed by detection with substrate (BCIP/NBT; Roche Diagnostics, Indianapolis IN) or chemilluminescence with substrates (Supersignal, Pierce, Rockford, IL).

Fluorometric Measurement of FITC-Peptide Incorporation

FITC-labeled peptide was incorporated into CRCL as described above, and peptide retention in CRCL was measured using a Fluoroskan Ascent Microplate Fluorometer (Thermo Electron Corporation) with an excitation at 488nm and an emission at 527nm. Quantitation of peptide incorporation was done by generating a fluorescence standard curve with known amounts of FITC-peptide.

Flow Cytometry

Chapter III

Dendritic cells were experimentally treated with either CRCL, FITC-peptide (AnaSpec, Inc, San Jose, CA), or both. The cells were washed in PBS and then fixed with PBS containing 1% paraformaldehyde (Polysciences, Warrington, PA). Using a FACScan (Becton Dickinson Immunocytometry, San Jose, CA), 10,000 cells were analyzed and gated on CD11c+ cells as a marker for DCs (CD11c-PE, Pharmingen, San Jose, CA).

Chapter IV

Immature dendritic cells were experimentally treated with cell supernatant debris from tumor cells after treatment with WA. The cells were washed in PBS and then fixed with PBS containing 1% paraformaldehyde (Polysciences, Warrington, PA). Using a FACScan (Becton Dickinson Immunocytometry, San Jose, CA), 10,000 cells were analyzed and gated on CD11c+ cells as a marker for DCs (CD11c-PE, Pharmingen, San Jose, CA) along with co-stimulatory molecules CD40, CD80, CD86 and MHCII (all FITC-antibodies, Pharmingen, San Jose, CA).

Confocal Microscopy

Dendritic cells were experimentally treated with either Texas Red-labeled (Molecular Probes, Eugene, OR) CRCL or FITC-peptide, or both, therefore antibodies were not required. The cells were washed in PBS and then fixed with PBS containing 4% paraformaldehyde (Polysciences, Warrington, PA). Cells were then washed and transferred onto microscope slides using Cytospin (Thermo Shandon, Pittsburgh, PA) centrifugation followed by examination at 100x magnification using a Nikon TE300 microscope (Tokyo, Japan) and Bio Rad 1024 MRC Confocal Imaging System (Bio-Rad, Hercules, CA).

IL-2 Reporter Bioassay

IL-2 production by antigen-stimulated B3Z T-cell hybridomas was measured in surrogate via a Beta-Galactosidase Production Assay using kit from Novagen (Madison, WI).

ELISPOT Assay

Enzyme-linked immunospot (ELISPOT) assays were performed to assess IFN-γ production by splenocytes from vaccinated mice following in vitro stimulation with CRCL or peptides. Splenocytes (106) were cultured with 50μg/mL CRCL, 50μg/mL peptide-embedded CRCL or 5μg/mL peptides for 48 hours on Millipore MultiScreen-HA 96-well plates (MAHA S45; Millipore, Bedford, MA) as described previously (87). Spots were visualized with the addition of BCIP/NBT which was

added to the plates and incubated for 30 minutes at room temperature in the dark. The plates were then rinsed with deionized water and allowed to dry at room temperature. Spots were examined using a dissecting microscope. Wells of interest were photographed with a microscope-mounted Leica DFC480, and images captured with Leica Fire Cam DFC Twain software (Leica Microsystems, Bannockburn, IL). The image of each well was electronically optimized to visualize the maximum number of spots.

In vivo Studies

Female BALB/c (H-2d) (National Cancer Institute, Frederick, MD) and C57BL/6 (H-2b) (National Cancer Institute, Frederick, MD) mice 6- to 10-weeks old were used for the experiments. The animals were housed in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines. For establishment of primary tumors, refer to tumor generation mentioned above. Following tumor injection on Day 0, the mice were subjected to different treatments regimens on Day 1 and Day 3. Mice were given subcutaneous injections in the opposite groin of tumor site with BCR/ABL peptide-embedded liver CRCL (20µg/injection in 100µl of PBS). The control groups consisted of mice injected with 100µl of PBS, BCR/ABL peptide alone or liver CRCL alone all at 20µg/injection in 100µl of PBS. Tumor growth was monitored by measuring the tumor length and width with calipers and calculating the tumor volume according to the formula V= (LxW2)/2.

Cell Proliferation/Survival Assay

To compare the sensitivity of various cell lines to Withaferin A (WA), EMT6, 4T1, RENCA, CT26 (10,000 cells/well) and dendritic cells were plated in 96-well flat-bottom plates (40,000 cells/well) and allowed to adhere for 24 hours. Cells were incubated in the presence of DMSO vehicle control, 10 μM to 0.04 μM WA serially diluted for 24 hours followed by addition of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for an additional 1.5 hours. The supernatant was aspirated and the formazan crystals were solubilized in dimethylsulfoxide, followed by determination of optical densities at 560 nm and 690 nm as an indicator of relative viable cell number.

Surface Expression of Inducible HSP70

To determine the amount of inducible HSP70 on the surface of tumor cells, 4T1, EMT6, RENCA and CT26 cells ($1x10^6$) were plated in 60mm tissue culture plates in complete media and allowed to adhere overnight. The media was then removed and replaced with fresh media containing either 2.5 μM WA, DMSO vehicle control or media alone for 24 hours. Plates were washed 2x with cold PBS containing calcium and magnesium. 3mL of the same cold PBS containing calcium and magnesium along with EZ Link Sulfo-NHS-LC-Biotin (Pierce) were incubated at 4°C for 1 hour. Next, plates were washed 3x with cold PBS containing calcium and magnesium. Cells were lysed in TNES by scraping and protein levels were quanitfied using a standard BCA assay (Pierce). 300 μg of

lysate was added onto Neutra-Avidin coated beads (Pierce Endogen, Rockford, IL) to pull down biotin-labeled material for 1 hour at 4°C. Supernatants were saved and beads were washed 3 times. Bound proteins were eluted by incubating beads in 2X loading buffer at 100°C for 5 minutes and size fractioned using SDS-PAGE. Western Blot analysis was performed and levels of inducible HSP70, actin (cytoplasmic control) and IGF1R (cell surface control) were analyzed.

Lymphocyte Proliferation Assay

BALB/c mice (4 mice/group) were immunized by intraperitoneal (IP) injection with 2mg/kg WA or DMSO vehicle control in a total of 50µl/mouse/injection. After 5 days, mice were sacrificed and the spleen cells were processed aseptically into a single cell suspension by passing through a mesh filter. The spleen cells from treated and untreated mice were cultured (200,000 cells/well) in 96 well plates in the presence and absence of Con A (0, 0.1, 0.5, 1 and 5µg/ml) for 48 hours. Cells were then labeled by adding 1µCi of ^{3}H Thymidine to each well and further incubated for 18-20 hours at 37°C. After the incubation, the cells were harvested and radioactivity levels were measured using a Rack Beta Liquid Scintillation Counter.

Co-Culture of Dendritic Cells and Tumor Cells Pre-Treated with Withaferin A

CT26 and 4T1 tumor cells were plated at 5 x 10^5 cells/well in 24 well tissue culture plates in RPMI with 10% FBS. Twenty four hours later, culture media was removed and replaced with fresh medium containing 2.5 μM WA (WA) or DMSO alone. After 24 hours the supernatant containing the non-adherent cells was collected and centrifuged at 22,000 x g for 10 minutes. The pellet obtained was re-suspended in complete medium and co-incubated with immature DCs for 24 hours in 24 well plates. DCs were collected 24 hours later, and phenotypic analysis was performed. DCs were collected and washed with PBS and stained for the expression of CD11c, MHC II and the co-stimulatory molecules CD40, CD80 and CD86.

III. CHAPERONE-RICH CELL LYSATE EMBEDDED WITH EXOGENOUS PEPTIDES DEMONSTRATES ENHANCED ANTI-TUMOR ACTIVITY

Introduction

Chaperone proteins are ubiquitous in all living things. Molecular chaperones include members of the heat shock protein (HSP) families and the glucose-regulated protein (GRP) families, as well as other members. Some are constitutively expressed and active, and others are induced further under stressful conditions. Molecular chaperones such as the HSPs are considered part of a superfamily based on their sequence homology and molecular weight, i.e. HSP110, HSP90, HSP70, HSP65, HSP27 etc (39, 124, 125). Heat shock proteins interact with other proteins to help maintain appropriate protein-protein contact, particularly during translation of nascent polypeptides. When other proteins are in non-native conformations, heat shock proteins will recognize and bind them (126). Often heat shock proteins function as hetero- and homo-oligomers, forming complexes with different HSPs, co-chaperones, and client proteins. HSPs are also responsible for preserving proper protein folding and unfolding and refolding during intra-organelle import and/or export, and for labeling aggregated or non-native proteins for destruction or transport out of a cell. A controversial topic in chaperone biology is whether chaperone proteins also have the ability to bind and shuttle intracellular peptides from one compartment to another within a cell (66, 68, 126-130).

When chaperone proteins are purified from a tumor source and utilized in a vaccine setting, they have the ability to mediate specific anti-tumor immunity. Specifically, the chaperone proteins HSP70, HSP90, GRP94, GRP170, HSP110 and calreticulin, when purified and used as vaccines individually, are capable of driving immune responses against their tumors of origin as demonstrated previously (85, 131-133). Antigenic peptides associated with HSPs (66, 128, 134) have been demonstrated to be responsible for the immunogenicity of the HSP preparations rather than the HSPs themselves, although there is substantial debate over the true nature of the peptide/HSP complex (118, 130, 135). By removing HSP-bound peptides, the immunogenic capabilities of HSP preparations have been shown to be abrogated (128, 134, 136), while others have shown that the HSPs themselves posses potent innate immune stimulus (102, 118, 137, 138). A growing body of data suggest that dendritic cells (DCs) take up chaperone-peptide complexes through specific receptors, such as CD91 (131-133), the scavenger receptors (139, 140), CD40 (101), LOX-1 (141), and the Toll-like receptors (142). DCs re-present the peptides on MHC-I and MHC-II molecules as stated above, although the mechanisms are not fully defined (119). Thus, exogenous HSPs may be a means of directing peptides into DC antigen presentation pathways, while also providing activation or “danger” signals to the DCs. This would culminate in the activation and stimulation of antigen-specific T-cells.

Through the use of free solution-isoelectric focusing (FS-IEF), we are capable of enriching for multiple immunogenic chaperone protein complexes from tumor-derived or normal tissue-derived lysates (86). Given the capabilities of chaperones to effectively interact with exogenous free peptide (130, 135), CRCL may present an effective and novel method of transport for exogenous tumor-associated or tumor-specific peptides.

We have previously demonstrated that CRCL preparations from normal tissue have potent adjuvant effects when combined with apoptotic tumor cell material as a vaccine (94). We have also shown that CRCL, from tumor-derived material, contains known peptide antigens such as a fusion peptide from a BCR/ABL+ murine leukemia (119). Since there is an emphasis on identifiable specificity in immune monitoring (143, 144), we hypothesized that we could add tumor peptides exogenously to CRCL that could enhance the anti-tumor immunity in a T-cell specific fashion. In this report, we show that an exogenous BCR/ABL peptide (GFKQSSKAL) and OVA257-264 peptide (SIINFEKL) can indeed incorporate into leukemia-derived and liver-derived CRCL. Upon pulsing the peptide-laden CRCL onto DCs, these peptides are then taken up and presented by DCs with high efficiency to stimulate T-cells in *in vitro* assays. Our findings also show that when peptide-embedded CRCL is administered in vivo, it has significant anti-tumor effects. Thus, these results indicate that CRCL could be the carrier of choice for exogenous tumor-related antigenic peptides. The novel

concept of creating a peptide-“designer” CRCL will offer the ability to personalize enhanced vaccines to those afflicted with cancers containing known antigens, such as the BCR-ABL fusion peptide, or to generate vaccine with known antigens where autologous tumor is not available.

Results

Exogenous Peptide Can Be Efficaciously Incorporated into CRCL Vaccine During Free Solution-Isoelectric Focusing (FS-IEF)

We had previously reported that tumor-derived CRCL has antigenic peptides associated with it (119), although the mechanism for this association is unclear. We speculated that we could exploit CRCL's peptide binding capacity by adding exogenous peptide to the unrefined lysate preparation (i.e., prior to the isofocusing procedure). We would then have to determine if the specific peptide co-separated with the chaperone proteins in the fractions that would be pooled to make the CRCL vaccine. To that end we chose the 9-mer ovalbumin peptide, SIINFEKL (OVA257-264), which was added to C57BL/6 mouse liver homogenate before subjecting the mixture to FS-IEF. Proteins from the 20 harvested fractions were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose to probe for the four main chaperone proteins of interest in generating CRCL vaccine; HSP70, HSP90, GRP94 and calreticulin. One or more of those four chaperone proteins was present in fractions 9-14 and 18. The 20 fractions were separately prepared as vaccines (dialyzed, concentrated, detergents removed). Each of the 20 fraction "vaccines" were individually pulsed onto 20 different groups of C57BL/6 mouse DCs overnight and were washed thoroughly the next day (as shown in Figure 3). The pulsed DCs were incubated with B3Z T-cell hybridomas, which have been engineered to produce beta-

galactosidase upon transcription from the IL-2 promoter, following T-cell receptor stimulation with the H2Kb-presented SIINFEKL peptide. Thus, IL-2 secretion was measured in surrogate by beta-galactosidase output. This output was found to be significantly increased in B3Z cells that were incubated with DCs that had been pulsed with proteins from fractions 9-14 and 18 (Figure 4), which coincided with the fractions containing the four aforementioned chaperones. These results indicated that SIINFEKL peptide localized to chaperone-containing fractions corresponding to the fractions ordinarily chosen to generate the CRCL vaccine. Furthermore, these fractions had the ability to donate the SIINFEKL peptide into the presentation pathways of DCs leading to specific T-cell stimulation. SIINFEKL peptide alone subjected to FS-IEF and vaccine preparation did not elicit B3Z activity from any of the 20 fractions (data not shown), nor did liver CRCL prepared without addition of exogenous SIINFEKL peptide (Figure 4).

