GENERATION OF TUMOR-SPECIFIC IMMUNITY USING HER2/NEU POSITIVE TUMOR DERIVED CHAPERONE-RICH CELL LYSATE (CRCL)

By

Gang Li

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Gang Li

entitled Generation of tumor specific immunity using HER2/neu positive tumor derived chaperone rich cell lysate (CRCL)

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

_____ Date: (March 05, 2007)
(Emmanuel Katsanis, M.D.)

_____ Date: (March 05, 2007)
(Emmanuel T. Akporiaye, Ph.D)

_____ Date: (March 05, 2007)
(Samuel F. Schluter, Ph.D)

_____ Date: (March 05, 2007)
(Lee D. Cranmer, M.D.)

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: (March 05, 2007)
Dissertation Director: (Emmanuel Katsanis, M.D.)

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SIGNED: __ Gang Li __

ACKNOWLEDGEMENTS

This work could not be done without the help and support from a number of people. I would first like to thank my mentor, Dr. Emmanuel Katsanis. It is his kind support, patience, and understanding that make my scientific journey possible.

I thank my advisory committee members: Dr. Emmanuel Akporiaye, Dr. Samuel F. Schluter, Dr. Lee D. Cranmer, Dr. Molly A. Brewer, Dr. Douglas Lake, and Dr. John J. Marchalonis, for their insight and advice during the past years.

I would like to acknowledge Dr. Michael W. Graner, Dr. Yi Zeng, Dr. Samita Andreansky, and Dr. Nicholas Larmonier for their inspiring discussion and kindly instruction for my study and research work.

I thank fellow students and all other members in Dr. Katsanis' Lab: Dr. Xinchun Chen, Dr. Kerri Kislin, Jessica Cantrell, Marjan Sepassi, Angela Romanoski, Sylvia Thompson, Xuemei Cai, and Marilyn Marron, for the technical assistances and advices.

Barb Carolus for her technical assistance in the FACS assays.

My thanks also go to my many friends who help me one way or the other during the hard times.

Last but not the least; I thank all the volunteers and patients involved in this study.

DEDICATION

I would like to dedicate this work to my husband, Youpeng Cai, and my parents, Yongrang Li and Jei Li. Their love and support is always the driving force and source of strength in my life.

I would also like to dedicate this work to cancer patients and their families, with the wish that the advances made in the field of cancer Immunotherapy will be able to help them.

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ABSTRACT

HER2/neu is an oncogenic tumor-associated antigen over-expressed in several human tumors including breast and ovarian cancer. The selective expression of HER2/neu and its role in epithelial carcinogenesis makes HER2/neu an ideal target for immunotherapy. Tumor-derived chaperone-rich cell lysate (CRCL), containing numerous heat shock proteins, has successfully been used to generate tumor-specific immunity against a wide range of murine tumors and is a great candidate for an effective vaccine against HER2/neu positive tumors. In the first part of this study, the potency of human ovarian cancer-derived CRCL to activate dendritic cells (DCs) and to generate tumor-specific T cells *in vitro* has been investigated. Chaperone-rich cell lysate was generated from primary ovarian cancer tissues and SKOV3-A2, a HER2/neu, Wilm's tumor gene 1 (WT1) and HLA-A2 positive human ovarian tumor cell line. T cells from healthy donors and from ovarian cancer patients secreted higher amounts of interferon-γ following *in vitro* re-stimulation with ovarian cancer-derived CRCL compared to HER2/neu or WT1 peptide-pulsed DCs. We were also able to generate cytotoxic T lymphocyte activity against cancer-specific antigens such as HER2/neu and WT1 from all healthy donors, but from only one of the four ovarian cancer patients with bulky disease. In the second

part of the study, the potency of tumor-derived CRCL to elicit the humoral immune response against a murine HER2/neu positive tumor (TUBO) has been examined. Vaccination of mice bearing a palpable tumor efficiently delayed the development of the tumor. In the vaccinated mice, CRCL vaccination induced significant anti-HER2/neu antibodies. Using B cell deficient mice and antibody transfer experiments, we have shown that the induction of anti-HER2/neu antibodies is both necessary and sufficient for the anti-tumor effect. Further, we have demonstrated that serum from TUBO CRCL-vaccinated mice stimulated the internalization of the HER2/neu molecules, resulting in the down-regulation of their surface expression. Moreover, antibody-dependent cellular cytotoxicity has been observed against TUBO cells when presented with sera from vaccinated mice. These results indicate that CRCL may be a potent adjuvant for women suffering from HER2/neu positive ovarian or breast cancer and that this personalized vaccine may be a promising approach for active immunotherapy.

BACKGROUND

1. HER2/NEU POSITIVE CANCER

HER2/neu oncoprotein is encoded by the HER2/neu oncogene and is a member of the HER2/neu family of receptor tyrosine kinases (1). This is an intrinsic property of the HER receptor family where the activation leads to the dimerization of the receptor and triggers a complex network of signaling events that promotes cell proliferation and cell survival. Amplification of the HER2/neu gene leads to an over-expression of the protein, resulting in increased cell division and a higher rate of cell growth (2). Although widely expressed in normal epithelial tissues, HER2/neu is over-expressed in various cancers such as breast cancer, ovarian cancer, melanoma, kidney, colon, prostrate and bladder cancers (3-6). Over-expression of HER2/neu provides both growth and survival advantages for the tumor and is consequently associated with a poor prognosis (7, 8).

Traditional cancer therapeutic methods include surgery, chemotherapy, radiation, and hormonal therapy. Despite these modulations the overall long-time survival rate

of the disease is not satisfactory. HER2/neu over-expressing cancers frequently recur or progress to metastasis. It has been shown that women with HER2/neu positive breast cancer have a more aggressive disease and a greater likelihood of recurrence with 3 to 4 years shorter median survival than HER2/neu negative cases (9). The overall 15-year survival rate for invasive breast cancers in USA is around 60% and for ovarian cancer it is less than 30% (American Cancer Society 2006). Problems associated with these conventional treatment strategies include the inherent tumor cell resistance, the lack of specificity and the toxicity along with associated morbidity and mortality. Therefore, more precisely targeted therapies are required. Immunotherapy represents a new and potentially powerful tool against HER2/neu expressing tumors.

Cancer and T cell / B cell responses

The anti-tumor immune response represents the integrated sum of interacting components of the innate and adaptive immune system. It is well known that mature dendritic cells (DCs) loaded with tumor antigen activate both $CD4^+$ and $CD8^+$ T cells in the context of proper T cell co-stimulation. Activated $CD4^+$ T cells provide help to maximize the humoral (antibody) response mediated by B cells and the magnitude and durability of the $CD8^+$ cytotoxic T lymphocyte (CTL) response. The immune

response is further integrated by the engagement of antigen specific immunoglobulin (Ig) with specific receptors on neutrophils, macrophages, and natural killer (NK) cells, resulting in an alternative path to antibody or complement dependent cell-mediated cytotoxicity.

Humoral immunity is carried out by B cells, which represent the effector cell type involved in the production of antibodies. Recognition of an antigen, and the subsequent activation and differentiation of B cells into antibody-secreting plasma cells and longer lasting memory B cells, involves antigen-presenting cells (APCs) such as macrophages/monocytes and DCs. B cells themselves can also function as APCs. A subset of T cells expressing the CD4 molecule, designated T helper cells, are also involved in the activation and presentation of antigen to the B cell.

There exist several means by which antibodies that target tumor antigens can destroy tumors (7, 10-12). (1) Antibodies with specificity for a tumor antigen can recognize the tumor and activate other effector components of the immune system. Thus, an antibody bound to its tumor antigen can be involved via its Fc portion of in a tumoricidal phenomenon, referred to as antibody-dependent cellular cytotoxicity (ADCC). Fc receptors, which are expressed on phagocytic cells such as

macrophages and monocytes, on neutrophils, or on NK cells, can recognize the antibody-tumor cell complex and subsequently induce the internalization and destruction of the tumor cells. (2) Complement may also bind to the Fc portion of the antibody bound to tumor cells and the activated complement can cause tumor cells lysis, called CDC. (3) Antibodies have also been demonstrated to induce apoptosis resulting in DNA fragmentation and cell death. (4) Antibodies can also bind to specific key tumor antigen on the cell surface, such as HER2/neu, and induce signaling pathways that remove the tumor antigen from the cell surface (10, 11). (5) In addition, some specific antibodies can inhibit the tumorigenesis or proliferation of tumor cells by blocking tumor antigen molecules involved in the transduction of proliferation signals such as HER2/neu (13).

Activated CTLs are considered to be a very important population of effector cells in tumor immunity, since they can efficiently lyse tumor cells. Numerous vaccine strategies have been developed that are aimed at triggering specific anti-tumor CTL induction (14-17).

$CD4^+$ T helper cell (Th) response also plays a central role in the establishment of anti-tumor immunity. $CD4^+$ T cells are capable of activating antigen-specific

effector cells and of recruiting cells of the innate immune system such as macrophages and mast cells (Tumor immunology and cancer vaccines, Khleif Samir). $CD4^+$ T lymphocytes can be subdivided into functionally distinct subsets on the basis of their cytokine secretion profile, which determines the outcome of immune responses toward different pathways such as cell-mediated cytotoxicity or the neutralization of antigens. Th1 cells characterized by secretion of interferon-γ (IFN-γ), interleukin-2 (IL-2), and lymphotoxin are primarily responsible for the development and persistence of cell-mediated effector responses, such as CTL cytotoxicity and ADCC. Th1 cells also promote production of antibodies by B cells that can enhance the uptake of tumor cells (type I antibody such as IgG2a, IgG3). In addition, Th1 cells can directly kill tumor cells via release of cytokines such as TNF-α that bind to death receptors on the tumor cell surface (18). Th2 cytokines such as IL-4, IL-5 and IL-10 play an important role in the regulation of the Th2 immune response (18). Th2 cells are mainly helper cells that influence B cell development and augment the secretion of type II antibodies, such as neutralizing IgE, IgA, and IgG1. Both types of $CD4^+$ cells also influence each other by the cytokines they secrete. Interferon-γ has been shown also to inhibit the proliferation of mouse Th2 cells but not that of Th1 helper T lymphocyte clones, while Th2 cytokines such as IL-10 can suppress Th1 functions. It thus appears that

these functional subsets are mutually antagonistic so that the decision of which subset predominates may determine its outcome.

Current immunotherapy for HER2/neu cancer

HER2/neu protein is present in a single copy in normal epithelial cells and is amplified by gene amplification in the epithelial cancers (1). The differential levels of HER2/neu expression in normal verses tumor cells, together with the involvement of HER2/neu in tumor progression make such cancers ideal targets for immunotherapy. The existence of natural HER2/neu immunity in patients suggests that the generated antibodies and T cells in response to HER2/neu over-expression do not recognize its expression on normal epithelial cells (19). Reports from animal experimental models and clinical trials confirm that HER2/neu is immunogenic and antibody and peptide specific CTL and helper T cells can be generated with active immunization strategies (19-21).

Various immunological modulations have been tested in different murine HER2/neu positive tumor models (22-25). Vaccination with HER2/neu peptides (25-27) or protein (28) is a common method against cancer. Both T cell and B cell related epitopes have been investigated for their capacity to elicit HER2/neu specific

immunity. In these studies, the subsequent development of mammary tumors was significantly lower in vaccinated mice than in control mice, and vaccine treatment was associated with a significant increase in their survival (25-27). Some other groups have utilized also DNA vaccines encoding either the whole or partial HER2/neu protein. The degree of protection provided by these vaccines varied from a delay in tumor growth to complete protection from tumor onset (29-32). Tumor prevention was reported to be mediated by anti–HER2/neu antibodies, $CD4^+$ T cell and T cell-derived cytokines (29, 33). In another study, the authors combined the heat shock protein (HSP) with the intracellular domain (ICD) of human HER2/neu (34). The HSP110-ICD complex was capable of breaking tolerance against the rat HER2/neu protein and inhibiting spontaneous mammary tumor development. This vaccine induced ICD-specific IFN-γ and IL-4 production. $CD8^+$ T cells are involved in protection against challenge with mouse mammary tumors, whereas $CD4^+$ T cells provide partial protection. Moreover, it has been demonstrated by another group that passive infusion of HER2/neu-specific monoclonal antibodies (mAbs) combined with whole cell vaccination significantly improves tumor-free survival (35). *In vivo* lymphocyte subpopulation depletion experiments demonstrate that the efficacy of antibody, alone or combined with vaccine, is dependent on both $CD4^+$ and $CD8^+$ T cells (35). Collectively, all these observations above suggest that similarly increased

efficacy could be obtained by HER2/neu specific immunological strategies. These studies in the murine models provide a logical base for vaccination in HER2/neu positive cancer as a means to block aggressive carcinogenesis.

More importantly, several clinical trials investigated the HER2/neu related immunotherapies in the humans as well. First of all, anti-HER2/neu monoclonal antibody trastuzumab (Herceptin) has already been approved for treatment of patients (5, 36, 37). Herceptin binds to the outer segment of the HER2/neu protein (5) and selectively inhibits the growth of HER2/neu over-expressing cancer cells. Several mechanisms have been proposed to account for the therapeutic effect of Herceptin, including the blockade of signaling pathways, down-modulation of the HER2/neu receptor (38), activation of tumor cell apoptotic signals (39), and the induction of ADCC (38, 40). Multiple synergistic mechanisms that can directly target other pathways in the immune system have also been identified with the administration of Herceptin. For example, Herceptin can recruit innate immune effectors for the development of ADCC (41). It can also increase susceptibility of tumor cells to lysis by HER2/neu-specific CTLs by enhancing major histocompatibility complex (MHC) class I-restricted antigen presentation (42, 43). Clinical trials indicate that there is a clear therapeutic benefit when Herceptin is

given before (44), after (37) or during chemotherapy treatments (5) in women with HER2/neu over-expressing metastatic breast cancer. Combination of Herceptin with other chemotherapeutic agents before surgery was also effective for early stage breast cancer (45-49). However, the optimal duration of treatment is not known and patients who achieved an initial response to Herceptin-based regimens generally acquired resistance within one year (5, 50). Additionally, the increased risk of cardiocytoxicity when used in conjunction with chemotherapy such as anthracyclines remains a major threat for patients (51, 52).