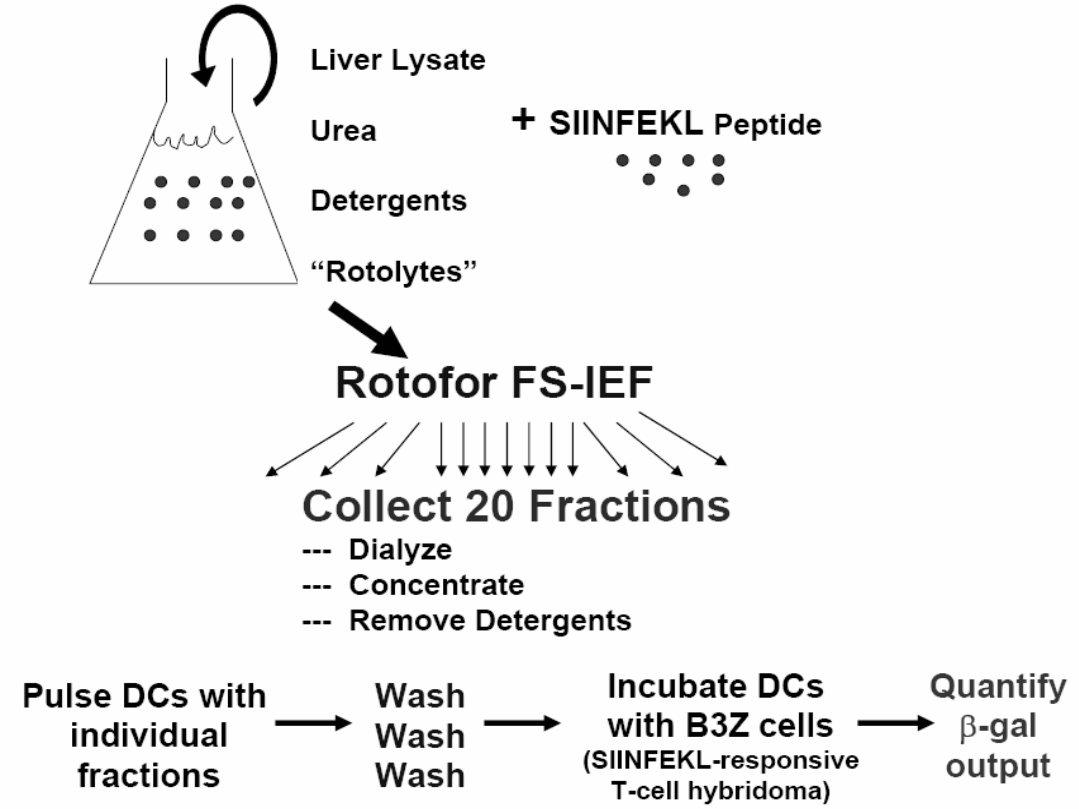


Figure 3. *FS-IEF of Liver Lysate Loaded with Exogenous Peptide.*

SIINFEKL peptide loaded into C57BL/6 liver lysate was subjected to free solution-isoelectric focusing (FS-IEF) and the subsequent 20 fractions were harvested individually and each produced into vaccines. The 20 fractions were pulsed onto C57BL/6 mouse DCs and further incubated with B3Z T-cells to determine T-cell activation through β-Galactosidase output.

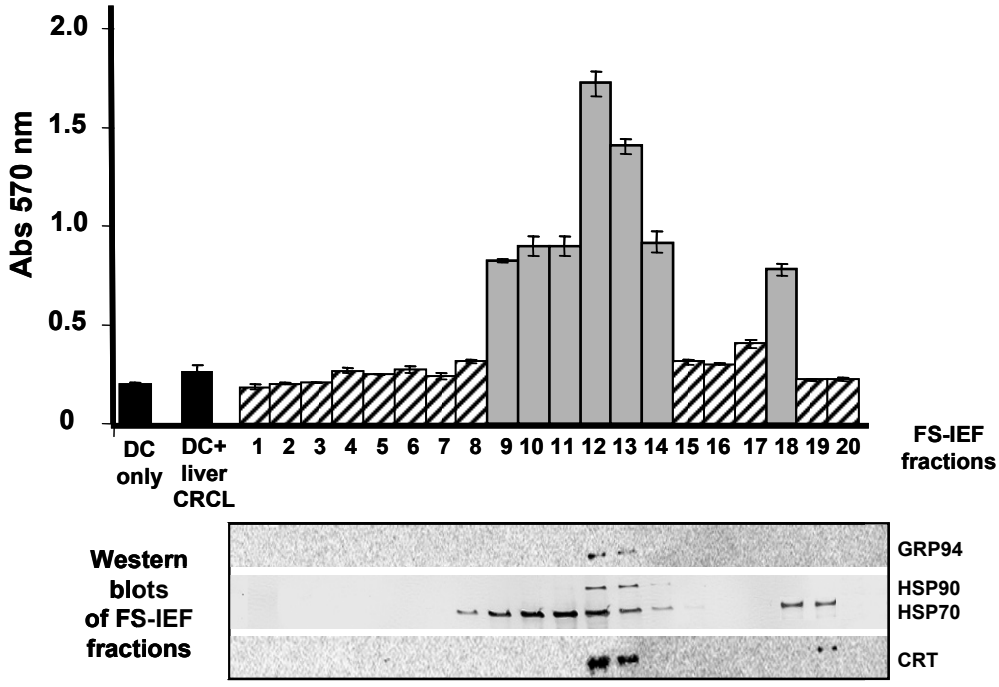


Figure 4. *Peptide Loading of Homogenate via FS-IEF Elicits T-cell Activation in Fractions Containing Chaperone Proteins of Interest.*

T-cell activation assays were executed using β-Galactosidase in surrogate for IL-2 production and corresponding Western Blot analysis was performed to identify chaperone proteins (HSP70, 90, GP94 and calreticulin) in each fraction. After processing all 20 FS-IEF fractions separately, it was determined that fractions 9-14 and 18 supplied SIINFEKL peptide to be presented by APCs to B3Z cells, which lead to IL-2 secretion/B-galactosidase activity. These fractions also corresponded to the chaperones of interest that are typically harvested to

produce the CRCL vaccine. Data are representative of three independent experiments.

High Levels of Exogenous Peptide are Incorporated into, and Retained by CRCL

As a model, SIINFEKL peptide demonstrated co-localization into the chaperone-containing fractions selected to produce CRCL vaccine. It was further determined that peptide incorporation could be accomplished by simply combining CRCL as an end product with a peptide of interest and allow it to incubate at room temperature (refer to methods). However, it was critical to know how much peptide, post-incorporation, remained bound to CRCL (or cell lysate as a control) after several washes and filtration through a size-specific membrane cut-off filter to release any free peptide present. We used a fluorescein-labeled BCR/ABL (FITC-GFK) peptide to measure the amount of peptide bound post-incubation to 50µg 12B1 tumor-derived CRCL compared to 12B1 lysate, mouse IgG or peptide alone (Figure 5). We also measured peptide incorporation in 50µg liver-derived CRCL versus liver lysate, mouse IgG or peptide alone (Figure 6). Proteins were incubated with a 1:1 microgram ratio of peptide and incubated at room temperature for 40 minutes. The peptide mixtures were centrifuged at 9.5 x 10^3 rpm through a 10kDa cut-off membrane filter to remove any unbound peptide and were then washed three times with 1x PBS by centrifugation of buffer through the filter. After a final spin the retentate mixtures were collected for use. FITC-peptide retention was measured using a microplate fluorometer with an excitation at 488nm and an emission at 527nm. The microgram amounts of peptide bound were derived based on a FITC-peptide standard curve. We used these values to calculate that a readily detectable 1-

2μg of peptide bound to both 12B1 CRCL (Figure 5) and liver-derived CRCL (Figure 6) vaccine, while only 0.2-0.3μg of peptide bound to either lysate. Since the amount of CRCL vaccine used was 50μg total, this suggests that between 1-2μg of peptide, or approximately 3% of total polypeptide content, may be incorporated into every 50μg of CRCL vaccine indicating a 5-10 fold increased peptide carrying capacity when compared to standard lysate.

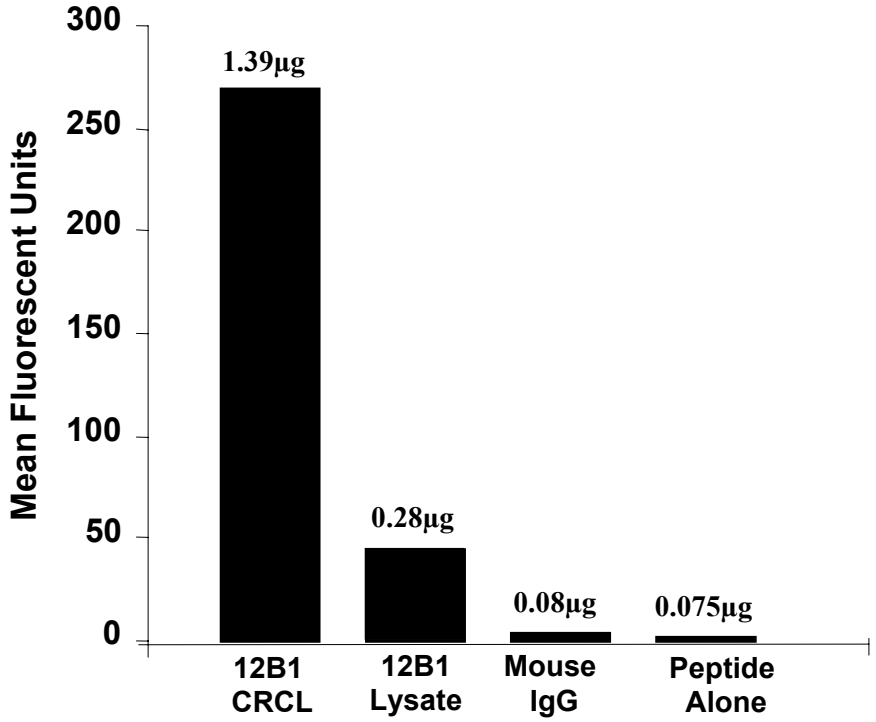


Figure 5. *High Levels of Peptide Retention Observed When Incorporated into 12B1 CRCL; Assay for quantifying fluorescent peptide retained in CRCL.*

FITC-tagged GFK peptide was embedded into 12B1 tumor-derived CRCL, lysate, mouse IgG and GFK peptide alone as indicated. After 40 minutes, all samples were washed to remove free peptide. Quantification of FITC-GFK peptide incorporated in each sample was performed using a microplate fluorometer in fluorescent units (exitation: 488/emission: 527) compared to a FITC-peptide standard curve. Microgram quantities of peptide retained are indicated above each of their corresponding bars. Data are representative of three independent experiments.

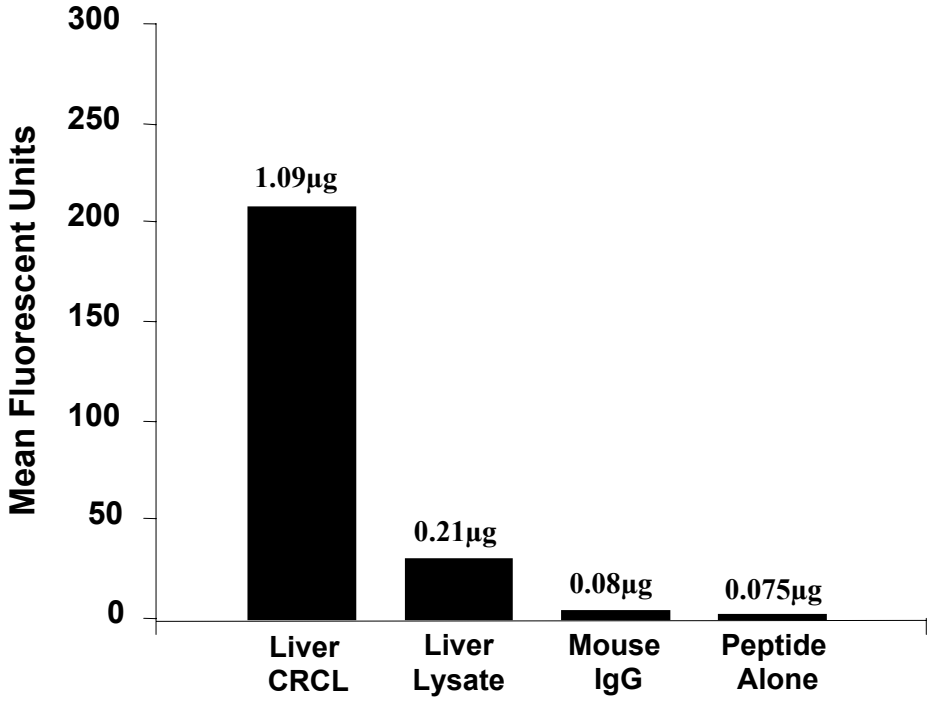


Figure 6. *High Levels of Peptide Retention Observed When Incorporated into Liver CRCL; Assay for quantifying fluorescent peptide retained in CRCL.*

FITC-tagged GFK peptide was embedded into BALB/c Liver-derived CRCL, lysate, mouse IgG and GFK peptide alone as indicated. After 40 minutes, all samples were washed to remove free peptide. Quantification of FITC-GFK peptide incorporated in each sample was performed using a microplate fluorometer in fluorescent units (exitation: 488/emission: 527) compared to a FITC-peptide standard curve. Microgram quantities of peptide retained are indicated above each of their corresponding bars. Data are representative of three independent experiments.

Identification of a Peptide-Binding Partner in CRCL Using Mass Spectrometry and Western Blot Analysis

We sought to identify the protein(s) responsible for the binding of peptide following peptide incorporation into CRCL. We chose to initially separate the FITC-GFK embedded liver CRCL on an agarose gel system run under native conditions. The fluorescence was detectable in a high molecular weight region of the agarose gel. Following the excision of a band (Figure 7A), the protein(s) associated with the fluorescence was/were separated from the agarose gel piece. The now liquid sample was denatured and run on a 10% acrylamide gel to separate the possible proteins. The gel was then stained with Sypro Ruby to visualize any bands that were present (data not shown). These bands were excised and subjected to in-gel trypsin digestion, and the internal peptide sequences were determined by LC-MS/MS. The database search result showed that two of the differentially expressed protein internal sequences matched that of murine heat shock cognate 70, a main component in the CRCL vaccine (Figure 7B, top panel). To confirm that the 70 kDa protein identified by LCMS/MS was HSC70, we performed Western Blot analysis using a specific rat monoclonal HSC70 antibody. These results strongly imply that HSC70 is one of the major peptide binding proteins in liver CRCL, or at least is one of the most abundant (Figure 7B, bottom panel).

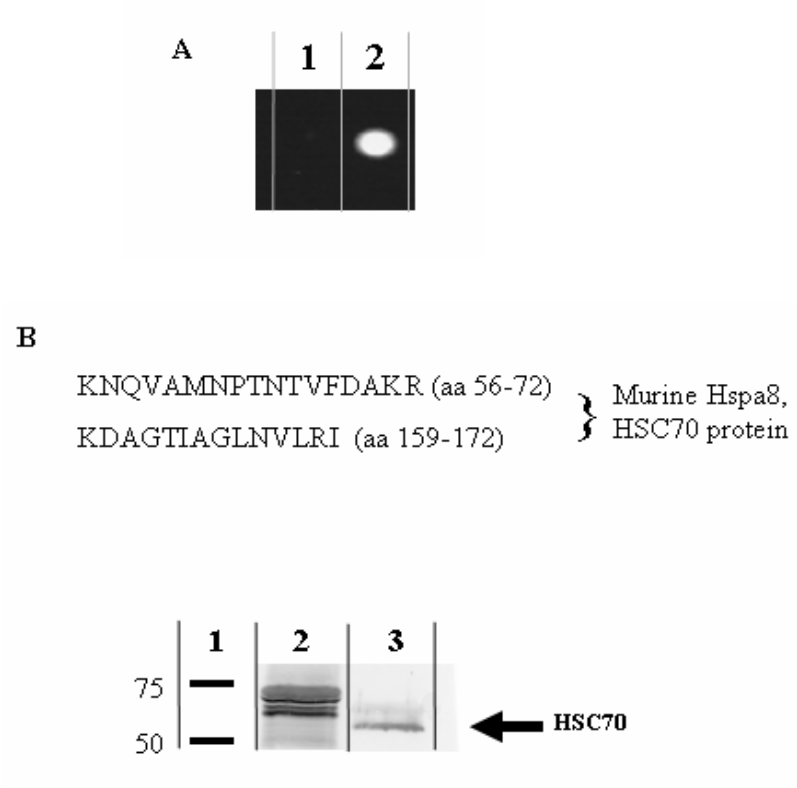


Figure 7. *Identification of HSC70 as a Peptide Binding Target within CRCL.*

A, Liver CRCL incubated with FITC-labeled GFK peptide was run on an agarose gel under native conditions. A prominent band tracked by the FITC label was excised for analysis by LC-MS/MS. *Lane 1* represents FITC-peptide alone and *Lane 2* represents FITC-peptide embedded in liver CRCL. *B (top panel)*, Protein(s) from the fluorescent region of the agarose gel were removed and subjected to SDS-PAGE separation. Proteins bands detected by Sypro staining were identified by LC-MS/MS techniques as heat shock cognate 70 kDa protein (HSC70). *B (bottom panel)* To verify LC-MS/MS results, the same process shown in *panel A* was executed and the subsequent Western Blot was probed for

the protein target, HSC 70, obtained from the MS database. *Lane 1* represents the molecular weight marker, Lane 2 represents liver CRCL alone and *Lane 3* represents the sample extracted from the agarose gel in *lane 2* of *panel A*. Data are representative of three independent experiments.