HER2/neu has also been targeted with either DCs (53) or peptide based vaccines (54-56). The majority of clinical trials have focused on peptide-based immunizations (57, 58). Patients with Stage III or Stage IV HER2/neu over-expressing breast, ovarian, or non-small cell lung cancer were vaccinated with candidate peptide epitopes capable of eliciting either MHC Class II-restricted $CD4^+$ T, MHC Class I-restricted $CD8^+$ T cell responses, or both (59-61). Some of these trials have also evaluated peptide vaccines in conjunction with either Granulocyte/Macrophage colony stimulating factors (GM-CSF) or Flt-3 as adjuvant (55, 62). HER2/neu-specific responses were also demonstrated in patients with various vaccination strategies such as protein-based vaccines composed of the

HER2/neu ICD and extracellular domains (ECD) (63), DCs pulsed with HER2/neu class I epitope, HER2/neu protein along with adjuvant such as GM-CSF (53, 64), with whole tumor cells genetically modified to express co-stimulatory molecules or immune-activating cytokines (65). Proliferative responses were detected in most of the studies, but no cytotoxicity or significant clinical responses to treatment were reported. A growing body of data indicates that antibody, T helper and CTL immunity concur in HER2/neu immunotherapy. These trials suggest that HER2/neu vaccines are safe and can exert anti-tumor effect by eliciting host specific immunity, but more efficient strategies need to be developed and merit further investigations.

2. TUMOR DERIVED CRCL ELICITS ANTI-TUMOR IMMUNITY

As mentioned above, immunological strategies offer multiple advantages for cancer therapy. However there are still several difficulties hindering this approach. One challenge of cancer vaccination has been to find better tumor antigens. Additionally, due to the antigenic variability that arises from the genetic instability, tumor cells can escape the immune attack induced by certain tumor antigens. A single tumor antigen or a single epitope vaccine may lead to tumor escape variants and tolerance to therapy due to the immune pressure (66, 67).

To achieve a better vaccine outcome, a polyvalent vaccine comprised of multiple immunogenic tumor antigens or epitopes should be a more efficacious approach. However the identification of immunogenic antigens of individual cancers from patients is not feasible. A promising approach that consists of the use of tumor derived HSPs may help to circumvent this limitation. The observation that HSPs chaperone antigenic peptides of the cells from which they are derived makes them an attractive and personalized approach against cancer, which allows for vaccination of the host against a large repertoire of individual tumor epitopes.

Immunogenicity of Tumor Derived HSPs

Heat shock proteins are highly conserved proteins and their expression is up-regulated during stress such as heat, UVB radiation and infection. One of the major roles of HSPs is to chaperone peptides and proteins. Tumor derived HSPs bind an array of peptides including immunogenic epitopes and can be applied as a polyvalent tumor vaccine without identifying specific tumor antigens (68). This would enable the vaccination of the host against a large set of individual tumor antigens (69, 70). HSPs can virtually bind all peptides present in tumor cells regardless of the MHC type of the cells from which they are isolated(71). The basis of the immunological specificity of HSP therefore lies not in chaperone itself but in the array of peptides chaperoned by these molecules.

In addition it has also been shown that HSPs enhanced tumor immunogenicity by increasing the abilities of APCs to process and present antigens to both $CD4^+$ and $CD8^+$ T cells (72). Chaperone proteins, regardless of their tissue source, activate APCs as part of an innate immune response, where the chaperones act essentially as pro-inflammatory cytokines (73, 74).

Srivastava *et al.* have shown that the vaccination with tumor derived HSPs protect

mice against the tumor from which the HSP were purified. This phenomenon has been demonstrated with several individual HSPs such as gp96, HSP70, and HSP90. Moreover, mice vaccinated with HSPs in a therapeutic setting often showed delayed progression of primary cancers and reduced metastatic load (75, 76). Currently, the HSP vaccine application has already entered the phase II clinical trial (76, 77).

Immunogenicity of Tumor Derived CRCL

Instead of individual HSP, we have utilized a free-solution isoelectric focusing technique (FS-IEF) to obtain fractions rich in multiple chaperone proteins from clarified tumor lysates for CRCL generation. It has been well demonstrated that at least four of the known immunogenic HSPs: HSP70, HSP90, GRP94/gp96 and Calreticulin are present in CRCL (78).

It has been demonstrated via immunological and non-immunological methods that there are numerous peptides confined within CRCL, and that some of those peptides are antigenic (79). The presence and immunogenicity of tumor-derived peptides peptide undoubtedly contribute to CRCL vaccine efficacy (79).

As a complex of multiple HSPs, CRCL is more effective than individual HSP in activating DCs and in stimulating tumor-specific CTLs (80). Dendritic cells

incubated with tumor derived CRCL have higher expression of CD40 and MHC-II on their cell surface, produce more IL-12, and have superior immunostimulatory capacity in a mixed leukocyte reaction when compared with DCs exposed to unfractionated tumor lysate or purified HSP70 (80). Furthermore, tumor derived CRCL is capable of providing protective immunity in mice and has been shown to be an effective anti-tumor vaccine as demonstrated in a variety of murine models (78, 80-85).

CRCL and T cell / B cell responses

In theory, as a polyvalent vaccine, CRCL/HSP can chaperone not only T cell epitopes but also B cell epitopes. Consequently, both cellular and humoral immunity should be elicited by CRCL/HSP vaccine. The role of T cells in CRCL/HSP vaccination has been well documented. It has been shown that the generated CTLs mediate the regression of tumor in animal models, and human CTLs generated *ex vivo* can recognize and respond to the tumor antigen expressed by the tumor of origin (86, 87). More importantly, the depletion of either $CD4^+$ or $CD8^+$ T cells significantly abrogated the efficacy of tumor derived CRCL/HSP vaccination, indicating that both $CD4^+$ and $CD8^+$ T lymphocytes are required for tumor cell rejection (88, 89). Vaccination with tumor-derived HSP96 induces activated $CD8^+$ T cells both in animal

models and in clinical trials. Recombinant fusion protein of HSP70L1 and carcino-embryonic antigen (CEA) fragment vaccination has generated CEA-specific CTLs either from patients or from splenocytes of vaccinated corresponding HLA/K^b transgenic mice, and adoptive transfer of splenocytes from the fusion protein vaccinated mice has inhibited tumor growth and prolonged survival in *nude* mice bearing CEA^+ human colon carcinoma markedly (90). Altogether, these data provide evidence that CRCL/HSP vaccine can present antigenic peptides to T cells and activate them both *in vitro* and *in vivo*. However, our T cell results concerning the tumor-derived CRCL vaccines have only been demonstrated in murine tumor models. Therefore the immunological T cell responses to CRCL vaccine need to be further defined in the humans.

On the other hand, the humoral response induced by HSP vaccines has not been adequately addressed, and the effects of CRCL on humoral responses have not previously been examined. In a limited number of studies with HSP vaccine, the detection of antibodies has been reported. For example, strong MAGE-3-specific IgG antibody secretion has been measured after vaccination of the fusion protein of M. *tuberculosis* HSP70 and MAGE-3 (91). Increased HER2/neu-specific IgG antibody has been induced *in vivo* by a DNA vaccine comprised of human HSP70 and

the extra-cellular domain of rat HER2/neu (92). Manjili *et al*. demonstrated higher IgG2a antibody titer in the sera of animals after vaccination with HSP110-HER2/neu complex. Despite the detection of antibodies to tumor antigen after HSP vaccinations (34, 91-93), it remains uncertain whether it is the humoral response that contributes to the anti-tumor effect of the HSP vaccine, or if the antibody secretion is simply an indicator of a Th1 response. Although high antibody titers were observed, $CD8^+$ T cell depletion completely abrogated the efficacy of anti-tumor immunity induced by the vaccine, which strongly suggests that antibody may only be a consequence of vaccination (92). Furthermore, if antibody responses do give rise to the anti-tumor effect, what are the mechanisms by which the antibody targets the tumor? These considerations prompted us to investigate whether the humoral response can be elicited by our CRCL vaccine.

CHAPTER 1

HUMAN OVARIAN TUMOR-DERIVED CRCL ELICITS T CELL RESPONSES *IN VITRO*

1.1 ABSTRACT

Tumor-derived CRCL, which is comprised of numerous HSPs, has successfully been used to generate tumor-specific T cell responses and protective immunity against a wide range of murine tumors. In this study, we have investigated the potency of human ovarian tumor-derived CRCL to activate DCs and to generate tumor-specific T cells *in vitro*. Chaperone-rich cell lysate was generated from primary ovarian cancers and SKOV3-A2, a HER2/neu, WT1 and HLA-A2 positive human ovarian tumor cell line. Peripheral blood mononuclear cells from HLA-$A2^+$ healthy donors and HLA-$A2^+$ ovarian cancer patients were stimulated weekly with autologous DCs loaded with ovarian tumor-derived CRCL. After four to six stimulations *in vitro*, specific cytokine secretion and cytotoxicity were measured. Chaperone-rich cell lysate promoted IL-12 secretion and enhanced the immuno-stimulatory capacity of DCs. T cells from healthy donors and from ovarian cancer patients secreted higher amounts of IFN-γ following *in vitro* re-stimulation with ovarian tumor-derived CRCL than with HER2/neu or WT1 peptide-pulsed DCs. We were also able to generate CTL activity against cancer-specific antigens such as HER2/neu and WT1 from all healthy donors, but from only one of the four ovarian cancer patients with bulky disease. These preliminary results further substantiate the concept that CRCL may prove to be a

potent adjuvant for women suffering from ovarian cancer and that this personalized vaccine may be a promising approach for active immunotherapy.

1.2 INTRODUCTION

Although ovarian cancer accounts for only 3% of all new cancer cases, it is the fifth leading cause of cancer-related death in women and the leading cause of mortality from gynecologic malignancies with approximately 15,000 deaths annually (94). Despite dose escalation of cancer chemotherapy and increasingly radical surgery, the overall survival has not significantly changed. Thus, if successful adjuvant treatment could be developed for ovarian cancer that would prevent the extremely high rate of recurrence, the survival from ovarian cancer could be improved. Cancer vaccines have minimal toxicity, can target the patient's immune response to tumors and may circumvent the intrinsic drug resistance that underlies present chemotherapeutic measures.

Ovarian cancer has been described as multiple diseases (95) and, in fact, more than 40 histological types contribute to the World Health Organization's classification of epithelial tumor categories (96). Thus, treating all ovarian cancers as if they were the same disease without tailoring treatment to each individual tumor has a high likelihood of failure. To develop effective immunotherapeutic strategies, the different immunogenic tumor antigens would need to be identified. Tumor-derived HSP vaccine is an individualized approach that circumvents the intricate hurdle of

identifying immunogenic antigens of individual ovarian cancers. The observation that HSPs chaperone antigenic peptides of the cells from which they are derived makes them an attractive and novel personalized approach against cancer, which allows for vaccination of the host against a large repertoire of individual tumor antigens (77, 97-99). In fact, tumor-derived HSPs (e.g., GRP94/gp96, HSP90, HSP70, calreticulin HSP110, and GRP170) have been shown to be effective immunogens in both animal models and in clinical trials (34, 77, 81, 86, 97, 98, 100-106).

Instead of utilizing individual HSP, our laboratory has focused its efforts on studying the potential benefits of tumor-derived HSP vaccines containing multiple HSPs. We have employed a FS-IEF that enriches for immunogenic chaperones from clarified tumor lysates to obtain CRCL (81, 107). Given the limitations of preparing tumor vaccines from an autologous tumor, CRCL offers an important advantage of high yield. With the same quantity of starting material we are able to obtain up to ten to twenty times as much CRCL-derived proteins as with conventionally purified individual HSP(107, 108). This can be achieved in less time and with less labor (82). Moreover, we have demonstrated that CRCL is a more effective adjuvant than purified HSPs (80, 85), and we were able to generate tumor-specific responses in

multiple murine models (80, 82). Thus, its rapid preparation, high-yield and potent anti-tumor activity makes CRCL a desirable immunogenic material for clinical use.

We had not previously studied the efficacy of CRCL in the humans. Preclinical studies particularly using human peripheral blood mononuclear cells (PBMCs) are needed to explore the feasibility of CRCL for immunotherapy in human cancers. This is necessary for advancing CRCL based vaccines in Phase I clinical trials. Therefore, the aim of this study was to investigate whether ovarian tumor-derived CRCL can be used to activate human T cells and elicit a cell-mediated immune response. Specific CTLs were generated from all HLA-$A2^+$ healthy individuals, but only from one ovarian cancer patient following stimulation with autologous CRCL-pulsed DC. These HLA-$A2^+$ specific CTLs were able to lyse original ovarian tumor cells but not irrelevant tumor cells in a MHC class I-restricted manner. Additionally, we were able to generate tumor-specific CTLs against HER2/neu and WT1 antigen, indicating that CRCL can chaperone multiple peptides. This observation correlated with the higher immuno-stimulatory capacity of CRCL-pulsed DC and secretion of IFN-γ by the tumor-specific CTLs.

1.3 MATERIALS AND METHODS

Cell lines

Ovarian cancer cells SKOV3 (HLA-A2$^-$) and SKOV3-A2 (HLA-A2$^+$) (109) were kindly provided by Dr. Mary L. Disis (University of Washington). SKOV3 cells were cultured in complete RPMI 1640 medium (Cellgro, Herdon, VA) supplemented with 10% heat-inactivated FBS (Omega Scientific, Tarzana, CA), 1,000 U/ml penicillin-streptomycin, and 2-mM of L-glutamine. SKOV3-A2 was cultured in complete RPMI 1640 in the presence of G-418 selection medium (500 µg/ml G-418, (Invitrogen, Rockville, MD). T2 cells (CRL-1992; American Type Culture Collection, Manassas, VA) were used as an APC cell line for ^{51}Cr release assays. T2 cells, K562 (NK-sensitive chronic myelogenous leukemia cell line), K562-A2 (HLA-A2$^+$) cells kindly provided by Dr. Lonnnie P. Lybarger (University of Arizona) were cultured in complete RPMI 1640 medium.

Generation of ovarian tumor-derived CRCL

Human ovarian tumor tissues were collected from chemotherapy naïve patients undergoing cytoreductive surgery for metastatic ovarian cancer. All patients signed an IRB approved consent form prior to surgery for procurement of tumor

samples for research purposes. SKOV3-A2 cells were also harvested as another source of CRCL. The FS-IEF enrichment of CRCL was performed as described (81, 82). Briefly, tumors were homogenized in lysis buffer and a 100,000*g* supernatant was obtained and quantified by BCA protein assay (Pierce Endogen, Rockford, IL). The high-speed supernatant was subjected to FS-IEF in a Bio-Rad Rotofor cell (Hercules, CA) for 5 hours at 15 W constant power. Twenty fractions were harvested, and each fraction was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot probing with specific antibodies for the chaperone proteins HSP70, HSP90, GRP94/gp96, and calreticulin. Fractions selected to be pooled for vaccines were those that contained all four of the above HSPs. Pooled fractions were then concentrated using Centricon devices (Millipore, Bedford, MA). Detergents were removed by passage over an Extractigel matrix (Pierce Endogen). The fractions were reconstituted in phosphate-buffered saline (PBS), quantified and stored at -70°C until use. The endotoxin level of the CRCL was lower than 0.01 endotoxic units (EU)/μg of CRCL as examined by Limulus amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD).

Generation of DCs

Leukopheresis was performed on HLA-$A2^+$ healthy donors or from ovarian patients

after undergoing cytoreductive surgery and prior to receiving chemotherapy according to the guidelines set forth by the Human Subjects Committee at the University of Arizona. All patients and healthy donors signed IRB approved consent prior to leukopheresis. Peripheral blood mononuclear cells were purified using standard white blood cell separation by density centrifugation with Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Piscataway, NJ). Aliquots of PBMC were cryopreserved in liquid nitrogen until use. Dendritic cells were generated from PBMCs by a modification of the methods described by Romani et al (110). In brief, PBMC were cultured in T75 flask in 10 ml of medium (AIM-V; Gibco BRL, Carlsbad, CA) and were allowed to adhere for 2 hr at 37°C in 5% CO_2. To remove the non-adherent PBMC fraction, flasks were washed several times with PBS. X-Vivo 15 (BioWhittaker, Walkersville, MD) supplemented with 1000 IU/ml of GM-CSF (Berlex, Wayne, NJ), and 1000 IU/ml of IL-4 (Peprotech, Rocky Hill, NJ) was added to adherent PBMC and the culture was continued for 5-6 days.

Generation of SKOV3-A2-specific CTLs

HLA-$A2^+$ CTLs were generated by repeated stimulation of autologous T cells with CRCL or peptide-pulsed DCs. Briefly, immature DCs were incubated for 24 hours with SKOV3-A2 CRCL or HER2/neu peptide at a final concentration of 25 μg/ml or 10

μg/ml at 37°C in 5% CO_2 followed by addition of 100 ng/ml tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN) and 10 μM prostaglandin E_2 (PGE_2; R&D Systems, Minneapolis, MN) for 48 hours to induce DC maturation. On day 0, non-adherent PBMCs (1×10^6/ml) were plated in 2 ml of Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Rockville, MD) with 25-mM HEPES, supplemented with 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA), 1,000 U/ml penicillin-streptomycin, 2-mM L-glutamine, and with pulsed mature DCs ($1x10^5$/mL) in 24-well plates at 37°C in 5% CO_2 (10:1 ratio of effector cells to stimulator DCs). Interleukin-7 (R&D Systems, Minneapolis, MN) was added at a concentration of 10ng/ml. Starting on day 1, 300 IU/ml of IL-2 (R&D Systems, Minneapolis, MN) was added every 2-3 days. The T cells were restimulated weekly with pulsed autologous mature DCs beginning 7 days after the first stimulation. At the end of the third or fourth stimulation, IFN-γ Enzyme-linked immunospot (ELISPOT) assays were performed. After four to six weeks of *in vitro* stimulation and selection, cytotoxicity assays (based on ^{51}Cr release) were performed. The CTL cultures were further separated into $CD4^+$ and $CD8^+$ populations after sorting using a FACScan (Becton Dickinson, Franklin lakes, NJ).

Enzyme-Linked Immunospot assays

Enzyme-linked immunospot assays were performed to measure the IL-12 secretion by DCs and IFN-γ production by PBMCs. For IL-12 detection, $1x10^6$ day 5 DCs were cultured with 50 μg/mL lipopolysaccharide (LPS), ovarian tumor-derived lysate, or CRCL in the presence of 1000 IU/ml GM-CSF and IL-4 for 48 hours on Millipore MultiScreen-HA 96-well plates (Millititer, Millipore, Bedford, MA). The plates were previously coated overnight with anti-IL-12 p70 capture antibody (10 μg/mL, BD PharMingen, San Diego, CA). Dendritic cells were then washed out with copious amounts of PBST (PBS + 0.05% Tween 20). Biotinylated anti-IL-12 antibody (2 μg/mL, BD PharMingen, San Diego, CA) was added for 2 hours. Free antibody was washed out, and the plates were incubated with horseradish peroxidase (HRP)–linked avidin (ABC Elite reagent, 1 drop each of Reagent A and Reagent B per 10 mL PBS; Vector Laboratories, Burlingame, CA) for one hour, following extensive washing with PBST, and then washing with PBS. Spots were visualized by the addition of the HRP substrate 3-amino-9-ethylcarbazole (AEC; Sigma Chemical, St. Louis, MO) prepared in acetate buffer (pH 5.0) with 0.015% hydrogen peroxide. Spots were counted using a dissecting microscope. Wells of interest were photographed with a microscope-mounted Cool SNAP CCD camera (RS Photometrics, Tucson, AZ), and images were captured with RS Image, Version 1.07 (Roper Scientific, Tucson, AZ). The image of each well was electronically optimized to visualize the maximum number

of spots. Enzyme-linked immunospot assays were also performed to measure IFN-γ secretion from stimulated PBMCs. Dendritic cells (2.5×10^4 cells/well) were loaded with the indicated peptide or CRCL (25 μg/ml) and incubated with effector cells (5×10^4cells/well) in a total of 200μl of X-Vivo 15 for 36 hr at 37°C in 5% CO_2. A mouse anti-human IFN-γ monoclonal capture antibody (10 μg/ml/well; Pharmingen, San Diego, CA) and a biotinylated anti-human IFN-γ monoclonal capture antibody (2.5 μg/ml/well, Pharmingen, San Diego, CA) were applied to visualize spots.

Mixed leukocyte reaction (MLR)

In order to assay for allogeneic or autologous lymphocyte proliferation, varying numbers of day 5 DCs were plated in a 96-well round-bottom plate (Falcon, San Jose, CA) and were pulsed with 25 μg/ml of lysate, CRCL; or 100ng/ml TNF-α and 10μM PGE_2; or were left untreated for 48 h. Dendritic cells were plated in triplicate in X-vivo medium containing, 1000 IU/ml of GM-CSF, and 1000 IU/ml of IL-4. Dendritic cells were treated with 100 μg/mL mitomycin-C (Sigma Chemical, St. Louis, MO) for 20 minutes, and then washed 3 times with PBS. Allogeneic PBMC (2×10^5 cells) were added to the DCs in a total volume of 200 μL of medium. Dendritic cells were serially diluted and incubated with the ratio of PBMC to DCs ranging from 5:1 to 40:1. On day 3 of culture, cells were pulsed with 1 μCi/well [^{3}H] thymidine (MP

biomaterial, Costa Mesa, CA) for 18 h and then were harvested using a 96-well Packard cell harvester and the radioactivity measured on a Packard beta counter (Packard Biosciences, Meriden, CT) (80).

Cytolytic activity of stimulated PBMCs

^{51}Chromium release cytotoxicity assays were performed to evaluate the ability of stimulated PBMCs to lyse the following target cells: SKOV3-A2, SKOV3, K562, K562-A2, or T2 cells pulsed with 10 µg/ml of HLA-A2 restricted peptides; HER2/neu (KIFGSLAFL), Wilm's tumor gene 1 (WT1, RMFPNAPYL) or hepatitis B virus (HBV) peptide (FLPSDYFPSV). Targets (5×10^3 cells/well) were labeled with 100 µCi of ^{51}Cr (Amersham Pharmacia Biotech, Piscataway, NJ) in complete IMDM for 1h. Percentage of specific lysis was calculated as ([experimental ^{51}Cr release-spontaneous release] / [maximum release – spontaneous release]) x100.

Flow cytometry

Fluorochromes fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated mAbs (BD PharMingen, San Diego, CA) were added to cell pellets, incubated for 30 minutes on ice, and washed before analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin lakes, NJ).

1.4 RESULTS

Effect of ovarian tumor-derived CRCL on human DC maturation

To generate CRCL-specific T cells from HLA-$A2^+$ PBMCs, autologous DCs were used as APCs. Previous data demonstrated that murine DCs incubated with tumor-derived CRCL had higher expression of CD40 and MHC class II on their cell surface, produced more IL-12, and had superior immuno-stimulatory capacity in MLR (80). In this study, we evaluated whether these findings would translate into the humans using CRCL from ovarian cancers. Chaperone-rich cell lysate was derived from ovarian tumors or from the SKOV3-A2 cell line. Dendritic cells were generated from either healthy volunteers or from ovarian cancer patient PBMCs as described in Materials and methods. Interleukin-12 secretion of the DC following stimulation with 50 μg/ml CRCL was evaluated and compared to LPS as the positive control. Compared with lysate and medium alone, CRCL-stimulated DCs had increased IL-12 production as assessed by ELISPOT (Figure 1A). We also examined whether DCs pulsed with ovarian tumor-derived CRCL would be more effective stimulators in a MLR assay when compared to DCs exposed to tumor lysate or to TNF-α and PGE_2. Our results indicate that ovarian tumor-derived CRCL has superior activating effects on DCs compared to tumor lysate (Figure 1B). However,

in terms of surface maturation markers, unlike our previous work in murine models, the expressions of CD40, CD80, CD86, and HLA-DR were not significantly up-regulated by CRCL alone (Figure 1C).

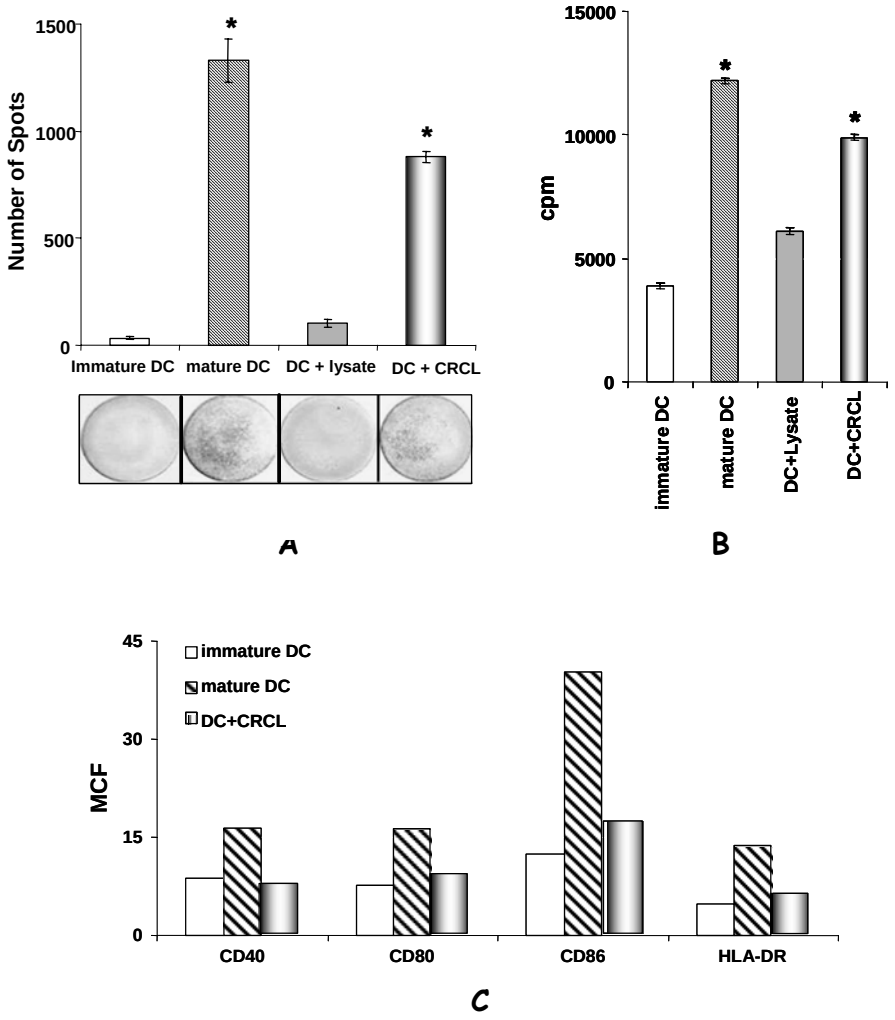
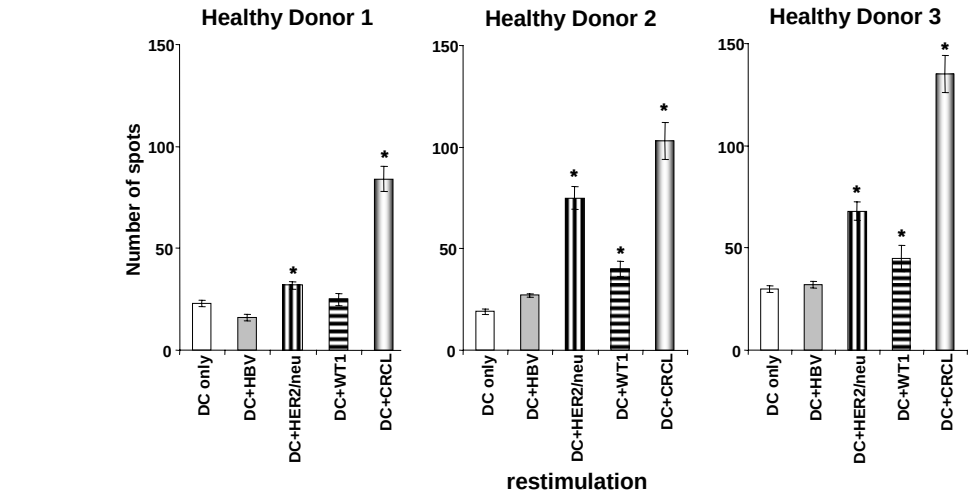