Peptides Incorporated into CRCL can Effectively Transfer to Dendritic Cell Surfaces

The data from Figure 4 indicated that peptides localizing into chaperone-containing fractions during FS-IEF could effectively be presented by DCs to T-cells. To verify the transfer and DC surface presentation of the exogenously added peptides incorporated into CRCL, we used unlabeled 12B1 CRCL and unlabeled liver-derived CRCL embedded with FITC-GFK peptide. Peptide-embedded 12B1 CRCL, peptide-embedded 12B1 lysate or 1μg of peptide alone, which is the approximate amount of peptide retained in CRCL, was produced and pulsed onto BALB/c mouse dendritic cells overnight (Figure 8). In the same manner, peptide-embedded liver CRCL and peptide-embedded liver lysate were prepared, and along with peptide alone, and were also pulsed onto DCs (Figure 9). The cells were washed thoroughly, then fixed and analyzed by flow cytometry. The dendritic cell population (gated for CD11c+) pulsed with FITC-GFK peptide-embedded CRCL (whether tumor-derived or liver-derived CRCL) showed significantly higher mean fluorescence intensity values than did DCs pulsed with peptide embedded into lysate or FITC-peptide alone (Figure 8 and 9).

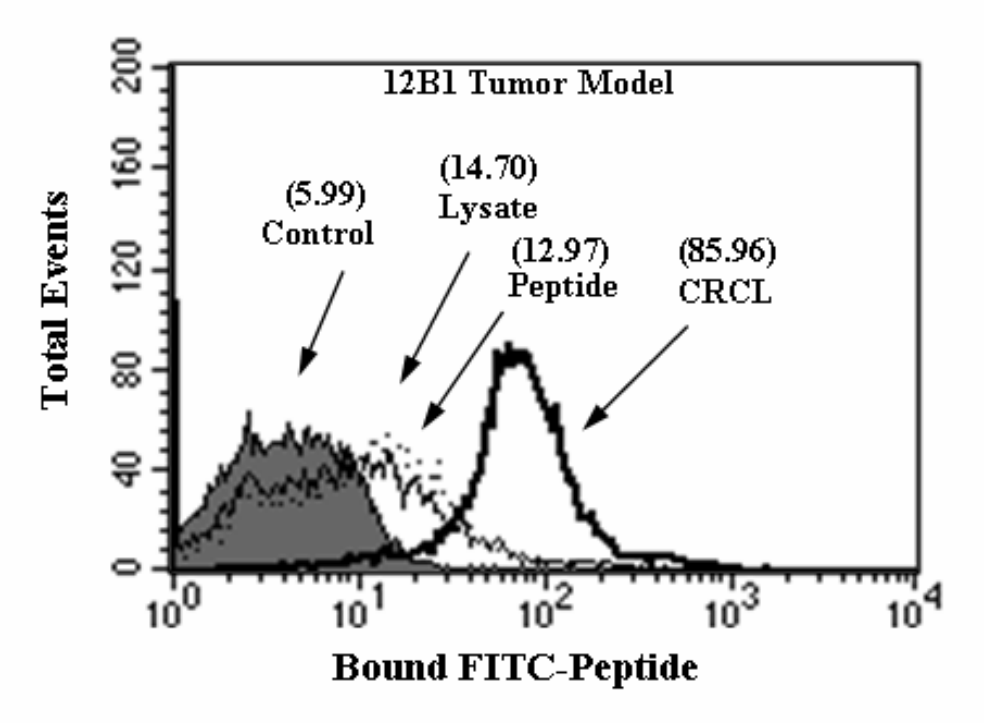


Figure 8. *Effective Peptide Binding Ability via 12B1 CRCL in Dendritic Cells.*

BALB/c mouse dendritic cells were pulsed with Peptide-Embedded 12B1 CRCL, Peptide-Embedded 12B1 lysate or GFK peptide alone. Fluorescently labeled GFK peptide was used for each condition. The flow cytometry analysis is based on the amount of FITC-peptide displayed on the surface of dendritic cells after overnight treatment. Values in parentheses represent mean fluorescence intensity of 10,000 events analyzed. Data are representative of three independent experiments.

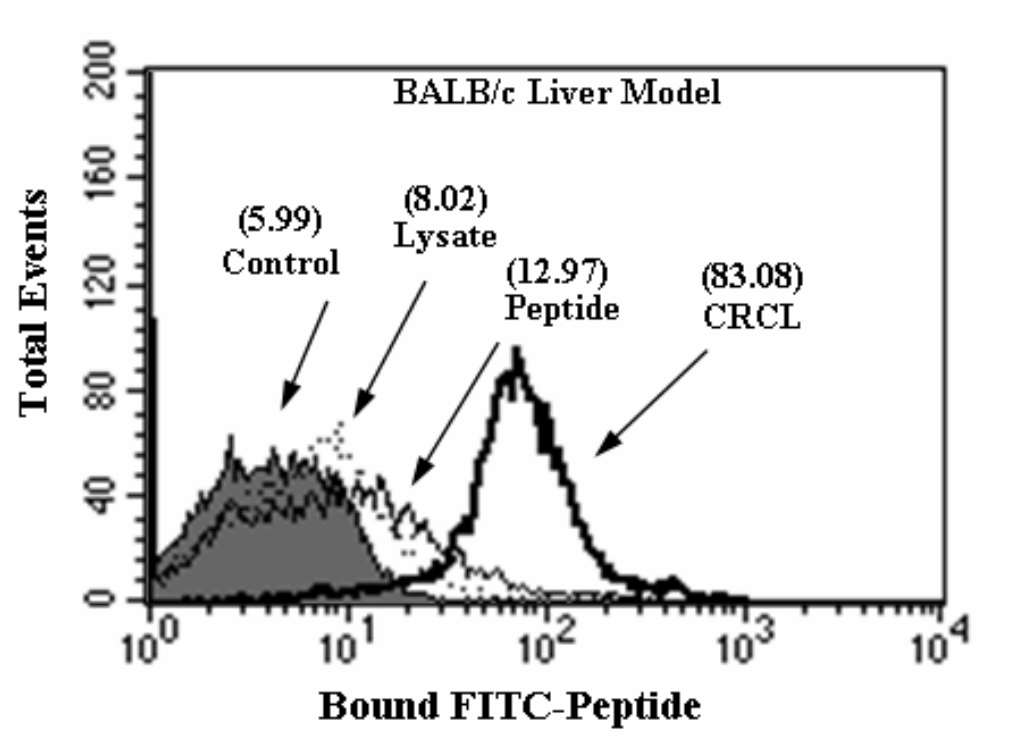


Figure 9. *Effective Peptide Binding Ability via Liver CRCL in Dendritic Cells.*

BALB/c mouse dendritic cells were pulsed with Peptide-Embedded liver CRCL, Peptide-Embedded liver lysate or GFK peptide alone. Fluorescently labeled GFK peptide was used for each condition. The flow cytometry analysis is based on the amount of FITC-peptide displayed on the surface of dendritic cells after overnight treatment. Values in parentheses represent mean fluorescence intensity of 10,000 events analyzed. Data are representative of three independent experiments.

CRCL Enhances Peptide Uptake into Dendritic Cells

From the flow cytometry studies (above) it was unclear if peptide that had been incorporated into CRCL was dissociated from CRCL and onto DC surfaces, while the other protein components of CRCL were washed away, or if there was still co-localization of CRCL and the peptide. To assess this, liver-derived CRCL, and its corresponding lysate were labeled with Texas Red. This labeled CRCL was embedded with FITC-labeled GFK peptide and pulsed onto dendritic cells overnight. The cells were then washed, fixed and transferred to microscope slides for confocal imaging to determine peptide delivery and uptake by dendritic cells with respect to CRCL proteins. As shown in Figure 10, imaging of CRCL proteins and labeled peptide showed a high degree of internalization into the pulsed DCs, along with co-localization of protein and peptide signals. Labeled lysate, while bound and internalized by DCs, did not enhance exogenous peptide uptake to any detectable level in this microscopy assay, probably due to the inability of the lysate to retain much exogenously added peptide (Figures 5 and 6).

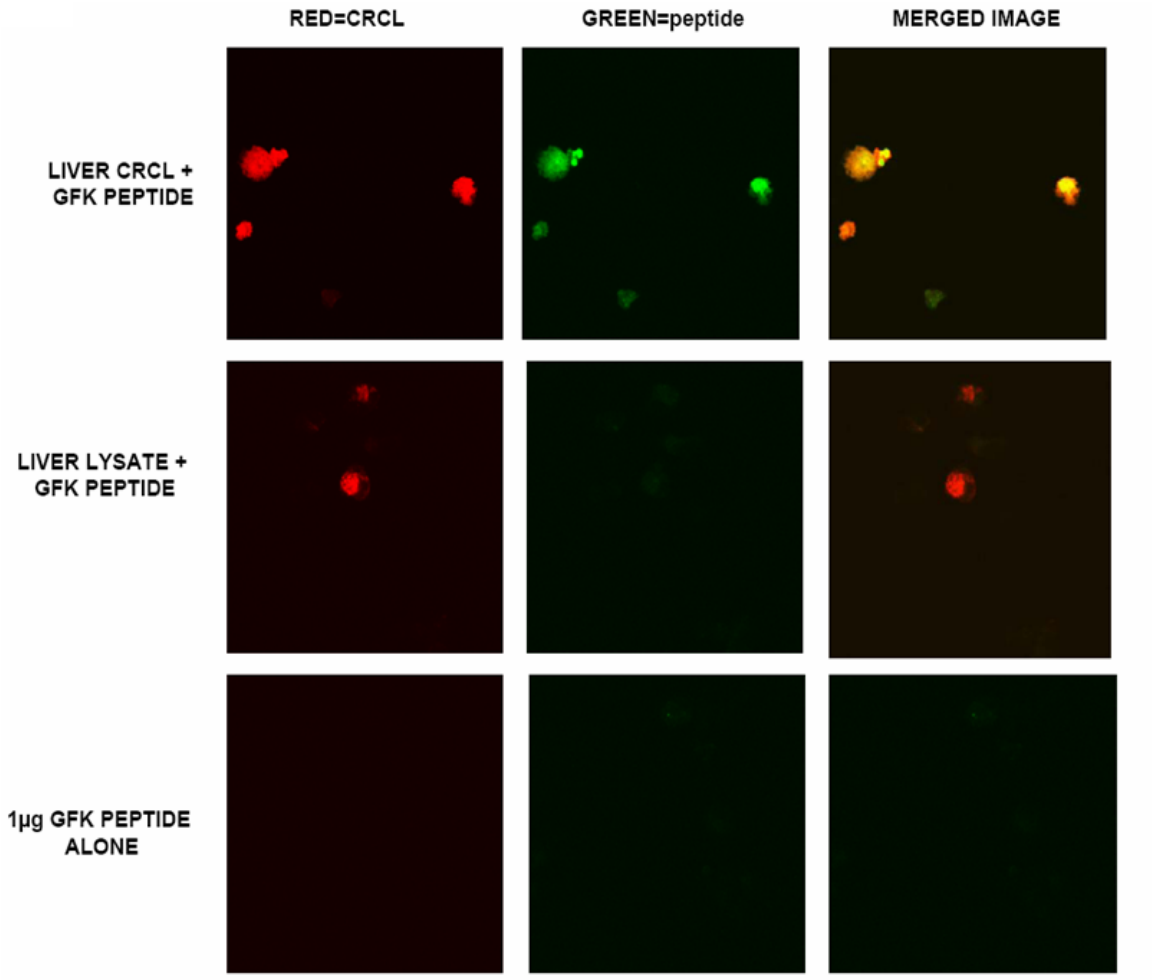


Figure 10. *Effective Peptide Uptake via CRCL in Dendritic Cells.*

Dendritic cells were experimentally treated with peptide-embedded liver CRCL, peptide-embedded liver lysate or GFK peptide alone overnight. CRCL and lysate were labeled with Texas Red before peptide incorporation and the GFK peptide was FITC-labeled for confocal microscopy. All images were acquired at the same 100x magnification using the oil immersion objective. Data are representative of three independent experiments.

Peptide-Embedded CRCL Elicits Potent Antigen-Specific T-cell Activation

To establish whether DCs pulsed with SIINFEKL peptide-embedded liver CRCL, peptide-embedded liver lysate, peptide combined with mouse IgG or peptide alone generated an antigen-specific T-cell response, we performed a T-cell activation assay. In brief, peptide-embedded CRCL, peptide-embedded lysate, peptide-embedded mouse IgG or peptide alone (all utilizing the SIINFEKL peptide) were pulsed onto C57BL/6 mouse DCs overnight. The cells were then thoroughly washed to remove free peptide, and then incubated with B3Z cells. B3Z activation was quantified by beta-galactosidase output as a surrogate for IL-2 production. 50μg of peptide-embedded liver CRCL was determined to elicit more than a 2 fold greater IL-2 output compared to 50μg of peptide-embedded liver lysate, 50μg of peptide-embedded mouse IgG or 1μg peptide alone. Peptide-embedded CRCL containing the embedded SIINFEKL peptide was therefore effectively processed by dendritic cells, leading to a more effective stimulation of OVA-specific T-cells (Figure 11).

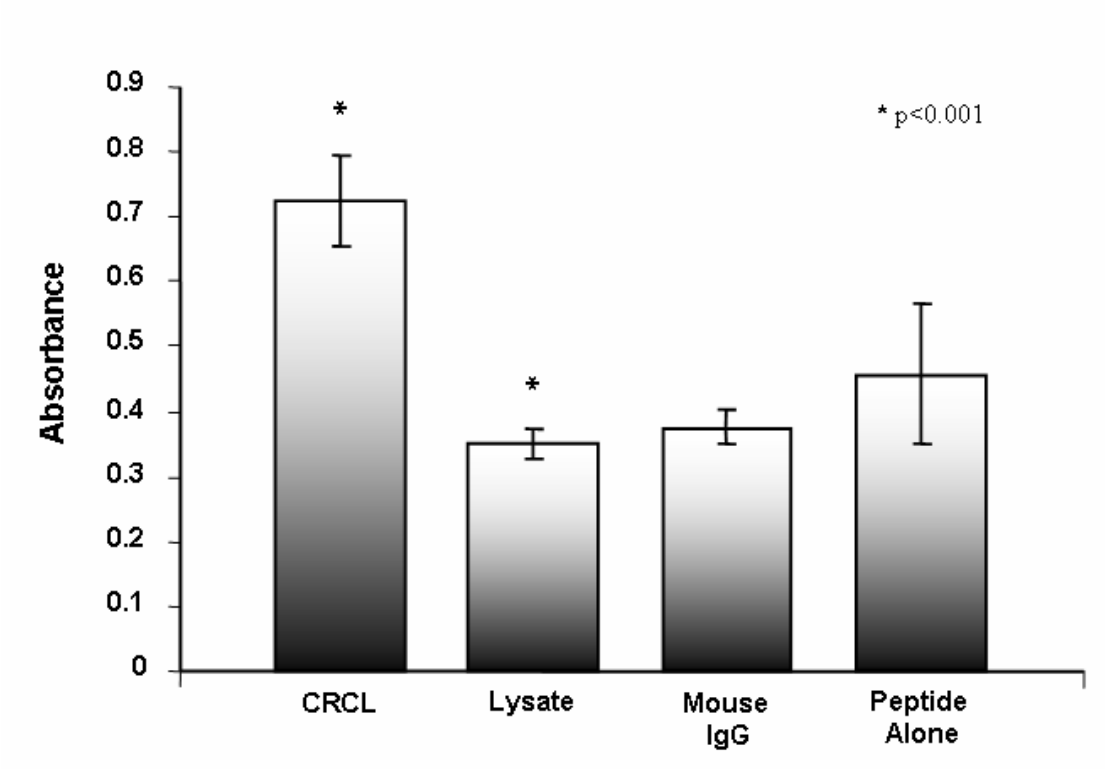


Figure 11. *Peptide-Embedded CRCL Elicits a Potent Antigen-Specific T-cell Response.*

Quantitation of IL-2 secretion via a T-cell activation assay. B3Z reporter T-cells that are stably transfected with a vector encoding LacZ under the transcriptional control of the IL-2 promoter were incubated in a 96-well plate with C57BL/6 mouse DCs that were pulsed with peptide-embedded CRCL, peptide-embedded lysate, peptide-embedded IgG or SIINFEKL peptide alone as indicated. The relative absorbance at 560nm (A_{560}) detected in each well was determined using a microplate absorbance reader. Data are representative of three independent experiments. Bars: S.D. of mean. Statistical analysis computed using student t-test.