Figure 1. Dendritic cell maturation induced by CRCL (A). Dendritic Cells pulsed with CRCL show increased IL-12 production. Interleukin-12 production by normal human DCs were measured by ELISPOT assays. At day 5 of culture, $1x10^6$ DCs were cultured with medium, 50 μg/ml tumor-derived lysate, CRCL or maturating LPS each for 48 hours. (B). Ovarian tumor-derived CRCL increases DC capacity to stimulate PBMC proliferation. Normal human DCs were cultured as described above, harvested, treated with Mitomycin-C, and washed. Allogeneic PBMC were added ($1x10^5$ per well) and cultured with the pretreated DCs for 3 days. [^{3}H] thymidine was added and the cells were cultured for an additional 18 hours before the incorporated radioactivity was counted (* $P < 0.05$ versus DC or DC + lysate; representative data from 3 experiments are shown). Cpm indicates counts per minute. (C). Normal human DCs were cultured as described above, harvested, and analyzed by flow cytometry for expression of the cell-surface markers indicated. These data are representative of DCs derived from both healthy donors and ovarian cancer patients and incubated with either patient ovarian tumor tissue or SKOV3-A2-derived CRCL.

Generation of tumor-specific T cells

Initially we tested the immunogenicity of the ovarian tumor-derived cell line, SKOV3-A2 in DC/PBMC combinations from healthy HLA-A2$^+$ individuals. To maximize the stimulatory capacity of DCs, we generated mature DC by two rounds of stimulation. Firstly, immature DCs were stimulated with CRCL for 24 hr and this was followed by TNF-α and PGE_2 exposure for another 48 hr. Peripheral blood mononuclear cells were monitored for cytokine production by IFN-γ ELISPOT assays after four rounds of CRCL pulsed DC stimulation. Additional experimental groups included DCs that had been pulsed with irrelevant HBV peptide or relevant HER2/neu, or WT1 peptide. SKOV3-A2 CRCL-pulsed DCs elicited the greatest antigen-specific IFN-γ responses from responding PBMCs derived from healthy individuals (Figure 2A). This suggests that improved DC stimulation and/or a larger antigenic repertoire were provided by CRCL. The stimulated healthy donor PBMCs demonstrated IFN-γ secretion following encounters with HER2/neu peptide in all cases, albeit less than CRCL, and WT1 peptide elicited only modest responses in two out of three healthy donors. Likewise, stimulation of patient PBMCs with CRCL resulted in specific responses in three of the four ovarian patients demonstrated by the increased IFN-γ secretion (Figure 2B). The IFN-γ responses from individual peptides clearly were not as strong as those from broader antigen exposure driven

by SKOV3-A2 CRCL re-stimulation (Figure 2B). These results suggest that T cells generated in the presence of DC/CRCL stimulation from both healthy donors and ovarian cancer patients could recognize either individual tumor antigen or a broader antigen repertoire encompassed in the tumor CRCL.



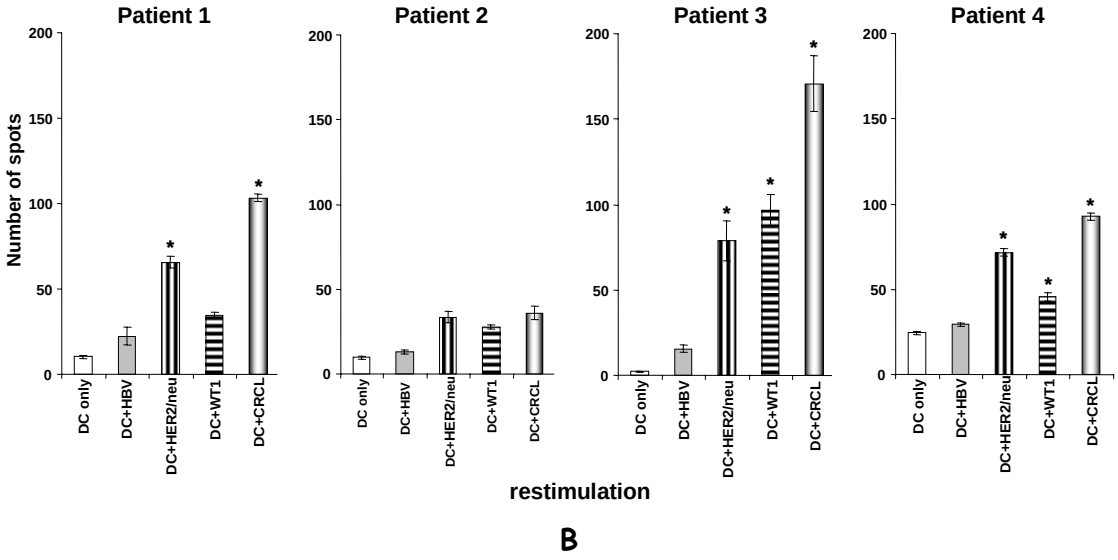
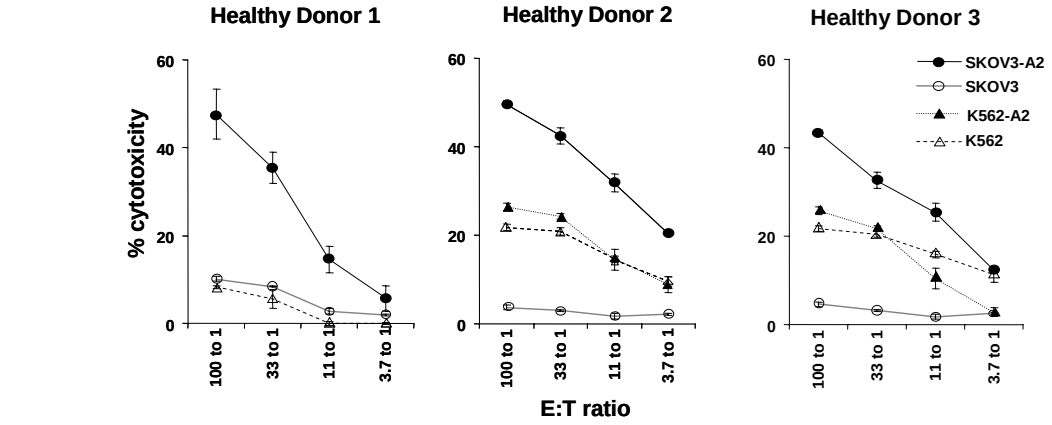


Figure 2. Tumor-specific responses detected from HLA-A2$^+$ healthy donors and ovarian cancer patients by IFN-γ ELISPOT assays. Healthy donors (*A*) and ovarian cancer patients' PBMCs (B) were stimulated for 4 weeks by autologous DCs loaded with SKOV3-A2 CRCL. Dendritic cells (2.5×10^4 cells/well) pulsed with SKOV3-A2 CRCL and indicated peptides (25μg/ml) were used to stimulate bulk PBMC cultures (5×10^4cells/well). Dendritic cells pulsed with HBV peptide and un-pulsed DCs were used as control stimulation. (Results from healthy donor 1-3 and patient 1-4 are shown). (* $P < 0.05$ versus DC or DC + HBV)

Cytolytic activity of tumor-specific T cells

After six weekly stimulations with CRCL-pulsed mature DCs, cytotoxicity assays were performed to address whether antigen-specific CTLs could lyse the SKOV3-A2 targets. Stimulated PBMCs from all three healthy individuals (Figure 3A) lysed the SKOV3-A2 targets. To determine MHC restriction of these T cell lines, we examined the cytotoxicity against cells not expressing HLA-A2 (SKOV3) and no lysis was observed. There was some NK mediated cytotoxicity as indicated by lysis of the NK sensitive target K562. K562 and HLA-A2 transfected K562-A2 cells over-express WT1 tumor antigen as SKOV3-A2 cells do. Some killing was observed on K562-A2 also. These data demonstrated that tumor-derived CRCL is capable of generating CTLs specific to the tumor of origin. In contrast, CRCL loaded DC stimulated PBMCs from ovarian cancer patients demonstrated specific cytotoxicity in only one of the four patients studied (Figure 3B). Although recognition of tumor antigen was observed as documented by IFN-γ secretion (Figure 2B), lysis of target tumor cells was not detected in patients 1 and 4 (Figure 3B). Patient 3 whose T cells demonstrated substantial lysis of SKOV3-A2 targets also demonstrated the highest IFN-γ secretion by ELISPOT (Figure 2B).



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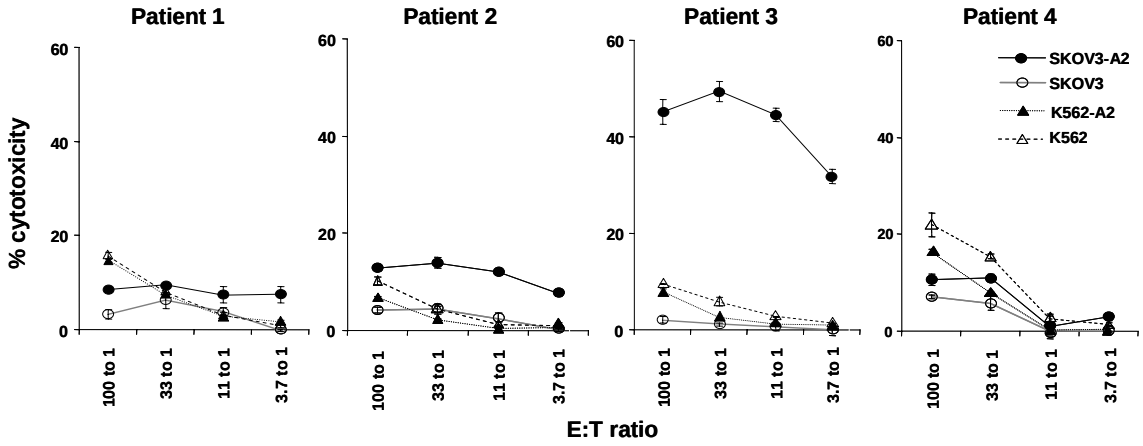

B

Figure 3. Cytotoxicity by stimulated PBMCs generated by weekly stimulation with SKOV3-A2 tumor-derived CRCL-pulsed DCs Specific T cells were generated by 6-week stimulation of DCs loaded with SKOV3-A2 CRCL from the PBMCs of healthy donor (A) and ovarian cancer patients (B). Effectors (at the indicated E:T ratios) were incubated with ^{51}Cr-labeled target cells (5×10^3 cells/well), and ^{51}Cr release from target cells was measured after 6–8 hr. Lysis of the NK-sensitive cell line, K562, and K562 expressing HLA-A2 served as background controls. Lysis of the HLA-$A2^+$ or HLA-$A2^-$ ovarian cancer cells (SKOV3-A2 and SKOV3) was also evaluated. (Results from healthy donor 1-3 and patient 1-4 are shown). (Error bars represent the mean±SEM of triplicate samples)

Cytolytic activity of CRCL-stimulated versus peptide-stimulated T cells

HLA-A2 restricted peptides have been shown to be potent CTL stimulators and antigens. We compared our CRCL stimulation with that by HER2/neu peptide. As shown in Figure 4A, HER2/neu-stimulated T cells lysed T2 cells pulsed with HER2/neu peptide and SKOV3-A2 cells which express and present this peptide. Importantly, CRCL-stimulated T cells lysed T2 cells pulsed with HER2/neu in addition to lysing the original SKOV3-A2 cells (Figure 4B), correlating with the results of our IFN-γ ELISPOT assays. This implies the presence of the HER2/neu antigen in the SKOV3-A2–derived CRCL preparations.