Immunization of Mice with Peptide-Embedded CRCL Induces Antigen-Specific IFN-γ Secretion

The *in vitro* assays suggested that peptide-embedded CRCL could stimulate T-cell activation from a T-cell hybridoma via DC presentation of peptide. To determine whether specific peptide antigenicity is maintained *in vivo*, BCR-ABL peptide (GFK) was embedded into 12B1 CRCL and liver CRCL, and BALB/c mice were immunized with GFK peptide-embedded liver CRCL, liver CRCL alone, or PBS as a control, on days 0 and 2. BALB/c mice were also immunized with GFK peptide-embedded 12B1 CRCL, 12B1 CRCL alone, or PBS as a control, on days 0 and 2. On day 7, splenocytes were harvested and restimulated *in vitro* with peptide-embedded liver CRCL, peptide-embedded 12B1 CRCL, liver CRCL, 12B1 CRCL, GFK peptide, an irrelevant peptide (HYLSTQSALSK) or media alone as background, and ELISPOT assays were performed. As expected, mice immunized with liver CRCL or PBS showed insignificant IFN-γ secretion with any restimulation due to the fact that no GFK peptide was present in the vaccine (Figure 12). Splenocytes from mice primed with 12B1 CRCL, GFK peptide-embedded liver CRCL and GFK pepide-embedded 12B1 CRCL produced significant amounts of IFN-γ when restimulated with the GFK peptide, and an even higher level of secretion when restimulated with GFK peptide-embedded liver CRCL (Figure 12) or GFK peptide-embedded 12B1 CRCL (Figure 13). These findings indicated that exogenous GFK peptide which is not

inherently found in liver CRCL alone, when embedded in the vaccine, is a major immunological component of CRCL. Splenocytes primed with peptide-embedded liver CRCL, liver CRCL, peptide-embedded 12B1 CRCL, 12B1 CRCL or PBS failed to secrete IFN-γ when restimulated with an irrelevant peptide or with liver CRCL alone (Figures 12 and 13). These findings confirm that *in vivo* GFKQSSKAL retains potent immunogenicity in GFK peptide-embedded liver or 12B1 CRCL.

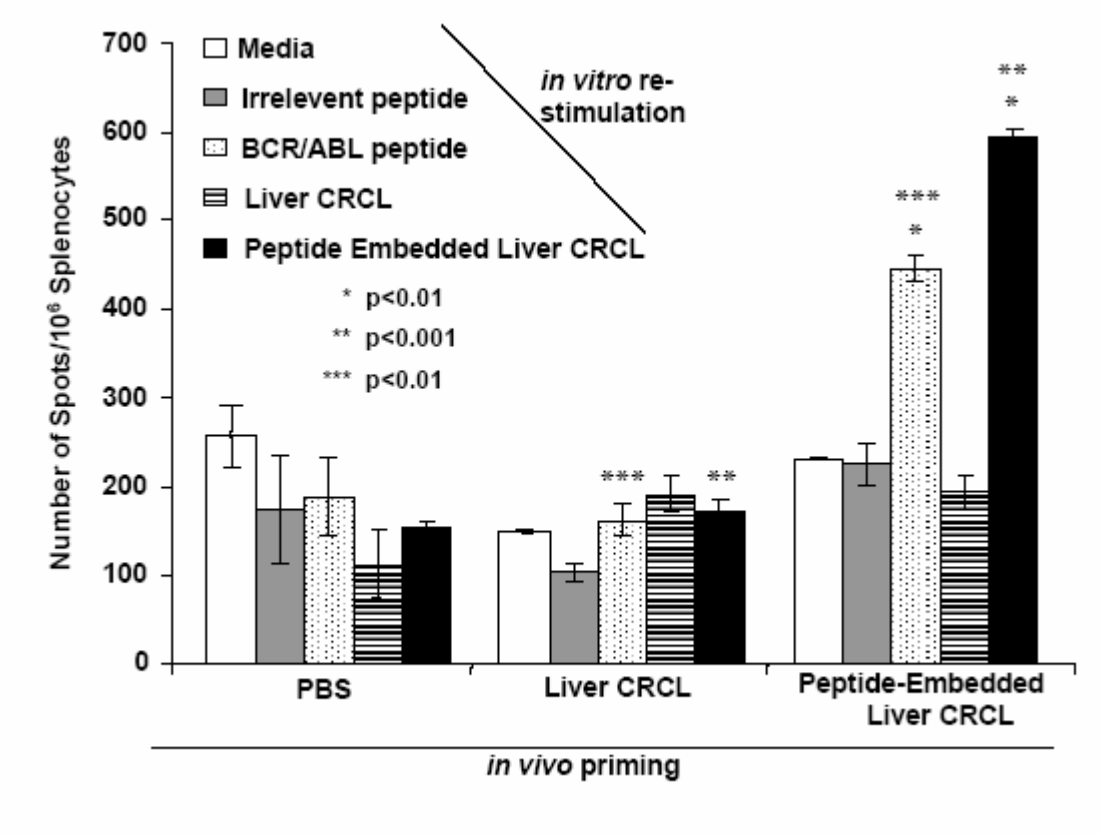


Figure 12. *Immunization of Mice with Peptide-Embedded Liver CRCL Induces BCR/ABL-Specific IFN-γ Secretion.*

Mice were immunized with PBS, liver CRCL or peptide-embedded liver CRCL. All immunizations took place on days 0 and 2. On day 7, splenocytes were collected and restimulated with indicated peptides or CRCLs for 48 hours. IFN-γ production was determined by ELISPOT assay. Error bars represent SEM. Statistical analysis computed using student t-test.

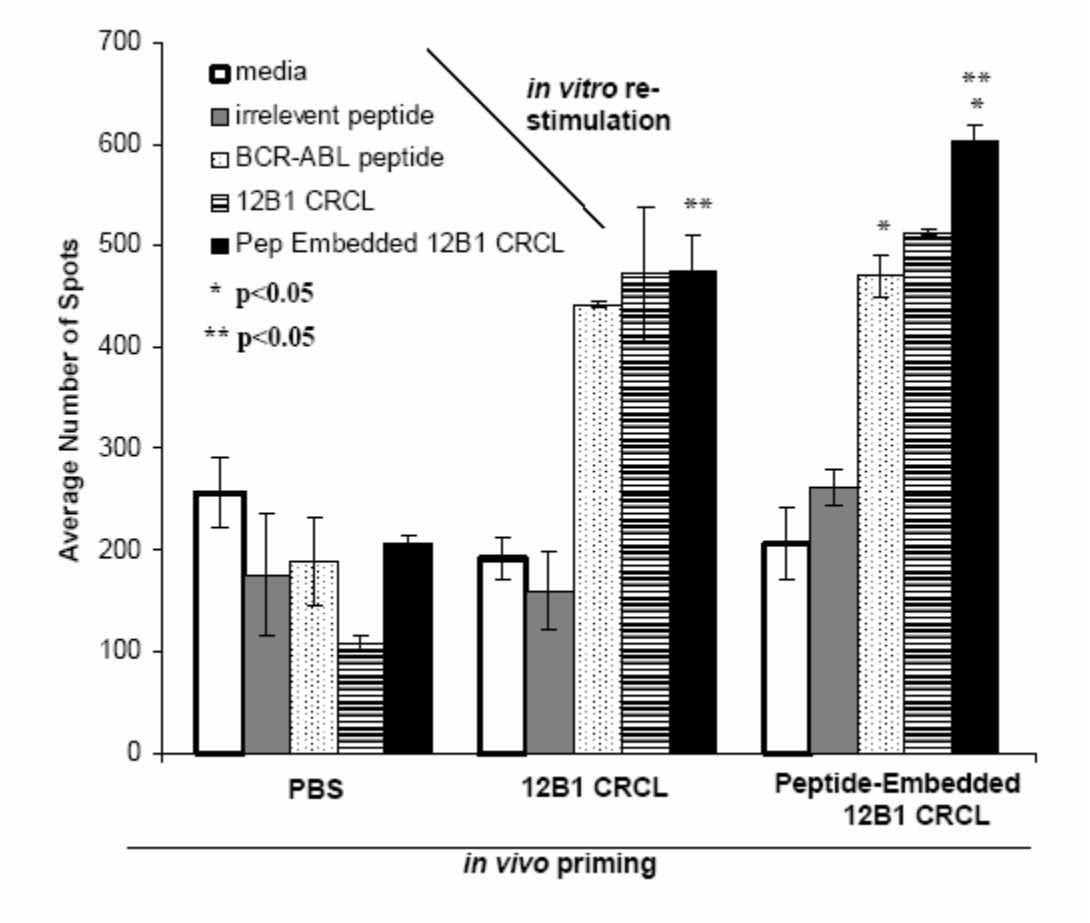


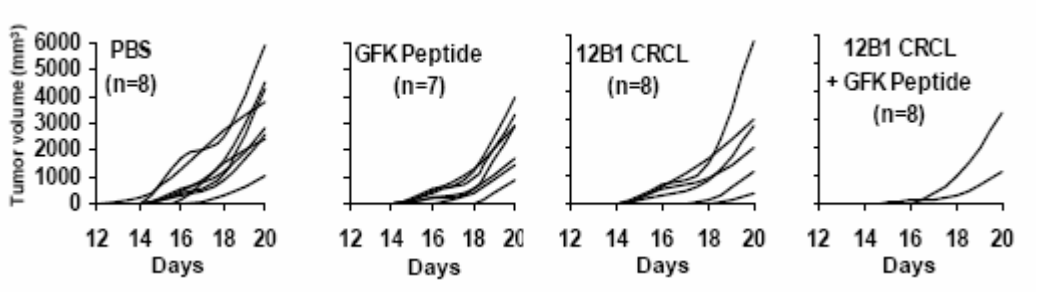
Figure 13. *Immunization of Mice with Peptide-Embedded 12B1 CRCL Induces BCR/ABL-Specific IFN-γ Secretion.*

Mice were immunized with PBS, 12B1 CRCL or peptide-embedded 12B1 CRCL. All immunizations took place on days 0 and 2. On day 7, splenocytes were collected and restimulated with indicated peptides or CRCLs for 48 hours. IFN-γ production was determined by ELISPOT assay. Error bars represent SEM. Statistical analysis computed using student t-test.

CRCL Vaccine Embedded with Exogenous BCR/ABL Peptide Has Enhanced Anti-Tumor Activity

In an earlier study (87), we showed that 12B1 CRCL, especially when combined with DCs could delay tumor growth when compared to individual heat shock proteins as vaccines in treating pre-existing 12B1 tumors. Given the *in vitro* and *in vivo* data indicating that peptide-embedded CRCL could generate specific T-cell responses, we asked whether CRCL embedded with GFK peptide would induce protection against in vivo 12B1 tumors. Thus, we treated mice with 12B1 or liver CRCL embedded with GFK peptide on days one and three following subcutaneous injection of 12B1 tumor cells. As shown in Figure 14 and 15, the addition of GFK peptide to 12B1- or liver-derived CRCL vaccine delayed tumor growth compared to groups of mice immunized with 12B1 CRCL or liver CRCL alone, GFK peptide alone, or saline. A portion of the GFK/12B1 CRCL and GFK/liver CRCL-treated mice were found to have rejected their tumors for more than 90 days and upon re-challenge with 12B1 tumor, a significant tumor growth delay was still observed (Figure 16).

A



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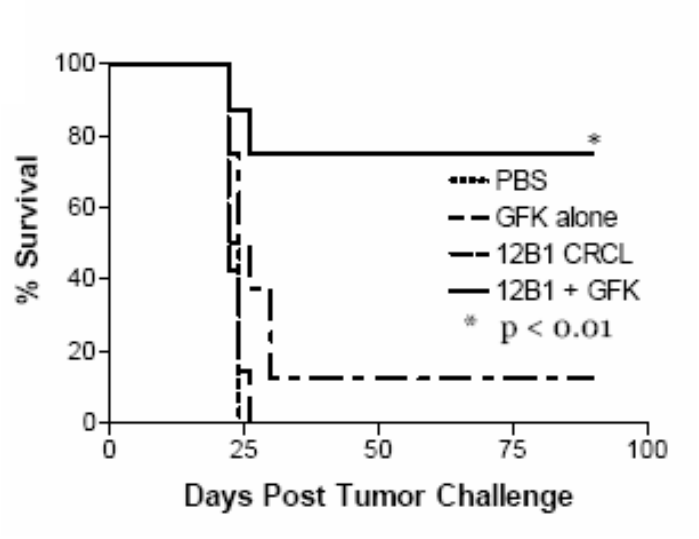
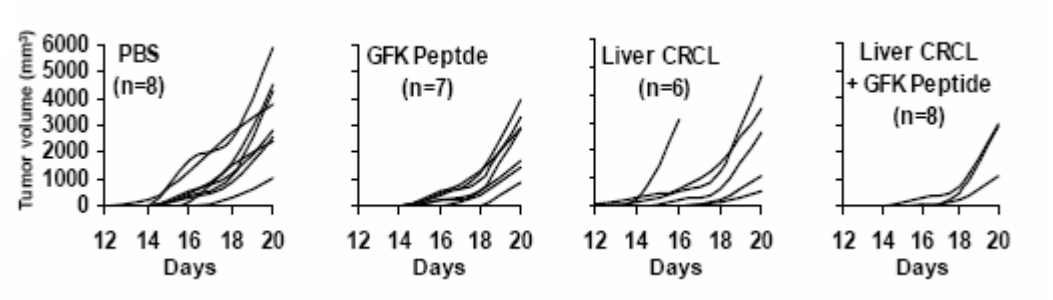


Figure 14. *Effects of Immunization with Peptide-Embedded 12B1 CRCL on Pre-Existing 12B1 Tumors.*

Mice were injected subcutaneously (s.c.) with 3000 *in vivo* passed 12B1 tumor cells. On day 1 and day 3, mice were injected with 20µg of GFK peptide-embedded 12B1 CRCL. Other groups of mice were also injected s.c. with either PBS, 1µg GFK peptide alone or 20µg 12B1 CRCL alone. The data in *A*,

represent tumor volume measurements of individual mice per group. Animals were sacrificed when tumor volume reached 3000 mm^3. *B*, represents Kaplan-Meier survival plots of the mice shown in *panel A*. Mice vaccinated with GFK peptide-embedded 12B1 CRCL (n=6) remained tumor free at day 90. Data are representative of two independent experiments and Log-Rank was used to determine statistical significance.