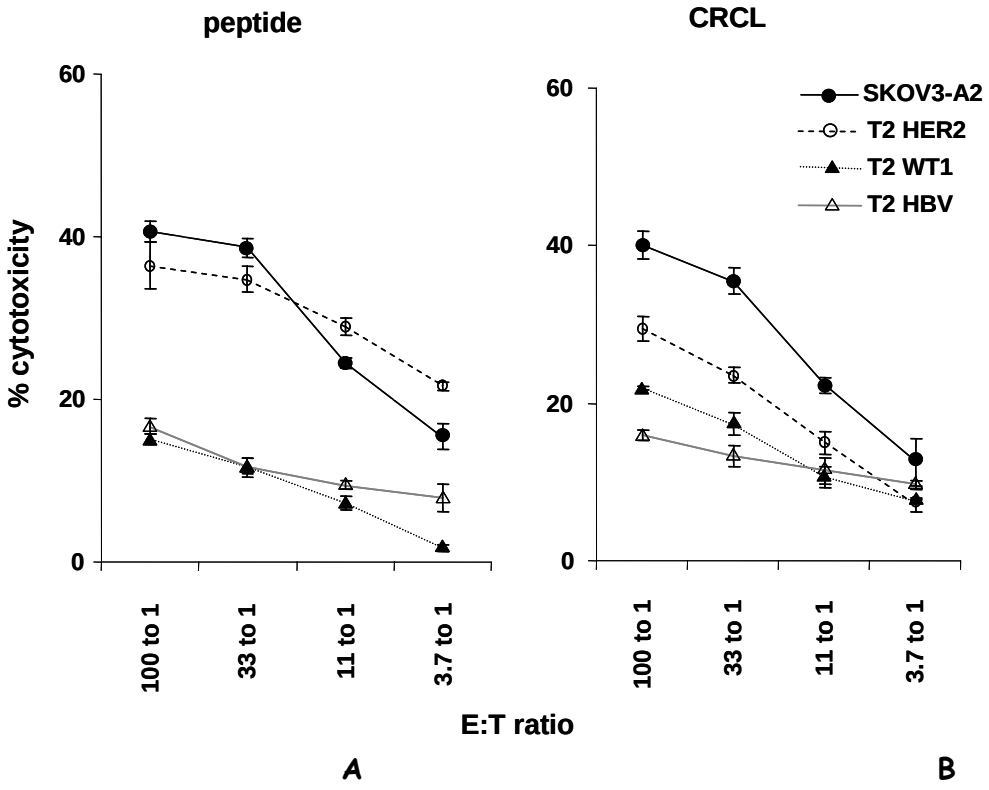

Figure 4. Cytotoxicity of target cells by stimulated PBMCs generated by SKOV3-A2 tumor-derived CRCL and by HER2/neu peptide Specific T cells of healthy donors were generated by 6-week stimulation of PBMCs by DCs loaded with HER2/neu peptide (A) or SKOV3-A2 CRCL (B). Effectors (at the indicated E:T ratios) were incubated with ^{51}Cr-labeled target cells (5×10^3 cells/well), and ^{51}Cr release from target cells was measured after 6–8 hr. Lysis of T2 cells pulsed with HBV peptide was the background control. Lysis of the ovarian cancer cells SKOV3-A2 or T2 cells pulsed with HER2/neu or WT1 peptide was evaluated. (Representative data from 2 experiments were shown. Error bars represent the mean±SEM of triplicate samples). These data are representative of both healthy donors and ovarian cancer patient 3.

Cytotoxicity of the stimulated PBMCs is mainly mediated by $CD8^+$ T cells

Flow cytometric analysis demonstrated that CRCL stimulated PBMCs were comprised of mainly $CD4^+$ and $CD8^+$ T cells. In all healthy donors and patient 1, 3 and 4, $CD4^+$ and $CD8^+$ T cells contributed to 60-70% and 20-30%, respectively, of bulk cell culture after six-weekly stimulations (Figure 5A and B). In contrast, the $CD8^+$ T cell population of patient 2 was dramatically lower at only 6% compared to 25% before stimulation (data not shown), suggesting that there were defects in the $CD8^+$ T cell growth in this patient. This lower number of $CD8^+$ T cells correlated with lower IFN-γ (Figure 2B) production and cytotoxicity (Figure 3B) in this patient. The percentages of $CD56^+$ (NK cells) and $CD14^+$ (monocytes) cells were low (1% and 1-3% respectively) in both healthy donors and ovarian patients.

To confirm that the cytotoxicity of the stimulated PBMCs was mediated by CTLs, we isolated $CD4^+$ and $CD8^+$ T cells from bulk cultures of healthy donors and patient 3, and performed ^{51}Cr release assays. As demonstrated in Figure 6, SKOV3-A2 targets were primarily lysed by $CD8^+$ T cells. There was some K562 cell lysis induced by bulk cell culture compared to $CD4^+$ or $CD8^+$ T cells, suggesting that the overall cytotoxicity of bulk stimulated PBMCs was also comprised of NK cell mediated lysis, but at much lower levels than that of $CD8^+$ T cells. These results

together with the absence of cytotoxicity against SKOV3 (HLA-A2 negative) target cells indicate that the cytotoxicity against the tumor, from which CRCL originated (SKOV3-A2), can mainly be attributed to MHC class I (HLA-A2)–restricted $CD8^+$ T cells.

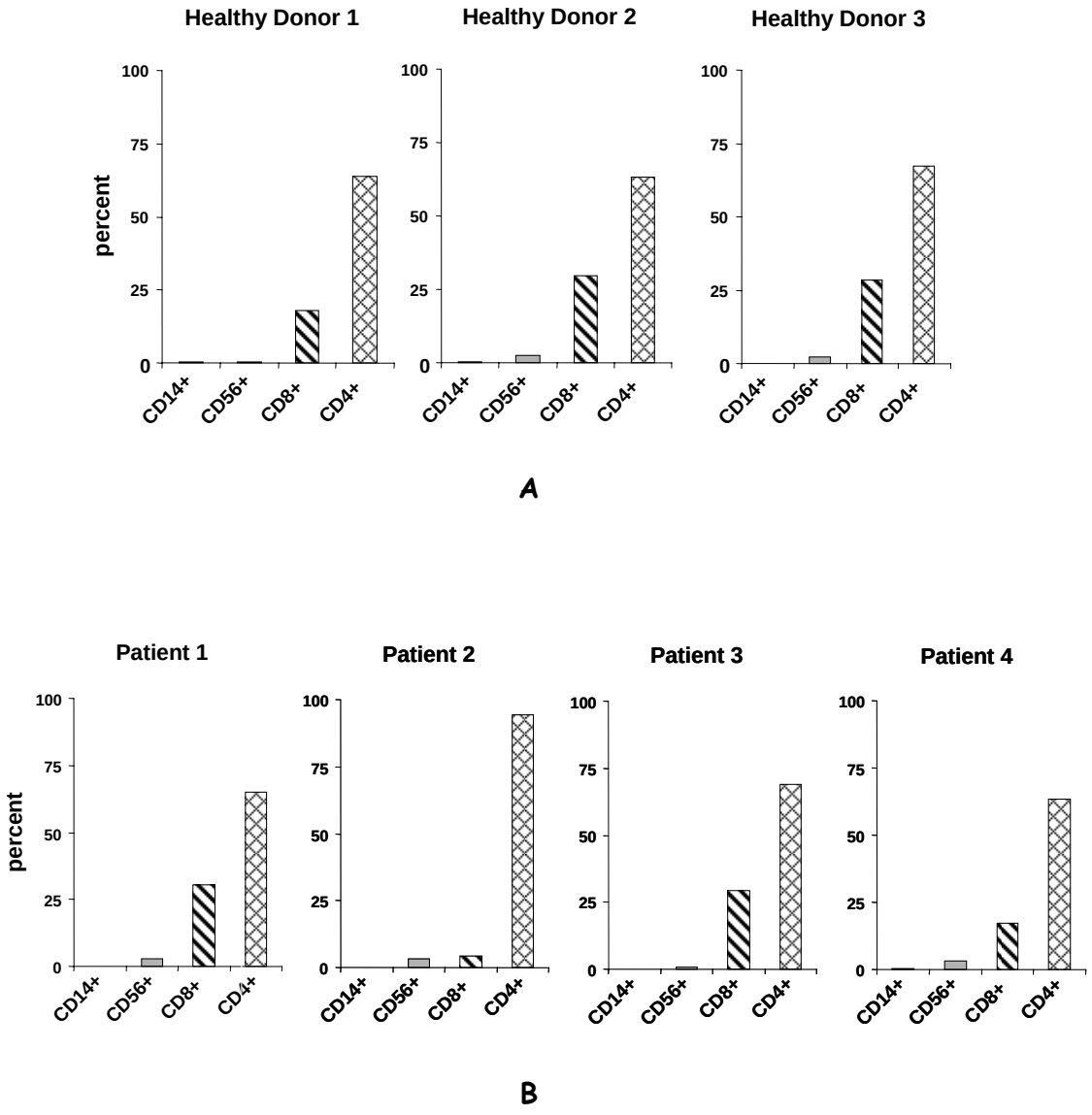

Figure 5. Phenotype of stimulated PBMCs. Stimulated PBMCs were generated from healthy donors (A) or ovarian cancer patients (B) by weekly stimulation with DCs loaded with SKOV3-A2 CRCL. Stimulated cells were double stained for $CD14^+$ (monocyte), $CD56^+$ (NK cell), $CD8^+$, and $CD4^+$ T cells. The percentage of positive cells was determined using flow cytometry.

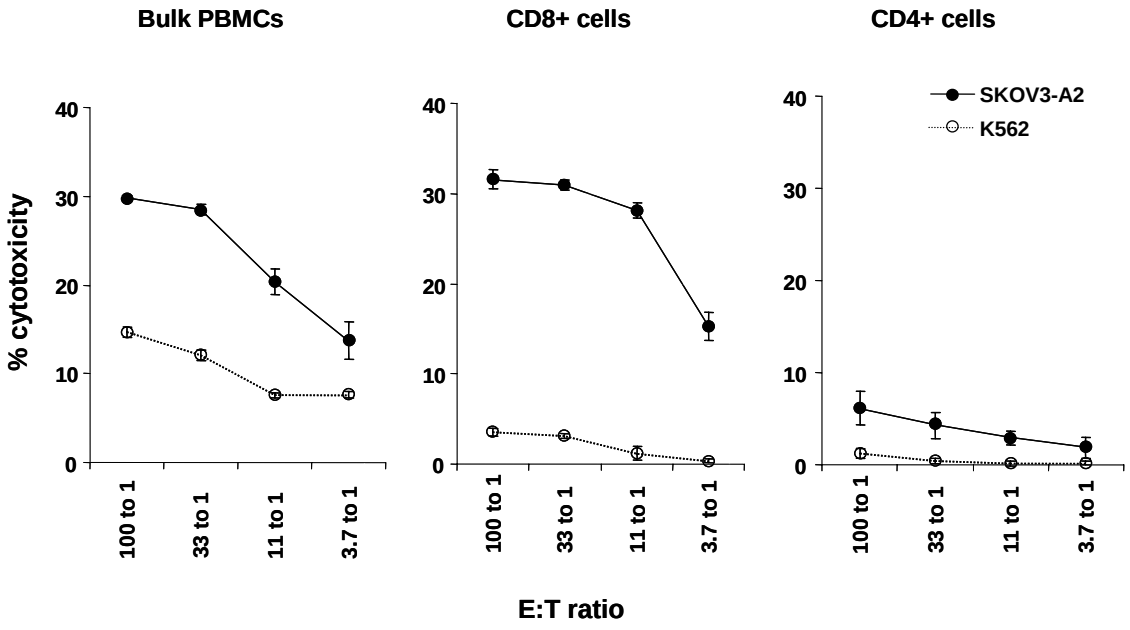

Figure 6. Cytotoxicity against target cells by bulk PBMCs, or by separated $CD8^+$ or $CD4^+$ T cells derived from stimulated PBMCs. Stimulated PBMCs from ovarian patient 3 were generated as previously mentioned. Target SKOV3-A2 and K562 cells were labeled with ^{51}Cr. The bulk PBMCs, or sorted $CD8^+$ and $CD4^+$ T cells were coincubated (at the indicated E:T ratios) with labeled target cells (5×10^3 cells/well) for 6–8 hr. The percentage of target cell lysis was then determined. (Representative data from 4 experiments were shown. Error bars represent the mean±SEM of triplicate samples.) These data are representative of healthy donors and ovarian cancer patient 3.

1.5 DISCUSSION

As described before in background, tumor-derived HSPs are personalized vaccines that carry multiple antigens of the original tumor (71, 111), circumventing the need to identify each tumor's antigens. Compared to individual HSP, CRCL is a multiple HSP complex which can chaperone a wide variety of tumor-specific antigens. In this pilot human study, we have demonstrated for the first time that CRCL can maintain the advantages of individual HSPs as vaccines (i.e. no need to identify antigens, multivalency of the antigen ensemble, and specificity of the class I restricted response) in a human *in vitro* system. We have focused on whether ovarian tumor-derived CRCL was able to generate HLA-A2 specific responses. Stimulation of autologous human DC with ovarian tumor-derived CRCL generated specific CTLs that were able to recognize and lyse ovarian tumor targets. Additionally we were able to generate CTLs against two different tumor antigens such as HER2/neu and WT1 in a class I restricted manner. Therefore as we have previously reported in mice, human CRCL is capable of generating a larger repertoire of tumor-specific CTLs (79, 112). Such T cells may respond to subdominant epitopes which would be an important consideration as high affinity T cells derived from self antigens can get deleted in the thymus (113, 114). Additionally, the chaperone proteins themselves

have potent natural adjuvant or cytokine-like activities on APC such as DC, thus enhancing the DCs' ability to process/present antigen and stimulate T cell responses, as demonstrated by IL-12 production and higher immuno-stimulatory capacity of CRCL stimulated DC. However, the surface expression of the co-stimulatory molecules was not significantly increased. This might be because the up-regulation was not elevated enough to be measured by the flow cytometry method.

We demonstrated that CRCL can be generated from patient tumor tissue. Compared to 1-2mg of CRCL from 1g of murine tumor, 0.5-1mg CRCL could be generated from 1g of human ovarian tumor tissue. However, the overall yield of CRCL is still much higher than that of individual HSPs. Given the limitations of preparing tumor vaccines from an autologous tumor, CRCL offers an important advantage in that with the same quantity of starting material we are able to obtain up to ten times as much CRCL-derived protein as with conventionally purified individual HSPs (112). This makes the method 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. Chaperone-rich cell lysate has also been successfully isolated from an ovarian cancer cell line culture SKOV3-A2. Instead of using solid tumor tissue, cell pellets from cell cultures after

centrifugation were homogenized and applied to FS-IEF. The vaccine generated in this way was as efficient in activating DC as that from a solid tumor, suggesting that cells in culture may provide another feasible source of CRCL if inadequate tumor is available.

To measure the potential efficacy of a tumor vaccine, CTL responses against tumor antigens have been regarded as an important indicator of activity. Cytotoxic T lymphocytes usually recognize cytoplasmic antigens of target cells that are processed and presented as peptide complexes with MHC class I molecules (115). Exogenous antigens can be cross-presented on MHC class I molecules of professional APCs to $CD8^+$ T cells in certain circumstances with the help of HSPs (71, 116-119). This mechanism had been utilized in our study to generate CTLs *in vitro* following stimulation with mature DCs pulsed with tumor-derived CRCL. Because immature DCs are highly effective in taking up and processing antigens compared with mature DCs (120), we pulsed immature DCs with CRCL and induced DCs to maturation after antigen pulsing. Indeed, CRCL alone could activate immature human DCs as indicated by augmented IL-12 secretion and immuno-stimulatory capacity in MLR. Because cancer patients are immunosuppressed, their DCs may have a functional defect which has been suggested to be one of the causes for T cell dysfunction in cancer patients

(121, 122). Following ovarian cancer CRCL stimulation, DC from both healthy donors and ovarian cancer patients revealed an increased cytokine secretion and T cell stimulatory capacity.

We have demonstrated that following CRCL stimulation of human DC, we were able to promote the expansion of both $CD4^+$ and $CD8^+$ T cells. Examining the ratio of $CD4^+$ *versus* $CD8^+$ T cells, there was preferential expansion of CD4+ T cells at the end of 6 weeks of re-stimulation. This is in sharp contrast to single purified HSP such as gp96, where an activated $CD8^+$ T cell bias was observed (123). This result suggests that there are qualitative differences in CRCL stimulation compared to single HSPs. However, the cytotoxicity was mainly attributed primarily to MHC class I–restricted $CD8^+$ T cell and therefore the exact contribution of $CD4^+$ T cells in CRCL stimulated culture needs further investigation. It is important to note that in spite of expansion of $CD4^+$ T cells in the patient PBMC cultures, the $CD8^+$ T cells were generally not able to kill tumor-specific targets indicating that these patients' may have ongoing immuno-suppression.

Unlike other preclinical studies which have isolated PBMC from vaccinated patients (77, 86, 97, 98, 124), we have not yet received the approval to immunize patients

with CRCL. Therefore, we examined the function of CRCL stimulated DC in stimulating anti-tumor T cell responses in an *in vitro* cell culture system. With the help of autologous DCs loaded with ovarian tumor-derived CRCL, we stimulated PBMC from both healthy donors and ovarian carcinoma patients *in vitro*.

Specific IFN-γ secretion was observed in the stimulated PBMC from healthy donors and from three of four ovarian cancer patients. However cytolytic function of stimulated T cells from ovarian cancer patients with untreated bulky disease was generally suppressed. Considering the relative normal secretion of IFN-γ by stimulated PBMCs from three ovarian cancer patients, it suggests that the defects of $CD8^+$ T cells in these patients are worse than those of other cell populations, such as $CD4^+$ T cells. Published preclinical studies using HSP vaccines have generally evaluated the IFN-γ secretion, without cytolytic assays, with the cytokine data correlating with the clinical responses to HSP vaccination (77, 86, 97, 98). One has to keep in mind that the absence of positive data in ^{51}Cr release assays does not predict lack of *in vivo* responses. Moreover, the experimental *in vitro* conditions used to generate CTLs are artificial given that other cells and cytokines that are normally present *in vivo* may be absent in culture. However, the difference in the generated cytotoxicity between healthy volunteers and patients was striking and can

not be ignored and suggests that chemo-naïve patients with widely metastatic ovarian cancer may be so immuno-suppressed that their T cells have a defective response to CRCL.

The nature of the defects in generating CTLs in these ovarian cancer patients are not clear and need to be defined. The host immunosuppressive environment associated with cancer may play a vital role in this defect. It has been known that cancer cells could escape immune responses by immunoselection, the selection of non-immunogenic tumour cell variants, such as the low expression of tumor antigens or MHC class I molecules (125). Another important mechanism of immuno-suppression is immunosubversion, which is the active suppression of the immune response, such as decreased co-stimulatory molecules on APC, blocking the granzyme-B–perforin pathway, the downregulation of death receptors, and increased T regulatory (Treg) cells (125). For example, high numbers of Treg were reported be increased in human ovarian cancer patients and to be associated with poor survival (126-128). T regulatory cells may suppress the immune responses primarily through contact-dependent mechanisms (129), or act through soluble cytokines such as IL-10 and transforming growth factor (TGF)-β (129). An additional possible source of the immunosuppression in ovarian cancer patient is plasmacytoid DC (DC2). Tumor

DC2s are reported to express low levels of costimulatory molecules, induce IL-10 secretion, and are poor in activating naive T cells (130). It has been demonstrated that ovarian epithelial tumors produce high-level SDF-1. This SDF-1 is a chemoattractant for DC2 precursors, and induces their migration into the tumor microenvironment. However, neither DC1s nor their precursors were detected in the tumor microenvironment (131). Therefore, ovarian tumors may dysregulate immunity by attracting DC2 precursors, and by hindering accumulation or differentiation of DC1. However, in our study, it is possible that neither DC2 nor Treg contributed to the defect of the cytotoxicity, because myeloid DCs were used to stimulate PBMCs and because we observed the IFN-γ secretion which should be less likely if Treg present in the culture. Therefore, the causes of immunosuppression in our study need to be defined further.

In addition to the immunosuppression associated with cancer, post-operative immunosuppression should also be kept in mind. The ovarian cancer patients recruited in this study received surgery shortly before the leukopheresis. It has been shown clinically and experimentally that surgery causes a transient period of immunosuppression (132, 133). However, the mechanism for post-surgical immune suppression is not very clear yet. Surgery is known to evoke a strong stress

response (134). This stress response is associated with changes in the levels of cytokines, such as a decrease in IL-2 and increases in IL-1β, TNF-α, IL-6, IL-8, IL-10 and PGE_2 (135). Concurrent with cytokine changes, changes in the phenotypic subpopulations of the immune effector cells occur, such as the increase in Th2 cells (136). These phenotypic changes influence the immune response and modulate cytokine secretion as well (136).

This study is the first to test the immuno-stimulatory effects of CRCL in the humans. The uniqueness of this system is that we were able to activate multiple antigenic determinants, including shared tumor antigens such as HER2/neu and WT1. Therefore it seems likely that CRCL could deliver a broader repertoire of tumor-specific antigens that would make CRCL vaccination desirable as an individualized therapy. Individual HSP such as gp96 have already been applied to the phase II clinical trial (97, 124). The objective of this study was not to compare CRCL with individual HSPs *in vitro*, since our data from the murine *in vivo* models indicate that CRCL has significantly more activity than individual HSPs. Our goal in undertaking this study was to examine the efficacy of obtaining CRCL from human tumors and generating immunologic responses *in vitro*. Our pilot data demonstrate that this approach may constitute a promising immunotherapeutic strategy to treat

patients with ovarian cancer and therefore warrants further investigation.

1.6 ACKNOWLEDGEMENTS

We are grateful to Dr. Douglas F. Lake, Dr. Sara O. Dionne for providing access to their laboratory resources; Roger Fiederlein for the valuable assistance during the study; and Lorry Velasco, Kathy Schmidt for their excellent nursing work.

CHAPTER 2

HER2/NEU POSITIVE TUMOR-DERIVED CRCL ELICITS TUMOR-SPECIFIC HUMORAL IMMUNITY

2.1 ABSTRACT

In the second part of the study, we have investigated the potency of HER2/neu positive tumor-derived CRCL to elicit humoral immune response against tumor. HER2/neu is an oncogenic tumor associated antigen that is over-expressed in several human tumors including breast and ovarian cancer. The efficacy and mechanism of CRCL vaccine to fight against the HER2/neu positive tumor was examined using a transplantable murine tumor model: TUBO in BALB/c mice. Vaccination of mice bearing palpable tumor efficiently delayed TUBO tumor growth. Chaperone-rich cell lysate vaccination elicited significant anti-HER2/neu antibody production in mice. Vaccinations of B cell deficient mice and antibody transfer studies have shown that the induction of anti-HER2/neu antibodies is both necessary and sufficient for anti-tumor effect. Further, we have demonstrated that serum from TUBO-CRCL vaccinated mice stimulated the internalization of the HER2/neu molecules, inducing the down-regulation of their surface expression. Moreover, ADCC has been observed against TUBO cells treated with sera from vaccinated mice and incubated with splenocytes. This is a significant observation since not much is known about the mechanism of the humoral responses induced by HSP-derived vaccines.

2.2 INTRODUCTION

Breast cancer is the most common cancer among American women, and an estimated 212,920 new cases will be diagnosed in 2006. Despite advances in early detection and treatment, breast cancer remains the second-leading cause of cancer death among American women, and the incidence rate has continued to increase in the United States since the 1980s. The average lifetime risk for breast cancer in American women is 13.22% (American Cancer Society, 2006). Traditional cancer treatment regimes including radiotherapy, chemotherapy improve survival of patients with breast cancer, but they are generally not selective, inducing cytotoxicity in normal as well as in malignant cells, and so they often are not well tolerated (137). Advances in the understanding of tumor biology have allowed targeted therapies against specific molecular targets to develop. Recently, these approaches have led to the development of certain immunotherapeutic strategies, for example, HER2/neu related vaccine.

As described previously, the HER2/neu gene encodes a tyrosine kinase growth factor receptor homologous to other members of the epidermal growth factor receptor family (138). Malignant transformation of epithelial cells is correlated with the

over-expression of HER2/neu protein and the poor prognosis of the disease (139, 140). In breast cancer patients, its over-expression has been reported to be 100-fold higher than in normal tissues (1), and it has been suggested to play a direct role in the aggressiveness of tumors (9). The selective over-expression of HER2/neu by tumor cells makes it an ideal target candidate for immunotherapy. Preclinical studies and clinical trials have shown some effectiveness of such strategies, such as specific antibodies and HER2/neu peptides (5, 62, 139, 141). Currently, an FDA approved humanized monoclonal anti-HER2/neu, Trastuzumab (Herceptin), is used to treat women with advanced HER2/neu-expressing breast cancer (5, 142). However, a positive response is observed in only a limited number of patients (142). HER2/neu-specific immunity can also be augmented in patients vaccinated with HER2/neu peptides, although no durable clinical response has been documented (139, 143, 144). The main problems with monoclonal antibody therapy have been the development of toxicity and resistance (36, 145). The major disadvantages with peptide vaccination are the magnitude and durability of responses along with the limitations of MHC restriction (5, 62, 146). Thus, more innovative approaches to treat HER2/neu cancers need to be developed.

Based on our previous experience, we propose to use our CRCL vaccine, a

multi-epitope based HSP as an active immuno-stimulant against HER2/neu positive breast cancers. We hypothesized that active immunization with CRCL derived from a HER2/neu positive TUBO tumor, a syngeneic breast cancer cell line expressing rat HER2/neu (147), would stimulate anti-HER2/neu tumor immunity. In this model, an anti-HER2/neu CTL response was not found in the vaccinated mice, whereas they display a significant titer of anti-HER2/neu antibody that may be responsible for the tumor delay, suggesting that CRCL can indeed present B cell epitopes to the immune system. The mechanism of anti-tumor effect was evaluated by assessing B cell immunity against HER2/neu protein. Vaccinated mice developed anti-HER2/neu antibodies and abrogation of this response in B cell deficient mice led to the loss of tumor delay. Additionally, the delay of tumor in SCID mice by the sera transfer of TUBO tumor-derived CRCL vaccinated mice has confirmed the importance of antibody production. The mechanisms of action of the antibodies were then investigated. The antibody has been shown to stimulate the internalization of the HER2/neu molecules, resulting in its surface expression down-regulation. Moreover, the ability of anti-HER2/neu antibodies to mediate ADCC by splenocytes was assessed. Cytolysis of TUBO tumor cells was increased significantly in the presence of anti-HER2/neu antibody. Taken together, this provides evidence for the first time that CRCL vaccine can mediate B cell immunity

against tumor specific antigens. Several vaccines have been demonstrated to be effective in this HER2/neu positive murine breast cancer model, such as antibody-cytokine protein (147, 148) and DNA vaccines (31, 32). The generation of anti-tumor humoral responses by CRCL vaccine argues for an alternative immunotherapy for HER2/neu positive cancer. Moreover, this study is the first to illustrate the mechanism of antibody response elicited by a HSP based vaccine.