A



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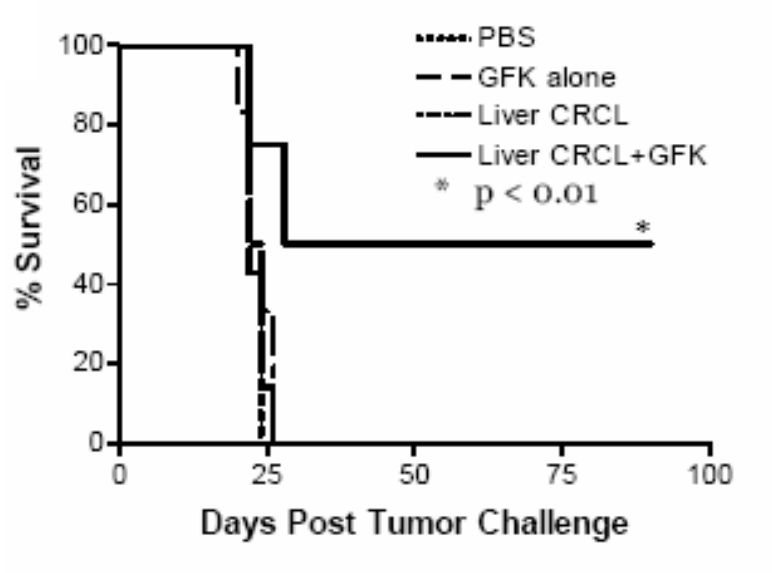
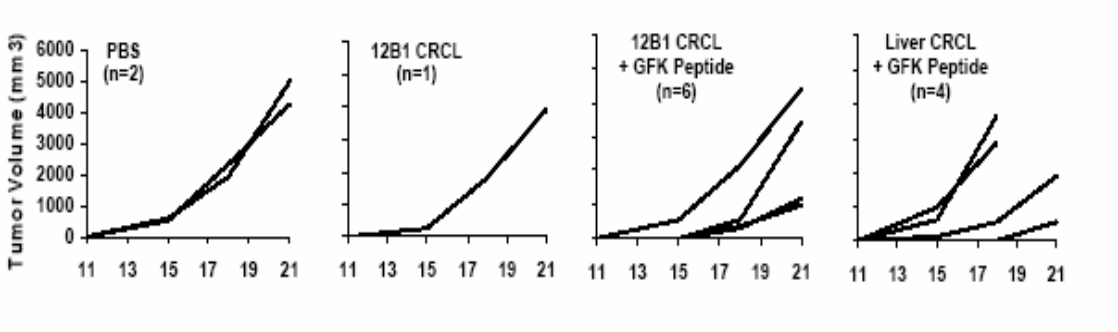


Figure 15. *Effects of Immunization with Peptide-Embedded Liver CRCL on Pre-Existing 12B1 Tumors.*

Mice were injected subcutaneously (s.c.) with 3000 *in vivo* passed 12B1 tumor cells. On day 1 and day 3, mice were injected with 20µg of GFK peptide-embedded liver CRCL. Other groups of mice were also injected s.c. with either PBS, 1µg GFK peptide alone or 20µg liver CRCL alone. The data in *A*, represent

tumor volume measurements of individual mice per group. Animals were sacrificed when tumor volume reached 3000 mm3. *B*, represents Kaplan-Meier survival plots of the mice shown in *panel A*. Mice vaccinated with GFK peptide-embedded liver CRCL (n=4) remained tumor free at day 90. Data are representative of two independent experiments and Log-Rank was used to determine statistical significance.

A



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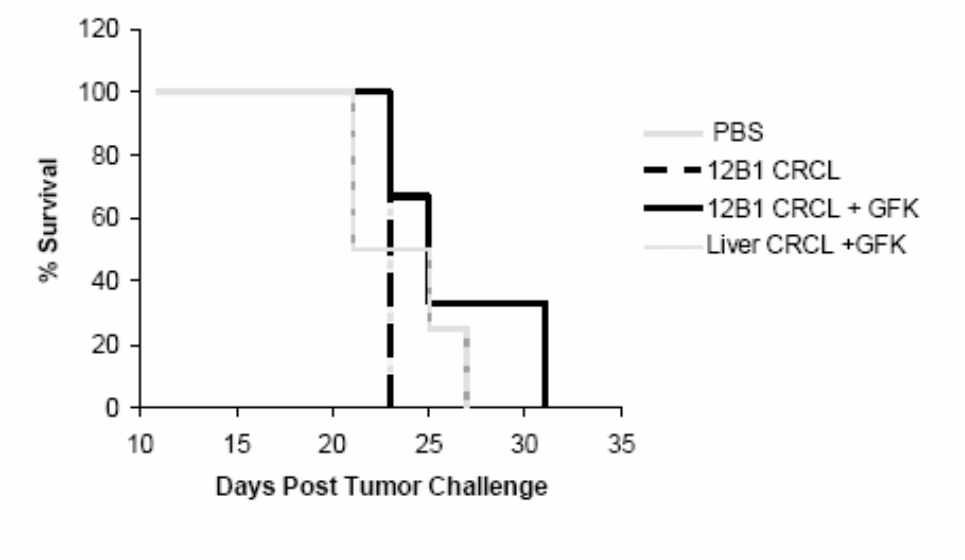

Figure 16. *Effects of Immunization with Peptide-Embedded Liver or 12B1 CRCL on Pre-Existing 12B1 Tumors Following Tumor Re-challenge.*

Panels A and B represent mice that had rejected their tumors following peptide-embedded CRCL vaccination (one mouse from 12B1 CRCL alone, six mice from peptide-embedded 12B1 CRCL and four mice from peptide-embedded liver CRCL) were again challenged with 5 x10^3 12B1 cells 90 days subsequent to the

initial tumor inoculation (s.c. in the groin, opposite the first challenge). *A*. Tumor growth was monitored, and individual tumor volumes and *B.* percent survival were determined over time (representative data from 1 of 2 experiments are shown). Naïve, age-matched mice (n=2) were injected with tumor in the same fashion as the treatment mice to ensure tumor take and viability.

Discussion

One of the major roles of intracellular heat shock proteins is that of chaperones for peptides and other proteins. As extracellular entities, chaperones are capable of potent innate immune modulation (69, 94, 102, 145). Chaperone proteins such as heat-shock protein 70, 90 (HSP70, 90), GRP94, and calreticulin, that have been purified from a tumor source, have been reported as effective vaccines, culminating in tumor-specific cytotoxic T-lymphocyte (CTL) responses and anti-tumor immunity in several in vivo models (51, 85, 87, 116). Yet, a frequent disadvantage to single HSP vaccines is the lack of total purified protein obtained from tumor sources to generate the vaccine (56, 135). We found that CRCL vaccine has a more pronounced immunologic effect per unit material of protein than any of the individual chaperone proteins used as a vaccine alone (85-87). Moreover, we have demonstrated that the antigenicity of CRCL can be augmented by loading it onto dendritic cells. CRCL has been shown to mature and activate DCs (85), thus enhancing antigen presentation and T-cell stimulation.

The basis for antigen-specific active immunotherapy is provided by the identification of tumor-specific and tumor associated antigens expressed by different human tumors (52, 72, 76, 117, 146, 147). The BCR-ABL protein is an excellent model to study tumor-specific antigens as it is found in the tumor cells of chronic myelogenous leukemia but not normal tissue. This fusion oncoprotein

contains several specific peptides including GFKQSSKAL (GFK), which has been previously shown to bind to major histocompatibility complex (MHC) class I and II molecules in mice and humans (1, 20, 148-153).

Due to the fact that heat shock proteins purified from tumor or other pathologic sources associate with antigenic peptides (154-156) and that CRCL vaccine is enriched for multiple HSPs, we sought to determine if CRCL has the ability to bind specific peptides of choice and enhance the overall effect as antigens in the vaccine; in essence, we wanted to create a “designer” CRCL. Such approaches have been difficult and sometimes inefficient when using purified chaperones in in vitro systems designed to bind antigens (peptides or proteins) (77, 146, 157-159). As proof of principle, SIINFEKL peptide was loaded into clarified liver lysate to determine if, and where peptide binding would occur during the FS-IEF process for the making of CRCL. Liver lysate was utilized since SIINFEKL peptide does not reside in normal tissue, and liver CRCL served to demonstrate that non-tumor sources could be useful as chaperone-based methods of antigen delivery. Following fractionation and harvest, each individual fraction was handled as we would normally treat pooled fractions for vaccine preparation. That is to say each fraction was dialyzed using 10kDa cut-off cassettes, concentrated using a 10kD cut-off membrane, and passed over a column used to remove detergents. Any of these steps should have resulted in the loss of peptide that was not tightly associated with the focused liver proteins within a

given fraction. Not only did the peptide localize to chaperone-containing fractions chosen to generate CRCL vaccine, but these fractions had the ability to donate the SIINFEKL peptide into the presentation pathways of antigen presenting cells (APCs) leading to specific T-cell stimulation (Figure 4). This was important because it strongly suggests that the chaperones somehow play a role in the trapping of the peptides during FS-IEF. It is also important to note that those chaperones could functionally deliver the peptide to DCs for presentation to T-cells. While this method of peptide incorporation into CRCL was essentially no more difficult than the normal preparation of CRCL vaccines, we later discovered that simply mixing peptide with pre-formed CRCL (i.e., CRCL which had already been processed into a vaccine form) resulting in surprisingly high levels of peptide incorporation, again in a functionally deliverable form for DC-based T-cell activation. From Figure 4 it can be seen that T-cell stimulation tracks with HSP/HSC70 content in the FS-IEF fractionation. Indeed, we have shown herein that one of the key components in CRCL which appears to bind to the exogenous peptide is heat shock cognate 70 protein (HSC 70) (Figure 7).

Our data presented here have shown that a significant amount of peptide of interest can be incorporated into tumor-derived or liver-derived CRCL as opposed to the nominal amount detected following incorporation into their corresponding lysates (Figures 5 and 6). Furthermore, we were able to determine that the considerably higher amount of FITC-peptide retained in both

liver and tumor-derived CRCL was effectively taken in by dendritic cells contrary to the minimal amount of FITC-peptide that was retained in both lysates (Figures 8 and 9). Moreover, this phenomenon was observed visually via confocal microscopy (Figure 10). Thus, even if the peptide of interest is added exogenously to CRCL, the peptide still traffics to DCs and is effectively presented by them. The stimulatory effect of CRCL over lysate or peptide alone likely contributes to this (85).

To solidify our earlier findings where peptide could be bound during the FS-IEF process and later elicit a T-cell response (Figure 4), pre-formed liver CRCL and liver lysate were incorporated with peptide and pulsed onto DCs along with peptide alone. After incubating the DCs with the B3Z cells (T-cell hybridoma line), a significant amount of T-cell stimulation was seen with the DCs treated with peptide-embedded CRCL compared to peptide-embedded lysate or peptide alone (Figure 11). These findings directly compared the peptide-carrying potential of both lysate and CRCL vaccine along with their ability to elicit a strong T-cell activation. All results in this study conclude that CRCL vaccine has an extraordinary peptide carrying capacity and, when embedded with specific peptide, leads to powerful specific T-cell stimulation (Figure 12 and 13). In contrast, no such enhancement was observed to the same degree with peptide-embedded lysate or peptide alone.

Importantly, we demonstrated through *in vivo* anti-tumor growth assays that peptide-embedded CRCL, when administered therapeutically, delayed tumor growth considerably when compared to CRCL alone or peptide alone (Figures 14-16). It is likely that the *in vitro* phenomenon of enhanced specific T-cell activation that we observed with the peptide-embedded vaccines plays a role in the tumor rejection observed *in vivo*.

Antigenic peptides or tumor lysate in combination with DCs are currently used as vaccine strategies (85, 160, 161). Although tumor-derived lysate has been determined to contain antigenic peptides, at the same time it can also contain inhibitors, such as TGF-β which may reduce the therapeutic outcome of the vaccine (162, 163). A further potential problem of tumor-derived lysate as a therapy may be the lack of tumor tissue required from the patient to make enough vaccine. The process of making CRCL requires little starting material and yields a larger quantity of vaccine when compared to individual HSP purifications. This line of thinking also brought about the concept of producing peptide-embedded CRCL derived from normal, non-tumor tissue, such as human placenta which is also rich in HSPs, in the event that minimal or no tumor tissue is available. Therefore, in this study, given the difficulty of obtaining mouse placental tissue, murine liver tissue was utilized along side 12B1 tumor-derived CRCL as a model for designing peptide-embedded CRCL. In a scenario where a vaccine is made from normal tissue, clearly one would have to add in antigens

artificially and exogenously, as we did with peptide incorporation into liver CRCL. Thus, a designer vaccine could be made from scratch, possibly with a cocktail of known antigenic peptides specific for the disease, either cancer or perhaps infectious disease. It is also conceivable to use peptides of unknown antigenicity, such as those acid-stripped from the cell surfaces of patient tumors (164, 165), thus recapitulating the personalized components of an autologous vaccine. Depending on the outcome of a clinical trial, this vaccine would be administered subcutaneously or initially pulsed onto DCs that would be harvested from the patient and then returned to the body. This process would more than likely be given more than once over an extended period of time. In addition, given the current state of the art in clinical trial immune monitoring, known antigenic peptides would offer a means of tracking and assessing the efficacy of the vaccine.

Studies are ongoing to determine the carrying capacity of CRCL vaccine incorporated with multiple peptides. Ideally, in a clinical setting this peptide-embedded vaccine would include a cocktail of human tumor peptides to strategically target various epitopes. Taken together, the results of this study lead us to postulate that the novel concept of creating a peptide-embedded CRCL using a wide range of known human peptides will offer the ability to administer high doses of various peptides that can be loaded onto MHC molecules on antigen presenting cells and furthermore personalize effective

vaccines to those afflicted with cancers containing known antigens, such as the BCR-ABL fusion peptide. These encouraging results with peptide-embedded CRCL may offer a practical and effective alternative for anti-cancer immunotherapy, and perhaps other vaccine formulations for infectious diseases.

IV. EFFECT ON CELLULAR STRESS AND IMMUNE ACTIVATION BY A NATURAL PRODUCT STEROIDAL LACTONE IN SEVERAL TUMOR TYPES

Introduction

Heat shock proteins (HSPs) are highly conserved proteins that are present in nearly all regions of the cell. The synthesis of HSPs is strongly induced in both, prokaryotic and eukaryotic cells following physical as well as chemical stress in order to protect the cells from lethal damage. Members of the HSP70 family are involved in folding of newly formed polypeptides, intracellular transport processes, and play important roles in the processing of antigens (48, 93, 94). Apart from their chaperoning functions (36, 166, 167), HSPs have been shown to elicit a specific, cellular anti-cancer immune response mediated by cytotoxic T-cells, APCs and natural killer cells (NK) (69, 78). The transport function of HSPs for tumor-specific antigenic peptides has been exploited in the majority of immunotherapeutic approaches (55, 70, 98). Tumor-derived Hsc70, Hsp70 and GRP94/96 have been shown to chaperone immunogenic peptides (70, 78) into the MHC class I antigen presention pathway (51, 98). The uptake and binding of HSP70- and HSP90-peptide complexes are thought to be mediated by several receptors, including CD91 (52, 72, 168), CD40 (101) and members of the Toll-like receptor family (TLR-2, TLR-4) (71, 169-171). More recently, human APCs have been shown to come in contact with HSPs through scavenger receptors LOX-1 and SRA- 1 (106, 127, 141) . The binding of HSPs to its receptor(s)

induces DC maturation (172-174) and facilitates the transfer of HSP-associated peptides from the extracellular compartment to MHC class I molecules for the recognition by antigen-specific $CD8^+$ T cells (52, 72, 175). HSP70 has the ability to stimulate the release of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α through a TLR-2/ TLR-4-associated pathway by APCs, even in the absence of tumor-derived peptides (71). A non-specific stimulation of the innate immune system is seen as a result of this "chaperokine" function of the HSP70 family members (176). Furthermore, peptide-free Hsp70 has been determined to elicit immunostimulatory effects upon NK cells (100, 177-179).

Membrane-bound Hsp70 has been selectively detected on the surface of tumors but not on corresponding normal tissues (95). One group showed that membrane-bound Hsp70 provides a tumor-specific recognition structure for NK cells (177, 180, 181). The sensitivity towards lysis mediated by NK cells correlated with the quantity of HSP70 bound to the plasma membrane (182, 183). Furthermore, the expression of tumor-derived membrane-bound HSP70 has been found to be selectively increased following several clinically employed treatments, including cytostatic drugs, γ-irradiation, insulin sensitizers and COX-1/COX-2 inhibitors (184-187).

Numerous studies suggest that heat shock proteins expressed on tumor cell surfaces have a strong influence on the priming, stimulation, and regulation of the immune response and contribute to the idea that the immune system might be extremely sensitive to conditions that enhance the surface expression of inducible HSPs.

Withaferin A (WA) is a small molecule natural product that is part of a larger group of compounds known as withanolides. Withanolides represent a group of steroidal lactones elaborated by a variety of solanaceous plants, especially *Withania somnifera* (also referred to as Ashwagandha). Use of extracts from this plant date back to over 3,000 years in Ayurvedic medicinal tradition, where they have been used as a tonic for several ailments, including cognitive and neurological disorders, inflammation, stress and insomnia. *Withania* extract has also been documented to possess anti-tumor (109-111), anti-oxidant (112) and immunomodulatory activities (113). Many of the medicinal properties attributed to *Withania somnifera* extracts can be linked to the activity of WA. Ayurveda is an intricate system of healing that originated in Sri Lanka and India thousands of years ago (Samhita, 1949). This form of treatment is mostly centered upon preparations derived from herbs and herbominterals (108). Specifically, immunologic studies have shown that *Withania* extract treatment has the ability to initiate CTL production in mice as well as increase the numbers of total white blood cells and bone marrow cells (113). *Withania somnifera* has also been

determined to augment cell mediated immune responses including natural killer (NK) activity (113). Moreover, the very few reports published on the immunomodulatory effects of WA reported a WA-dependent growth inhibition of Ehrlich Ascites Carcinoma in mice, and subsequently determined that WA rendered cured mice refractory to re-challenge with the same tumor due to induction of a marked anti-tumor immune response (111, 114).

Recently, WA has been characterized as an inducer of the cellular heat shock response, reaching extremely high levels (up to 25 fold above vehicle treated cells) at low micromolar concentrations (Figure 17, R. Falsey et. al. manuscript submitted). Furthermore, previous studies from our laboratory have indicated that induction of a stress response in tumor cells undergoing apoptosis dramatically enhances their immunogenicity (100).

In this study we showed that in several tumor types: renal carcinoma (RENCA), metastatic breast cancer (4T1, EMT6) and colon cancer (CT26), cells treated with WA resulted in a potent heat shock response and subsequent expression of inducible HSP70 on their cell surfaces. In addition, we determined that WA-treated tumor cells incubated with DCs caused an upregulation of co-stimulatory molecules on the DCs indicating that the surface HSPs may be playing a role in DC maturation. Lastly, administration of WA *in vivo* was found to enhance the proliferation of lymphocytes in response to the mitogen Con A.

A heat shock response serves as a biological adjuvant. It has the ability to activate APCs and tumor immunity through various mechanisms which appear to include both HSP-dependent and –independent effects (107). We believe that by inducing a significant cellular heat shock response within a tumor, the host immune system may be stimulated, resulting in an immune response against the otherwise unaffected tumor.

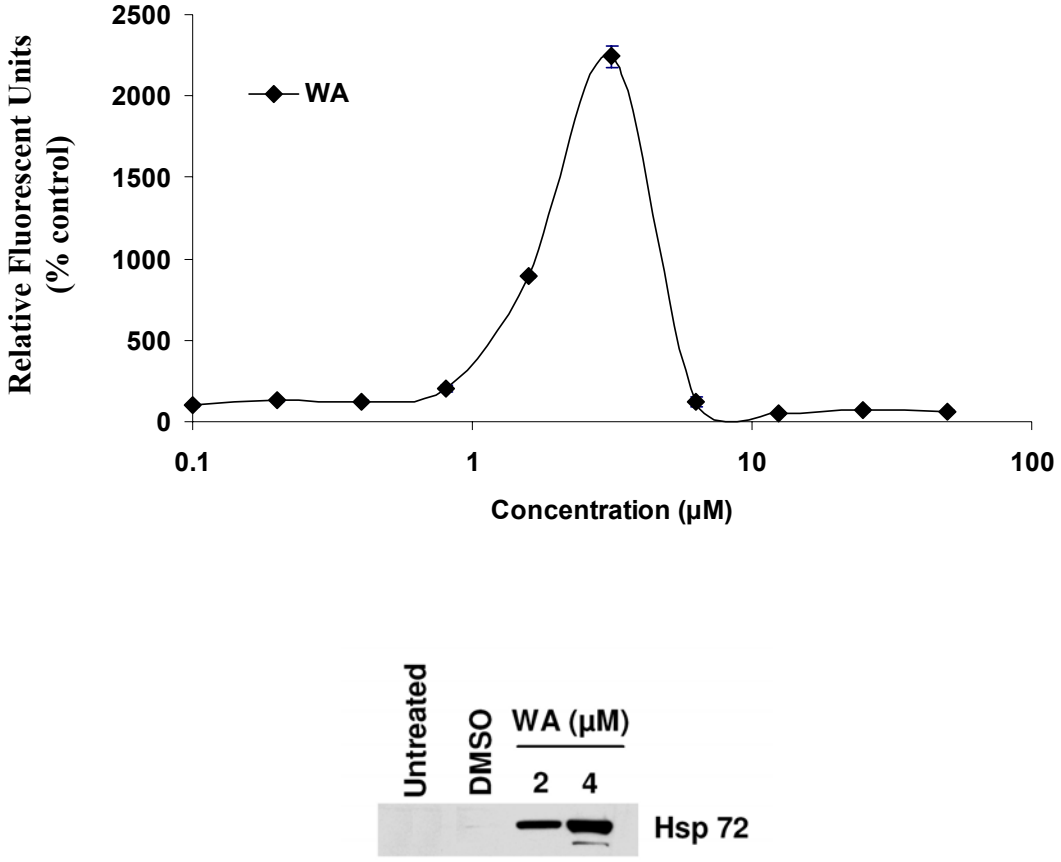

Figure 17. *Withaferin A Induces a Robust Heat Shock Response.*

Upper panel: Cell-based heat shock induction assay. 3T3-Y9-B12 reporter cells stably transfected with enhanced green fluorescent protein under the control of a minimal consensus heat shock response element were treated for 16 hours with increasing concentrations of WA. Fluorescent units were quantified using a microplate reader. *Points,* mean of triplicate determinations, expressed as percent of the solvent vehicle control (DMSO); *error bars*, s.d. Lower panel:

Immunoblot demonstrating increased expression of inducible HSP70 in 3T3-Y9-B12 reported cells after exposure to indicated concentrations of WA. Cells were treated for 16 hours, harvested and lysed in non-ionic detergent buffer and proteins were size fractioned by SDS-PAGE. Protein was subsequently transferred to a nitrocellulose membrane for Ponceau staining of total protein and blotted with anti-iHSP70 antibody and peroxidase-conjugated secondary antibody. Untreated and vehicle only (DMSO) were run as controls (Adapted from R. Falsey et. al. manuscript submitted).

Results

Inhibition of Tumor Cell and Dendritic Cell Viability by Withaferin A

We examined the extent to which cell survival in various tumor cell lines and primary culture dendritic cells were inhibited by WA treatment. The growth inhibition of 4T1 and EMT6 tumor cells (Figure 18) along with murine bone marrow-derived dendritic cells (Figure 19) by WA was measured by MTT assay. WA treatment for 24 hours continuous exposure resulted in a concentration-depended decline in viable cell numbers (Figures 18 and 19). 4T1 and EMT6 cell lines demonstrated IC_{50} values at approximately 2.5μM. Dendritic cells harvested from the bone marrow of BALB/c mice demonstrated an IC_{50} value of approximately 4μM WA.

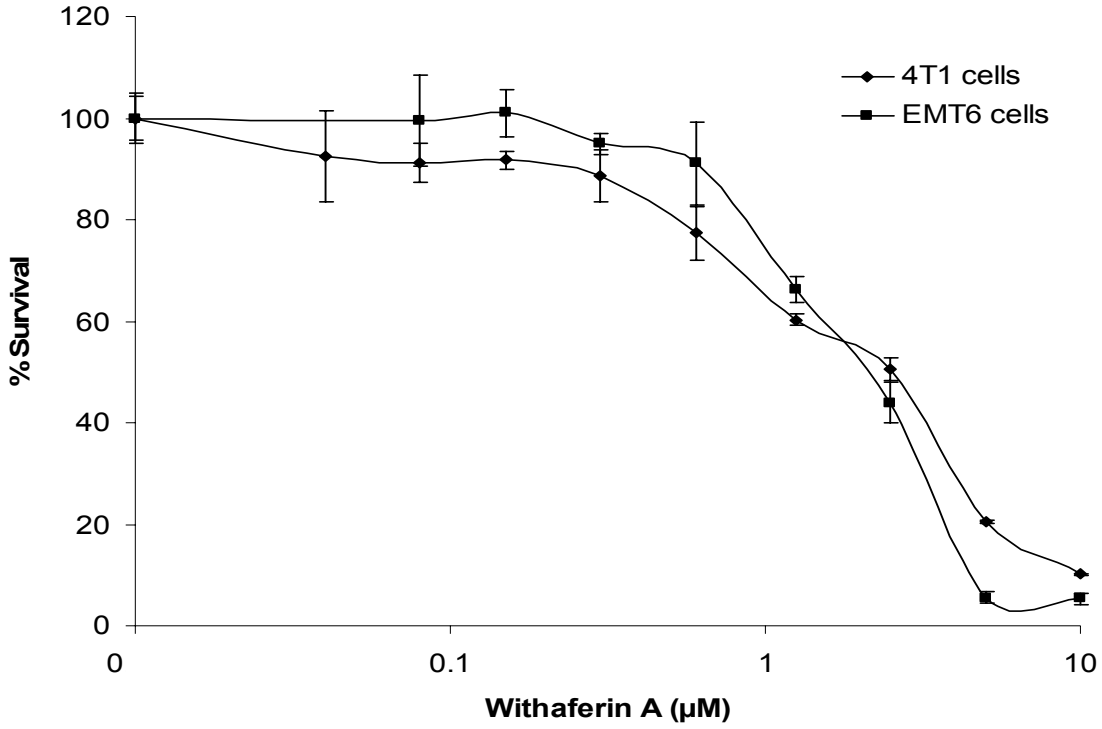

Figure 18. *Concentration-dependent Inhibition of Tumor Cell Growth in vitro by Withaferin A.*

4T1 (♦) and EMT6 (■) tumor cells in triplicate wells were treated with increasing concentrations of WA for 24 hours continuous drug exposure. Relative viable cell number was quantified by MTT assay and analyzed by a microplate reader at 560/690nm. Results are presented as a percentage compared to wells exposed to DMSO alone. *Points*: mean of triplicate determinations.

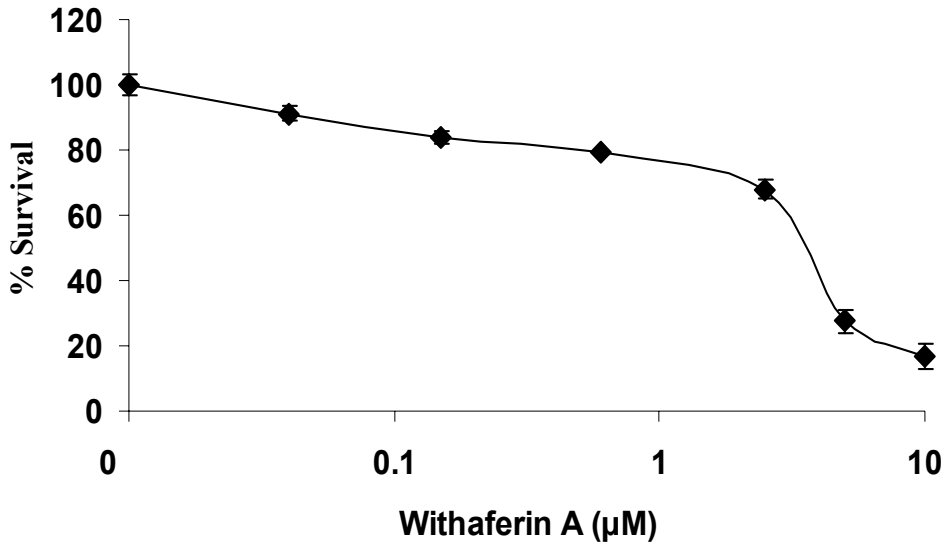

Figure 19. *Concentration-dependent Inhibition of Dendritic Cell Growth in vitro by Withaferin A.*

Bone marrow-derived dendritic cells (●) in triplicate wells were treated with increasing concentrations of WA for 24 hours continuous drug exposure. Relative viable cell number was quantified by MTT assay and analyzed by a microplate reader at 560/690nm. Results are presented as a percentage compared to wells exposed to DMSO alone. *Points*: mean of triplicate determinations.

Withaferin A Induces a Heat Shock Response that is Dose Dependent

WA was first identified as natural product of interest during a large scale screening program identifying compounds with the ability to induce a heat shock response at the transcriptional level (R. Falsey et. al. manuscript submitted). Given the ability of WA to induced a robust transcriptional heat shock response in this screening program, we examined the ability of WA to increase cellular levels of inducible HSP70 (iHSP70) in a series of cancer cell lines, including 4T1, RENCA, EMT6 and CT26 by Western Blot analysis. $5x10^5$ tumor cells were plated in 6 well plates. After 24 hours the medium was replaced with fresh medium containing 0μM, 0.6μM, 1.25μM or 2.5μM WA. Lysates were prepared from control or WA-treated cells and immunoblotted for iHSP70, which is typically expressed at very low levels under basal conditions. As evident in Figure 20, a marked increase in iHSP70 levels was observed when cells were exposed to low micromolar concentrations of WA for 24 hours. This data confirmed that at low micromolar concentrations, WA, induced a potent heat shock response in tumor cells.