2.3 MATERIALS AND METHODS

Mice

Mice were housed under specific pathogen-free conditions and cared for according to the guidelines of the University of Arizona Institutional Animal Care and Use Committee. Female 6–8 weeks old wild BALB/c or BALB/c SCID ($H2^d$) from the National Cancer Institute (Bethesda, MD), and B-cell deficient BALB/c ($H2^d$) mice from Taconic Farms Inc. (Germantown, NY) were used for the experiments. The B-cell deficient mice lack the J_H gene segment, and thus do not express immunoglobulin heavy chain and fail to generate mature B-cells (149).

Tumor cells line

TUBO, a cloned cell line over-expressing the rat HER2/neu protein, was established from a lobular carcinoma that spontaneously arose in a female BALB/c mouse transgenic for the transforming rat HER2/neu oncogene (32, 150). TUBO cells grow progressively in normal BALB/c mice and give rise to lobular carcinoma, histologically similar to that seen in BALB-neuT-transgenic mice (32, 150). TUBO cells were cultured at 37°C, 5% CO_2 in Dubecco's Modified Eagle Medium, (DMEM, GibcoBRL, Gaithersburg, MD), supplemented with glutamax, glucose, 25 mM Hepes

buffer, pyridoxine-HCl (GibcoBRL) with 20% fetal bovine serum (Gemini, Woodland, CA). 12B1 is a murine leukemia cell line 12B1 (kindly provided by Dr. Chen W, Cleveland Clinic, Cleveland, Ohio) with BALB/C background as an irrelevant tumor control (82). The cells were cultured in RPMI medium (Gibco/BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gemini). The cell lines were tested routinely and found to be free of *Mycoplasma* contamination.

Chaperone-rich cell lysate preparation

Tumor-derived CRCL was prepared as described previously (78, 82, 85). The endotoxin activity of CRCL was also determined using the limulus amebocyte lysate assay kit (Cambrex Bio Science, Walkersville, MD) according to the manufacturer's instructions. The level of endotoxin in CRCL was lower than that in media control (<0.01 EU/μg). TUBO- and 12B1-CRCL was used for *in vivo* vaccination of mice. Aliquots from the same batch of 12B1 or liver CRCL were used throughout the study.

***In vivo* tumor growth experiments**

Wild type BALB/c or B-cell deficient BALB/c mice were injected with 1×10^5 viable TUBO cells in the right groin on day 0. The mice received a subcutaneous (s.c) vaccination of 20μg TUBO tumor-derived CRCL in their left groins, 0, 2, 4, 7, 10 and

14 days after the tumor became palpable. Mice injected with PBS and 12B1-CRCL served as a control group. Tumor growth was monitored and measured with a caliper every 7 days after the vaccination. Mice with tumors 4,000 mm^3 in volume or greater were euthanized. The Kaplan-Meier product-limit method was used to plot survival and the log-rank statistic to test differences between groups (80).

Enzyme-Linked Immunosorbent Assay (ELISA) titration of the antibody response to HER2/neu

Mice were bled from a lateral tail vein prior to TUBO inoculation, vaccination or afterward from vaccinated mice, and sera were stored frozen at –80°C. Sera obtained were analyzed by ELISA for antibodies to ECD^{HER2} using 96-well microtiter plates coated with 50 µl of ECD^{HER2} (kindly provided by Dr. Penichet ML, University of California LA, Los Angeles) at a concentration of 1 µg/ml (147, 148). The plates were washed and blocked with 3% bovine serum albumin (BSA) (Sigma, St. Louis, MO) in PBS. After washing, dilutions of sera in PBS containing 1% BSA were added to the wells and incubated overnight at 4 °C. Bound IgG was detected by incubating for 1 h at 37 °C with Alkaline phosphatase (AP)-labeled rabbit anti-mouse IgG (Zymed, San Francisco, CA). After washing, *p*-nitrophenyl phosphate disodium dissolved in diethanolamine buffer (Sigma, St. Louis, MO) was added for 2 h and the

plates were read at 410 nm. Sera from naïve mice of the same age were used as a negative control. All ELISAs were made in duplicate. Anti-HER2/neu IgG1, IgG2a and IgG3 responses were analyzed by ELISA using 96-well microtiter plates. The plates were prepared as described above, with AP-labeled rat anti-mouse IgG1, IgG2a (Zymed, San Francisco, CA) or AP-labeled goat anti-mouse IgG3 (Southern Biotechnology Associates Inc., Birmingham, AL) used as detecting agents (147).

Transfer of immune sera

BALB/c SCID mice were randomized and distributed into 6 mice per group. Serum from unchallenged BALB/c mice vaccinated with PBS, 12B1 and TUBO tumor-derived CRCL, respectively, was pooled and stored at -80°C. The presence of HER2/neu-specific antibodies in the sera from vaccinated mice was verified by ELISA. At day -1, mice received an intravenous (i.v.) injection of 200 µl of pooled sera collected from the unchallenged vaccinated BALB/c mice. On day 0, $1x10^5$ TUBO cells were injected in the right groin (147). An untreated group of BALB/c SCID mice of the same age without any sera transfer was also challenged with TUBO cells as another control.

Cytotoxicity assay

BALB/c mice were vaccinated as indicated previously. Seven days after the last immunization, splenocytes from the vaccinated and control mice were harvested. The *in vivo* primed splenocytes were cultured for 6 days in the presence of 20 μg/ml TUBO-CRCL and 20 U/ml IL-2. Viable cells were then collected by Ficol density centrifugation and used as effector cells. ^{51}Chromium release cytotoxicity assays were performed to evaluate the ability of the cytotoxicity to lyse TUBO cells. Targets (5×10^3 cells/well) were labeled with 100 μCi of ^{51}Cr (Amersham Pharmacia Biotech, Piscataway, NJ) in complete DMEM for 1h. Stimulated effector cells were incubated with target cells at indicated ratios for 4-6 hours. Percentage of specific lysis was calculated as ([experimental ^{51}Cr release-spontaneous release] / [maximum release – spontaneous release]) x100.

Flow cytometry

The expression of HER2/neu was evaluated by using anti-HER2/neu mouse antibody, 7.16.4 mAb (Oncogene, Cambridge, MA) (32, 33). Monoclonal antibodies against H-2K^d (clone 31-3-4S) H-2D^d and Ia^d (clone 28-16-8S) were obtained from eBioscience (San Diego, CA). Cells were stained in a standard indirect immuno-fluorescence procedure with primary antibody followed by a fluorescein

(Alexa Fluor 488)-conjugated goat anti-mouse IgG (Molecular probes, Eugene, OR) (32). It has been shown that TUBO cells are highly positive for HER2/neu and class I glycoproteins of the MHC (32). To evaluate the presence of antibody capable of binding TUBO cells, sera were collected from naïve and tumor-bearing BALB/c mice. A total of 2 x 10^5 TUBO cells were stained in a standard indirect immuno-fluorescence procedure with 50 µl of a 1:10 dilution in PBS-azide-BSA of normal or immune sera followed by a fluorescein-conjugated anti-mouse IgG (Molecular probes) (32). The cells were re-suspended in PBS-azide-BSA evaluated using a FACScan (Becton Dickinson, San Jose, CA).

Cell internalization of HER2/neu

Expression of HER2/neu was evaluated by confocal microscopy. A total of 2 x 10^5 TUBO cells were suspended in DMEM, incubated with 50 µl of a 1:10 dilution in PBS-azide-BSA of normal or immune sera for 3 h at 4°C or at 37°C, and washed twice with cold PBS-azide-BSA. For detection of cytoplasmic HER2/neu, TUBO cells were incubated with 1 ml of PBS-4% paraformaldehyde at 4°C. After 20 min, TUBO cells were washed twice with cold PBS-azide-BSA and then incubated with 1 ml of PBS-0.3% Triton X-100. After 30 min at room temperature, TUBO cells were washed twice with cold PBS-azide-BSA. Membrane and cytoplasmic expression of

HER2/neu TUBO cells was assessed by staining with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Internalization of fluorescent mAb was then measured on a confocal microscope (LFM310; Zeiss, Jena, Germany) (488-nm argon laser). Green fluorescence was detected after excitation at 488 nm. Images were recorded as TIF files and processed (LSM Image Examiner; Zeiss) to subtract background and enhance lower and middle intensity fluorescence (32).

Antibody-Dependent Cellular Cytotoxicity

The ADCC test was performed as previously described (32, 33, 151). HER2/neu-positive tumor cells TUBO were plated $1x10^4$ cells/well in 96-multiwell plates and allowed to attach overnight at 37°C. Cultures were incubated for 2 hours on ice with sera from experimental and control mice at different dilutions. After washing, spleen cells from normal BALB/c mice were added at 50:1 E:T ratio and plates were incubated at 37°C overnight. Non-adherent cells were carefully washed out, 20 µl of WST-1 solution (Roche Diagnostics, Indianapolis, IN) was added to each well, and absorbance at 450 nm was read after additional 4-hour incubation.

Statistical analysis

All statistical analysis were made using the student's *t* tests to evaluate significance

between groups, except that the Kaplan-Meier product-limit method was used to plot survival and the log-rank statistic to test differences between groups. For all cases, results were regarded significant if *P*-values were less than 0.05.

2.4 RESULTS

HER2/neu expressing tumor-derived CRCL delay tumor growth *in vivo*

To determine if TUBO tumor derived CRCL enhances the anti-tumor response, female BALB/c mice were injected *s.c.* on day 0 with TUBO cells as described above. Mice bearing established palpable tumor were vaccinated (s.c.) in the opposite groin with TUBO tumor-derived CRCL. The results indicate that vaccination of HER2/neu tumor-derived CRCL caused a significant delay in tumor growth (Figure 7). More than half of the mice reached the same tumor volume as the control mice about 30 days later. In contrast, tumors grew progressively in both PBS and 12B1 tumor-derived CRCL treated mice. These results argue for an anti-tumor effect of TUBO tumor-derived CRCL against tumors expressing HER2/neu.

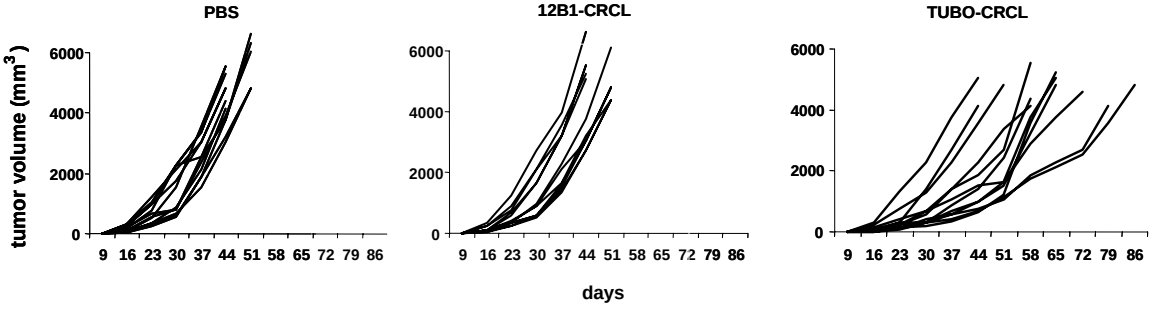

Figure 7 Tumor growth in vaccinated mice inoculated with TUBO. $1x10^5$ TUBO cells were injected to BALB/c mice s.c. on day 0. From day 9 onwards, mice were vaccinated in the opposite groin with PBS, 12B1 tumor-derived CRCL, or TUBO tumor-derived CRCL 0, 2, 4, 7, 10 and 14 days after the tumor became palpable. Individual tumor size was measured starting from day 9 every week. Mice with tumors exceeding 4000 mm^3 in volume at the time of inspection were euthanized and considered to have not survived the challenge. These data are from a representative experiment of three that were performed independently.

Vaccination with TUBO tumor-derived CRCL elicited specific antibody secretion

It has been demonstrated that antibody responses are critical in HER2/neu positive tumor immunity (33, 148, 150, 152). To study the humoral response in our model, BALB/c mice were vaccinated with TUBO tumor-derived CRCL as previously described. Anti-HER2/neu antibody response was markedly increased in the mice vaccinated with TUBO tumor-derived CRCL (Figure 8). The anti-HER2/neu antibody titers did increase one week after the first vaccination, and reached a higher level one week after all six vaccinations. In addition, within three weeks, high titers of antibody persisted in host blood. Besides the total IgG, we investigated the type I antibody, IgG2a and IgG3, and type II antibody, IgG1. Both IgG2a and IgG3 are increased after TUBO-CRCL vaccination. IgG1 response was also increased, but at 1:10 sera dilution, we saw non-specific binding (Figure 8).