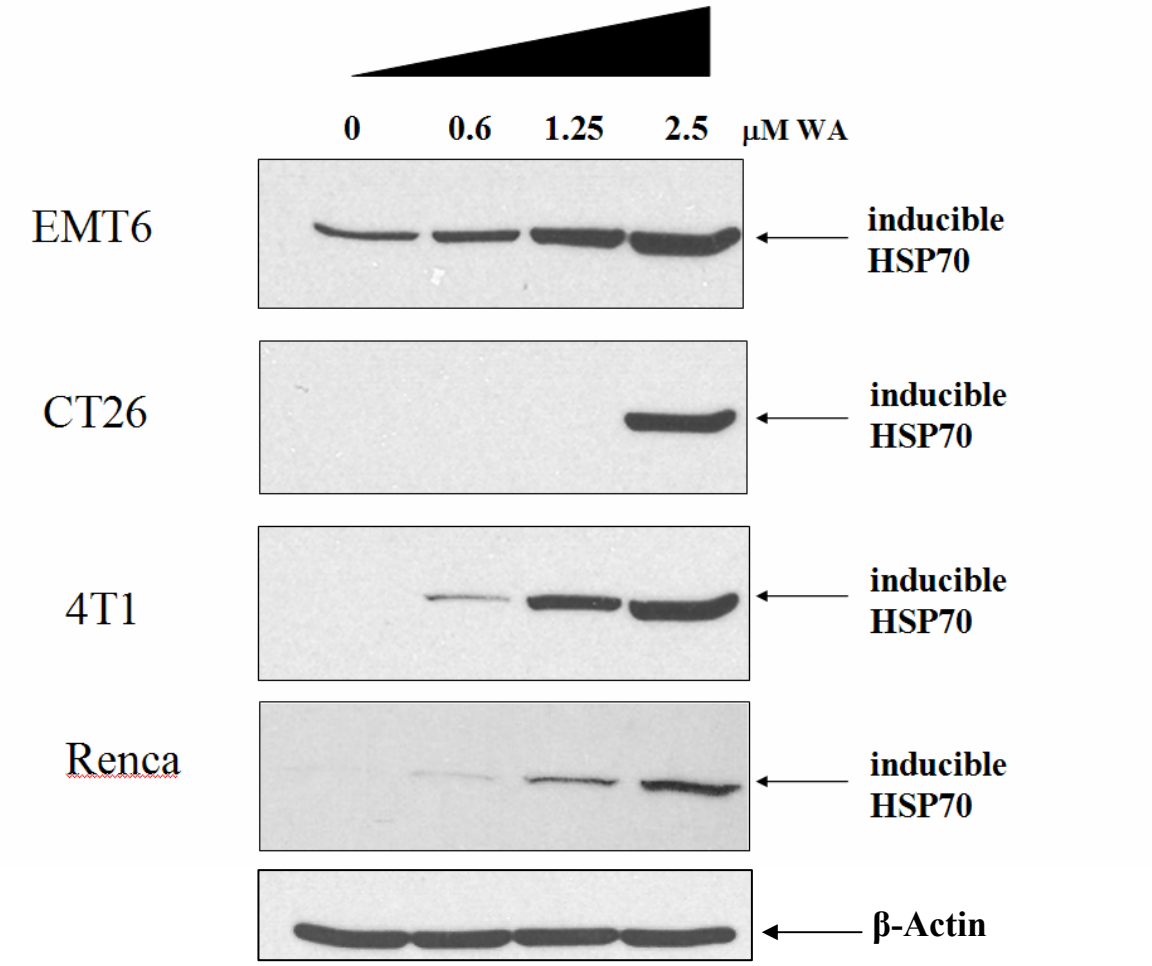

Figure 20. *Inducible HPS70 Expression in Tumor Cell Lines Following Withaferin A Treatment.*

All cells were treated with either 0μM, 0.6μM, 1.25μM or 2.5μM WA.for 24 h, harvested and lysed in non-ionic detergent buffer and proteins were size fractioned by SDS-PAGE. Protein was subsequently transferred to a nitrocellulose membrane for Ponceau staining of total protein and blotted with anti-iHSP70 antibody and peroxidase-conjugated secondary antibody. Untreated

and vehicle only (DMSO) were run as controls. iHSP70 can be seen at 70kDa where indicated. Beta Actin was run as a loading control. Results are representative of two experiments.

Surface Expression of Inducible HSP70 Following Withaferin A Treatment

Heat shock proteins are well-documented to be up-regulated by stress (93, 188) and have been suggested to provide “danger signals” that lead to the activation/maturation of dendritic cells (172, 189-192). To determine the extent to which iHSP70 is expressed on the cell surface after WA treatment, proteins were biotinylated using EZ Link Sulfo-NHS-LC-Biotin on the cell surface and affinity precipitated using Neutravidin-coated beads. Western blot analysis was used to determine the levels of iHSP70 on the cell surfaces of EMT6, CT26 (Figure 21), 4T1 and RENCA (Figure 22) cell lines. IGF-1R was probed for as a surface protein control and actin was probed for as a cytosolic control protein. This insured that the biotinylation was restricted to surface proteins and did not react with any cytoplasmic proteins. Supernatants were also collected as controls. Three conditions were tested for each cell line; a no biotin control where no reagents were added, cells alone with the addition of the biotin reagent, and cells treated with 2.5μM WA with the addition of the biotin reagent. The results indicated that all four cell lines expressed HSP70 on their cell surfaces after treatment with WA.

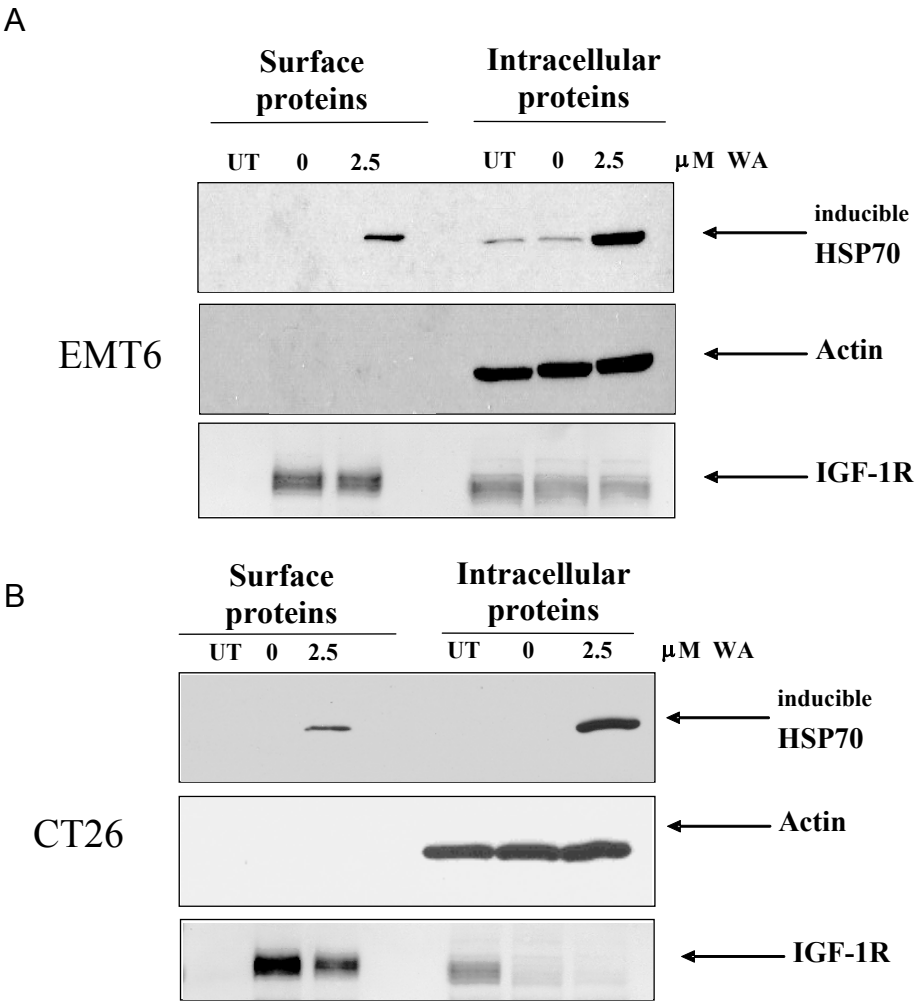

Figure 21. *Surface Expression of Inducible HSP70 after Withaferin A Treatment in EMT6 and CT26 Cell Lines*

EZ Link Sulfo-NHS-LC-Biotin was added to *A*. EMT6 and *B*. CT26 cells that had been previously treated with WA (2.5µM) or an equal volume of DMSO. Cells were lysed in non-ionic detergent buffer and biotinylated surface proteins were

captured on Neutra-Avidin beads. Bound proteins were eluted, size-fractioned and transferred to nitrocellulose for blotting with anti-iHSP70, anti-actin or anti-IGF-1R antibodies and peroxidase-conjugated secondary antibody. An aliquot of untreated cells without EZ Link Sulfo-NHS-LC-Biotin (untreated [UT] lane) and a sample eluted from beads that had been incubated with DMSO-treated whole cells (0μM lane) were run as controls. Data are representative of two independent experiments.

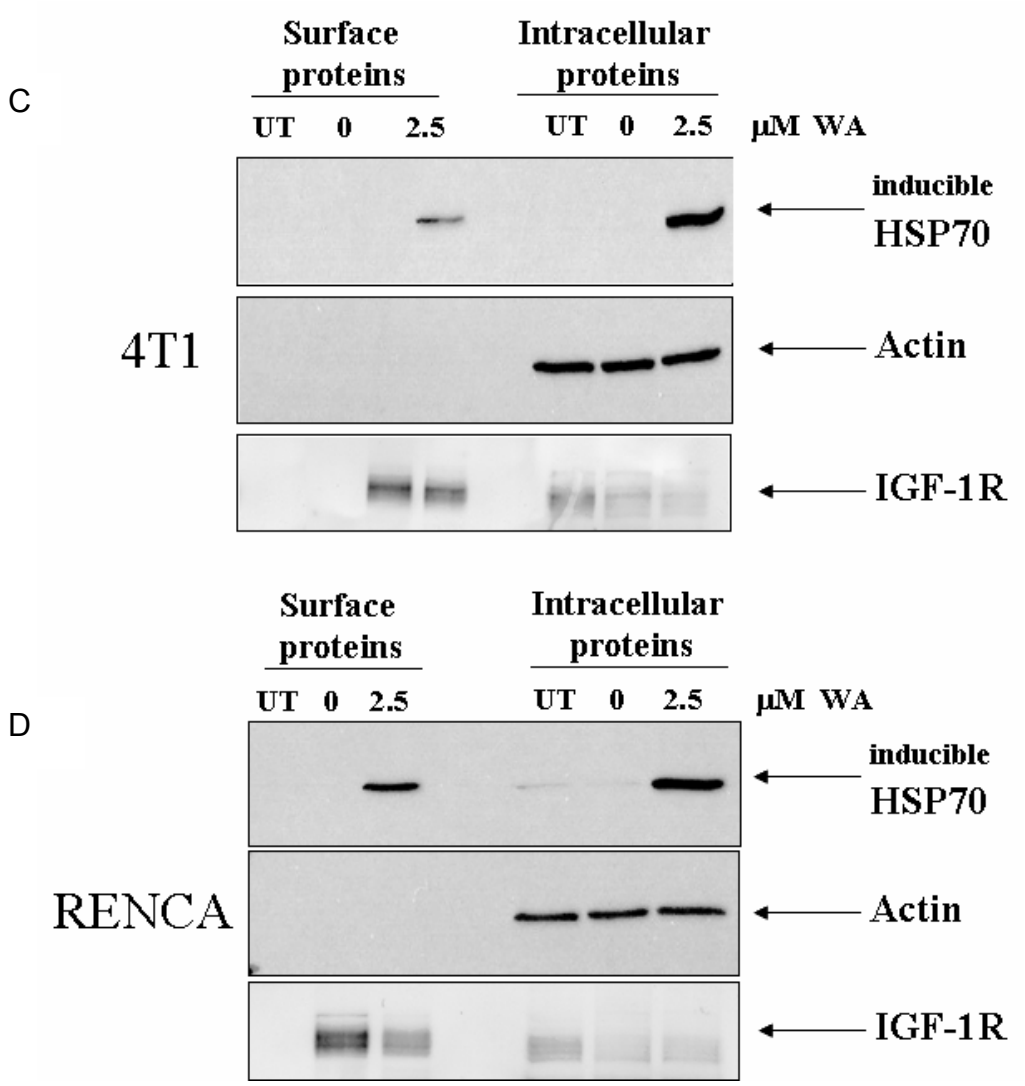

Figure 21 *Surface Expression of Inducible HSP70 after WA Treatment in 4T1 and RENCA Cell Lines*

EZ Link Sulfo-NHS-LC-Biotin was added to *C*. 4T1 and *D*. RENCA cells that had been previously treated with WA (2.5μM) or an equal volume of DMSO. Cells were lysed in non-ionic detergent buffer and biotinylated surface proteins were captured on Neutra-Avidin beads. Bound proteins were eluted, size-fractioned

and transferred to nitrocellulose for blotting with anti-iHSP70, anti-actin or anti-IGF-1R antibodies and peroxidase-conjugated secondary antibody. An aliquot of untreated cells without EZ Link Sulfo-NHS-LC-Biotin (untreated [UT] lane) and a sample eluted from beads that had been incubated with DMSO-treated whole cells (0μM lane) were run as controls. Data are representative of two independent experiments.

Effects of Withaferin A on Lymphocyte Proliferation

The effects of WA on mouse lymphocyte proliferation are indicated in Figure 22. DMSO or 2mg/kg WA was administered to BALB/c mice by IP injection for one day. Five days post-injection of WA or vehicle control, spleens were removed and splenocytes were isolated. The splenocytes were then incubated for 48 hours with increasing amounts of Con A (0, 0.1, 0.5, 1, 5µg/ml).
Cells were then labeled by adding 1µCi of ^{3}H Thymidine and further incubated for 18-20 hours. After the incubation, the cells were harvested and radioactivity levels were measured. Most notably, administration of WA *in vivo* was found to enhance the proliferation of lymphocytes in response to 5µg/ml Con A when compared to the DMSO control group. Further studies are needed to confirm the enhancement of lymphocyte proliferation specifically, rather than a possible depletion of other immune cells present in the spleen.

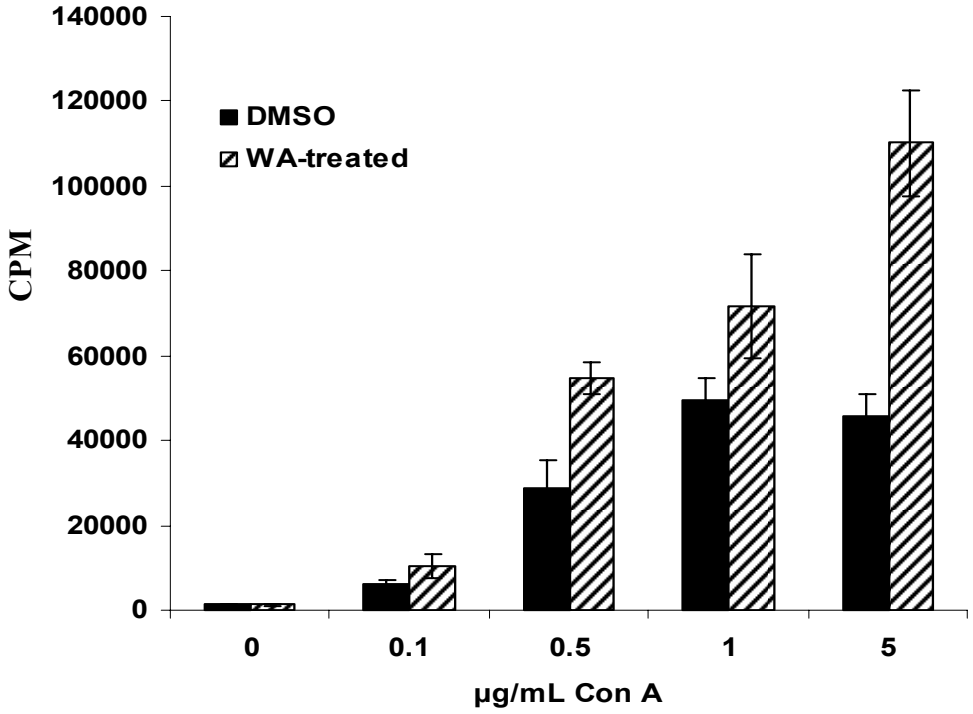

Figure 22. *Enhanced Lymphocyte Proliferation Following WA Treatment*

BALB/c mice were injected via i.p. administration with 2mg/kg WA for one day. Five days post injection, spleen cells were removed and stimulated with increasing amounts of Con A (0, 0.1, 0.5, 1, 5µg/ml) and subsequently incubated with ^{3}H Thymidine for an additional 18-20 hours. Black bars represent mice that were injected with DMSO. Striped bars represent mice that were injected with WA. ^{3}H Thymidine levels of the samples were analyzed by a liquid scintillation beta counter. Each condition was done in triplicate samples.

Withaferin A-Treated Tumor Cells Induce Maturation of DCs in vitro

Having shown that tumor cells treated with WA induce expression of iHSP70 on their cell surfaces, we hypothesized that WA-treated tumor cells could cause DC maturation. To examine this possibility, we incubated immature DCs with either CT26 or 4T1 tumor cells that have become non-adherent due to WA treatment for 24 hours. We then assessed the expression of maturation markers (CD40, CD80, CD86 and MHC II). The data (Figure 23) show that co-incubation of the CT26 or 4T1 tumor cells that have become non-adherent due to WA treatment with immature DCs caused up-regulation of the co-stimulatory molecules CD40, CD80 and MHC II on DCs as evidenced by the percent of cells that were gated for $CD11c^{+}$(FITC channel) along with the co-stimulatory molecules (PE channel). In contrast, direct incubation of immature DCs with WA for the same 24 hour period did not cause an increase in the expression of these markers above background (immature DCs alone or immature DCs incubated with DMSO). DCs were also treated with 0.1μg/ml LPS as a positive control along with DCs incubated with DMSO-treated tumor cells as negative controls.

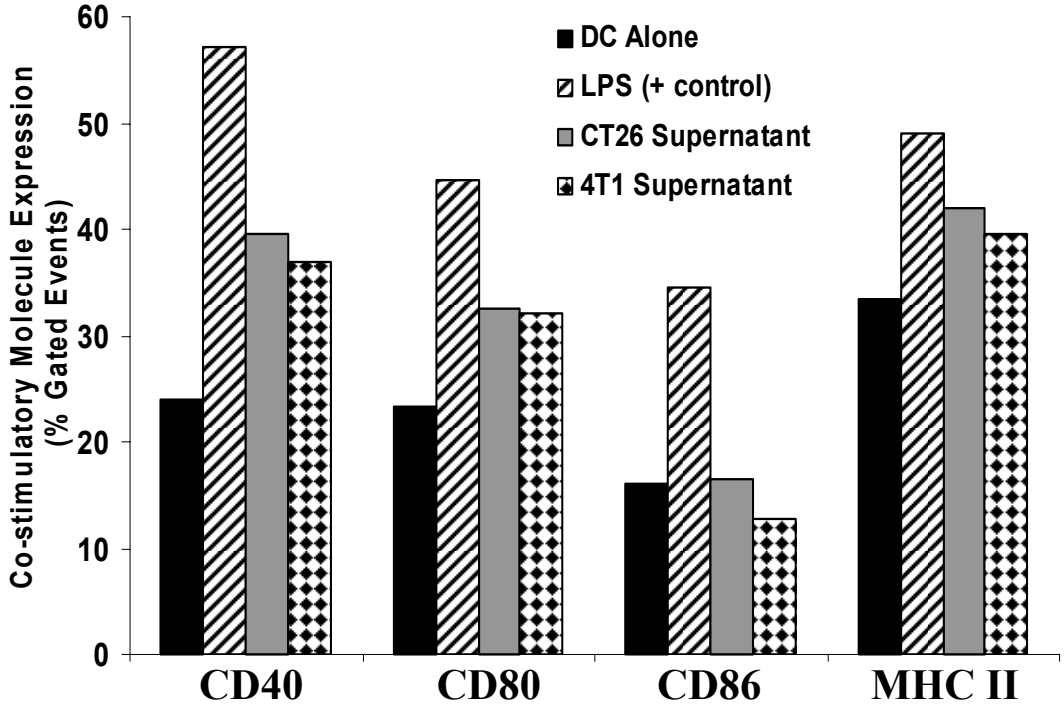

Figure 23. *Effect of WA-Treated Tumor Cells on Expression of Co-stimulatory Markers on DCs.*

CT26 and 4T1 tumor cells were treated with 2.5 μM WA for 24 hours. The supernatants were collected and centrifuged to collect non-adherent cells and membrane debris. The pellets obtained was re-suspended in media and incubated with immature bone marrow-derived DCs for 24 hours. DCs were collected and stained with FITC-conjugated CD11c antibody and PE-conjugated antibodies against CD40, CD80, CD86 and MHCII along with isotype controls and analyzed by flow cytometry. DC Alone (black bars) represents untreated DCs; LPS (+ control) represents DCs incubated with 0.1μg/ml LSP as a positive

control (striped bars); CT26 supernatant (grey bars) represents DCs incubated with non-adherent cells from WA-treated CT26 cells; 4T1 supernatant (checkered bars) represents DCs incubated with non-adherent cells from WA-treated 4T1 cells.

Discussion

Heat shock proteins are one of the most abundant intracellular molecules that function as chaperones. One of their critical responsibilities is to protect cells from the possibly lethal effects of stress (188). Heat shock proteins that are present in the extracellular milieu operate as “danger signals”. These HSPs signal antigen presenting cells, including DCs, of possible damage or infection which would lead to their activation (93, 188, 191, 193). Heat shock proteins, including iHSP70, have been shown to induce the maturation of DCs (172, 189, 192, 194, 195). The activation of DCs makes them effective antigen presenters which then travel to secondary lymphoid organs where they can initiate anti-tumor T-cell responses (136).

In a prior study, WA has been reported to inhibit the growth of Ehrlich Ascites Carcinoma in mice. This study also found that WA rendered cured mice refractory to re-challenge with the same tumor due to induction of a marked anti-tumor immune response (111, 114). Furthermore, a recent report determined that WA could induce a robust heat shock response *in vitro*, and mediate growth suppression of a human Ewing sarcoma cell line (CHP100) in SCID mice (R. Falsey et. al. manuscript submitted). In addition, previous work from our laboratory has shown that by inducing a heat shock response in tumor cells undergoing apoptosis, their immunogenicity is dramatically enhanced (100). In this study, we used renal carcinoma (RENCA), metastatic breast cancer (4T1,

EMT6) and colon cancer (CT26) cell lines to determine if WA-dependent heat shock induction of tumor cells may confer genuine anti-tumor immune activity. We observed that a low micromolar concentration of WA (2.5μM) could be used to induce a robust heat shock response in tumor cells without sacrificing the viability of normal bone marrow-derived dendritic cells. In addition, this WA-dependent heat shock response resulted in the expression of the highly inducible form of HSP70 (iHSP70) both intracellullarly and extracellularly.

Previous reports have determined that incubation of DCs with heat shocked apoptotic lysates, tumor cells or supernatants of necrotic transformed cell lines leads to the maturation of murine as well as human dendritic cells (172, 191, 193, 195, 196). In this study, WA was shown to induce a potent tumor cell heat shock response. Interestingly, the non-adherent heat shock cells generated by WA treatment had the ability to mediate DC maturation Furthermore, reports by Kuttan's group found that *Withania somnifera* root extract enhanced the proliferation of murine lymphocytes in the presence of a mitogen, such as Con A (113). In our hands, administration of WA *in vivo* was found to enhance the proliferation of lymphocytes in response to Con A when compared to DMSO vehicle control.

Overall, these results lead us to suggest that WA may contribute to several processes that prime the immune system against tumors including; enhanced

lymphocyte proliferation, the generation of activated/mature DCs by non-adherent WA-treated tumor cells and by direct killing of tumor cells whose antigens can be cross-presented to APCs. Additional studies are needed to confirm these mechanisms of immune activation.

Members of the HSP family are considered potent activators of the innate and adaptive immune response. Indeed, recent observations imply a crucial role for extracellularly localized and membrane-bound HSPs in inducing an efficient cellular immune response against cancer. Taken together, the present study suggests that HSPs may greatly influence immune activation and contribute to the idea that the immune system might be extremely sensitive to conditions that enhance expression of inducible HSPs.

VI. CONCLUDING STATEMENTS

Regardless of preliminary responses in advanced stages of cancer, relapse occurs often in patients due to drug resistance (e.g. Gleevec treatment for patients with CML) (10). Therefore, the exploration of other treatment options is needed. In the case of immunotherapy, many human solid tumors are antigenic but not immunogenic. This has been a major challenge in the development of cancer vaccines. Various methods have been employed to circumvent this, including the use of heat shock proteins that have been purified and administered individually, fused to other cells or HSPs complexed with other proteins and/or peptides. Many of these have been successful in animal models; however, very few clinical benefits have been determined (e.g. gp96 vaccine against melanoma).

In this report I have discussed the use of a novel technique focusing on exogenous tumor peptides chaperoned by multiple heat shock proteins in a complex. I also discussed the use of Withaferin A (WA), a natural compound possessing anti-tumor activity, as a potent activator of the heat shock response and modulator of immune function. Heat shock proteins (HSPs), including HSP70, 90 and 110, GRP94, and calreticulin, are considered natural adjuvants that show hope for cancer vaccination because they have the ability to bind antigens within the tumor cell and chaperone peptides to DCs in the lymph nodes (51, 116). Chaperone Rich Cell Lysate (CRCL) is a vaccine developed by our

laboratory which is composed of multiple chaperone proteins, including those mentioned above. Free solution-isoelectric focusing (FS-IEF) is used to enrich for these immunogenic HSPs and we have the capability of generating this vaccine from normal tissue or tumor sources. While CRCL is effective as a stand-alone therapy when pulsed onto DCs, an enhanced immunogenicity arising from exogenous peptides incorporated into CRCL, referred to as peptide-embedded CRCL in this report, indicates that CRCL could be the carrier of choice for antigenic peptides.

The first goal of these studies was to determine the carrying capacity of tumor-derived and normal tissue-derived CRCL for the BCR-ABL (GFK) peptide and to identify and validate the protein(s) in CRCL with which peptides bind. We synthesized a FITC-conjugated BCR-ABL (GFK) peptide that was incorporated into tumor (12B1) - or liver-derived CRCL. Fluorometeric analysis was used to measure the amount of peptide retained in CRCL after filtration of free, unbound peptide via a size-exclusion membrane. We found that CRCL vaccine had an increased peptide carrying capacity when compared to standard lysate. We have also used this same FITC-peptide to capture and identify the protein target(s) within CRCL utilizing mass spectrometry analysis. The database search results showed sequences matching that of murine heat shock cognate 70 (HSC70), a main component in the CRCL vaccine. In relation to immune activation, additional goals of this study were to determine the levels of

exogenous peptide taken up by dendritic cells and to determine CTL responses after treatment with peptide-embedded CRCL. We found that the considerably higher amount of FITC-peptide retained in both liver and tumor-derived CRCL was effectively taken in by dendritic cells and a significant amount of T-cell stimulation was seen with the DCs treated with peptide-embedded CRCL. Thus, even if the peptide of interest is added exogenously to CRCL, the peptide is still effectively taken up by DCs and is successfully presented by them. Given the *in vitro* data indicating that peptide-embedded CRCL could generate specific T-cell responses, we asked whether CRCL embedded with exogenous GFK peptide would induce anti-tumor activity against 12B1 tumors. The addition of GFK peptide to tumor- or liver-derived CRCL vaccine delayed tumor growth compared to groups of mice immunized with liver CRCL alone, GFK peptide alone, or saline, with a portion of the GFK/liver CRCL-treated mice rejecting their tumors.

Furthermore, in a wide variety of cancers, heat shock protein levels, including those of the HSP70 family, are elevated and may increase the immunogenicity of tumors (48). HSP70 can be seen on the plasma membrane when induced by heat or chemical stress in tumor cells but not in normal untransformed cells (95). In addition, circulating HSP70 have been shown to be released from dying cells or actively secreted from intact cells (83, 96-100). HSP70, released from an injured, dying or neoplastic cell, is often complexed with peptide and constitutes a ‘danger signal’ attracting and activating APCs (93, 94, 104). Our laboratory

has shown previously that the immunogenicity of tumor cells undergoing apoptosis can be greatly increased by inducing a heat shock response (100). The goal of this study was to determine if WA caused heat shock induction in several types of tumor cells that may lead to genuine anti-tumor immunity. First, we found that a low micromolar concentration of WA *in vitro* could be used to induce a strong heat shock response in tumor cells without greatly affecting the viability of normal bone marrow-derived dendritic cells. We were also able to determine that in the tumor cell lines tested, iHSP70 was expressed on the cell surfaces as well as intracellularly following treatment with WA. Furthermore, co-incubation of DCs with non-adherent cells generated by WA treatment lead to up-regulation of the co-stimulatory molecules CD40 and CD80 along with MHC II on the dendritic cell surface. This indicated that WA is indirectly capable of inducing DC activation via its biological affects on tumor cells. Moreover, administration of WA *in vivo* was found to augment lymphocyte proliferation in response to the mitogen Con A, when compared to DMSO vehicle control group.

Much effort is currently in progress to increase the efficacy of cancer therapy and decrease toxicity and the complexity of treatment administration. Immunotherapy as a treatment for cancer in humans is still in its early years of development, but current efforts appear to represent a base upon which significant gains in cancer treatment can be made. The findings presented in this report indicate that it may be possible to utilize novel therapeutic anti-cancer treatments that promote

immune stimulation through the use exogenous heat shock proteins and peptides or via induction of the heat shock response within the tumor itself. In particular, peptide-embedded CRCL derived from normal tissue, which has been shown here to be just as effective as tumor-derived CRCL, could be used to treat patients who would otherwise not have enough tumor tissue for a vaccine to be synthesized for them. In this report, we focused on one exogenous peptide to demonstrate the principle of the vaccine model, however, in the clinic this vaccine would most likely be administered subcutaneously with a cocktail of known antigens catered specifically for the patient (i.e. PR3, WT1 and GFK for CML). The hope is that such therapies can elicit potent anti-tumor immune responses that are both safe and effective for the patient.

APPENDIX: ANIMAL SUBJECTS APPROVAL

Institutional Animal Care and Use Committee

THE UNIVERSITY OF ARIZONA
TUCSON ARIZONA

P.O. Box 210101
Tucson, AZ 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#05-099 - "Chaperone Rich Cell Lysate (CRCL) Vaccine for Chronic Myelogeneous Leukemia"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Emmanuel Katsanis, MD - Pediatrics

GRANTING AGENCY:

NIH

SUBMISSION DATE: **June 23, 2005**

APPROVAL DATE: **July 28, 2005** APPROVAL VALID THROUGH*: **July 27, 2008**

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **August 3, 2005**

REVISIONS (if any):

- **Ibuprofen instead of acetaminophen will be used for palliative therapy**

MINORITY OPINIONS (if any):

Institutional Official: Leslie P. Tolbert, PhD
Vice President for Research

DATE: August 3, 2005

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

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