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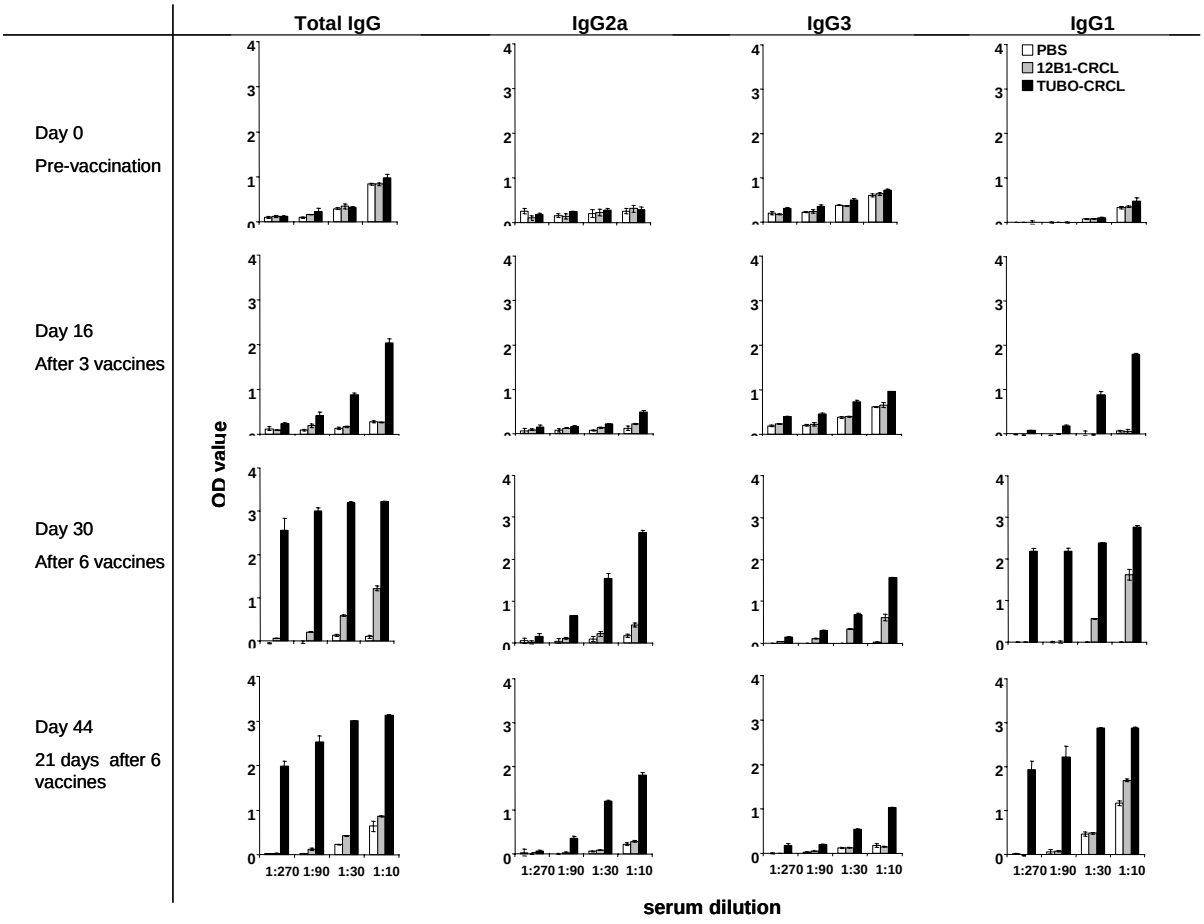

Figure 8 Characterization of anti-HER2/neu antibody secretion after vaccination with TUBO tumor-derived CRCL. Pooled sera from eight vaccinated mice were examined for anti-HER2/neu total IgG and the isotype IgG1, IgG2a and IgG3 levels by ELISA. Values represent the average intensity at $OD_{410\ nm}$ of duplicate wells at 1:270, 1:90, 1:30 or 1:10 dilutions, respectively. Sera from mice injected with PBS and 12B1 tumor-derived CRCL were used as control. The error bars represent the range of duplicate determinations. These data are representative of two experiments that were performed independently.

Anti-tumor activity can be transferred with immune sera

To determine if the observed anti-tumor activity could be transferred with the immune sera, BALB/c SCID mice were injected i.v. with pooled immune sera from TUBO-CRCL vaccinated naive BALB/c mice and then injected s.c. the next day with $1x10^5$ TUBO cells. No apparent anti-tumor activity was observed with sera from mice vaccinated with PBS or 12B1 tumor-derived CRCL. Conversely, in animals injected with sera from mice vaccinated with TUBO tumor-derived CRCL, the tumors were smaller than the control mice (Figure 9). The difference became significant beginning day 24. Therefore, the ability of the sera from TUBO-CRCL vaccinated mice confers an immune sera anti-tumor effect which correlates with the magnitude of the anti-HER2/neu response.

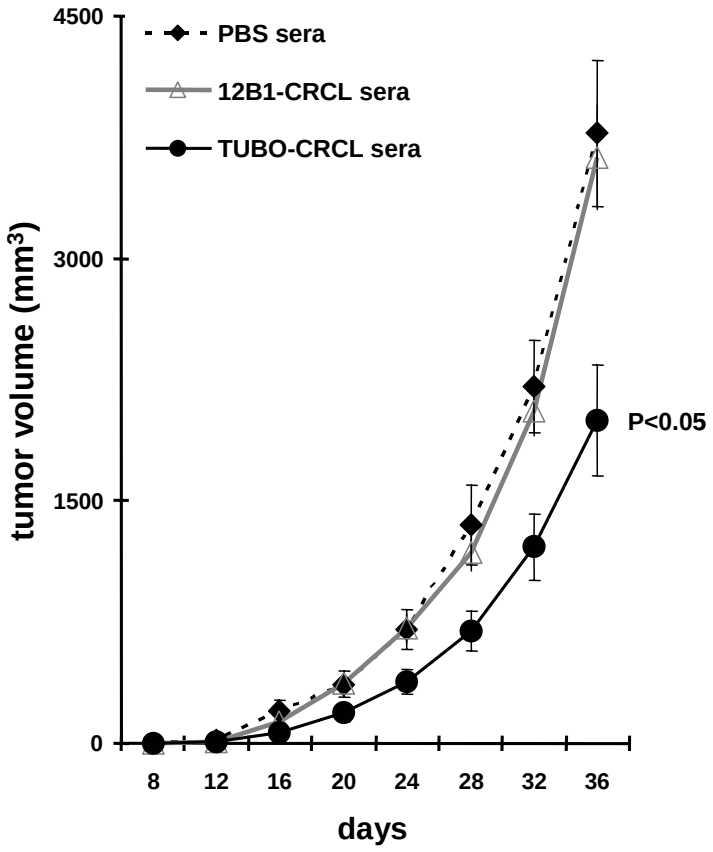

Figure 9 Anti-tumor activity was transferred with immune sera. At day -1, BALB/c SCID mice were injected i.v. with 200 µl of pooled sera. These sera were collected from naive BALB/c mice vaccinated with PBS or 12B1 or TUBO tumor-derived CRCL, respectively. On day 0, $1x10^5$ TUBO cells were injected in the right groin. The differences between the groups were statistically significant from day 24 ($p<0.05$). HER2/neu-specific antibodies in the sera from vaccinated mice were verified by ELISA.

TUBO tumor-derived CRCL-induced anti-tumor immunity requires B cells

In order to further investigate whether HER2/neu-specific antibody in CRCL vaccinated mice had any consequence in tumor delay *in vivo*, we used J_H knockout mice, lacking B cells, in the H-2^d background. B cell deficient mice were injected s.c. on day 0 with $1x10^5$ TUBO cells. After the tumor became palpable, the mice were injected s.c with TUBO tumor-derived CRCL and PBS as described in the methods. Tumor volume was measured weekly. Figure 10B indicates that mice vaccinated with TUBO tumor-derived CRCL were not protected against TUBO tumor growth. By comparison, in normal wild type BALB/c mice, TUBO-derived CRCL vaccination significantly delayed tumor progression (Figure 10A). No CTL activity was observed from the mice that received TUBO-CRCL vaccination (Figure 11). Taken together these results suggest that B cells and presumably antibodies are necessary for the observed anti-tumor effect in this cancer model.

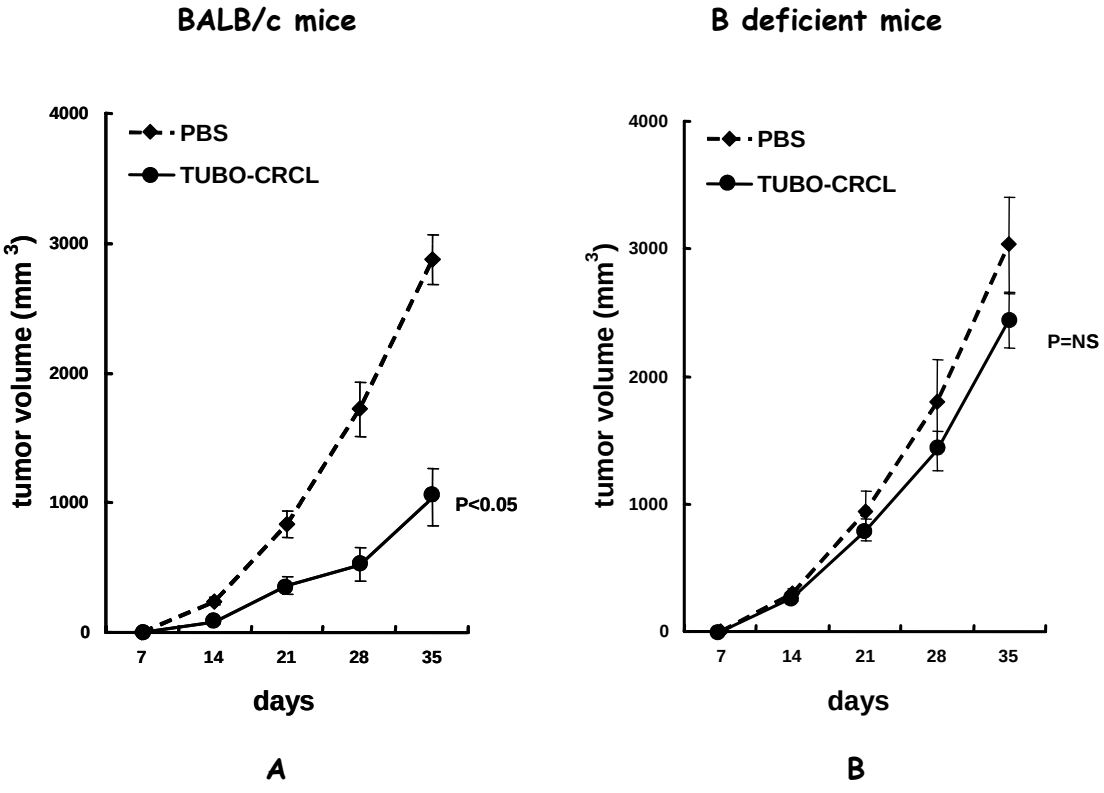

Figure 10 Effect of TUBO tumor-derived CRCL vaccine on tumor growth in B-cell deficient mice. (A) Wild type and (B) B cell deficient BALB/c mice were injected s.c. on day 0 with $1x10^5$ TUBO cells. After the tumor became palpable, the mice received the s.c vaccination with indicated vaccines. Tumor volume was measured weekly. There were no significant differences ($P>0.05$) between the two groups of mice in B cell deficient mice, but significant differences ($P<0.05$) in wild type mice. These data are representative of two experiments that were performed independently.

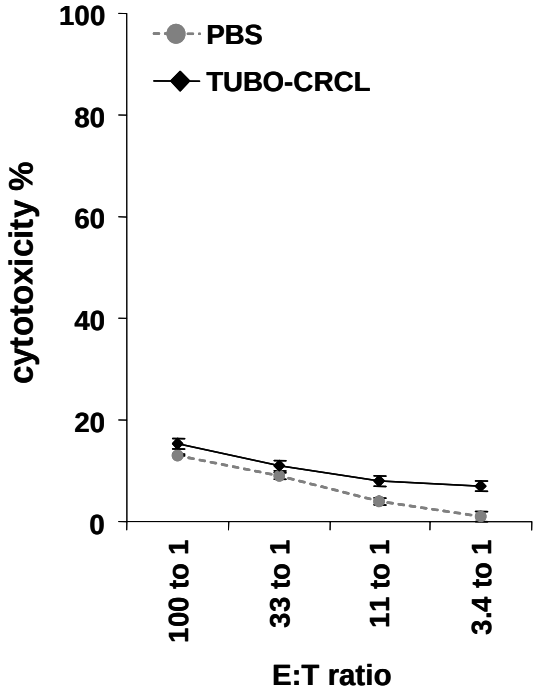

Figure 11 Immunization of mice with TUBO-derived CRCL induces no tumor specific CTL activity. Naïve mice were vaccinated as described previously. On day 0, splenocytes were harvested from TUBO-CRCL vaccinated mice and control mice. The cells were cultured in the presence of 20 μg/ml TUBO-CRCL and 20 U/ml IL-2 for 6 days. Viable cells were then harvested by Ficol density centrifugation and used as effector cells. TUBO cells (5×10^3 cells/well) were labeled with 100 μCi of ^{51}Cr in complete DMEM for 1h. Stimulated effector cells were incubated with labeled TUBO cells at indicated ratios for 4-6 hours. Representative data from 3 experiments are shown.

Serum from TUBO tumor-derived CRCL vaccinated mice induces down-regulation of TUBO cell surface HER2/neu expression

When incubated with TUBO cells, the sera from TUBO tumor derived CRCL vaccinated mice was able to bind to the cell membrane (Figure 12), and induced the down-regulation of HER2/neu membrane expression and its cytoplasmic re-localization (Figure 13). No similar phenomenon was observed when TUBO cells were incubated with control sera from PBS or 12B1 tumor-derived CRCL vaccinated mice. This suggests that this response is HER2/neu-specific and may be one of the tumor inhibition mechanisms induced by TUBO-derived CRCL vaccination.

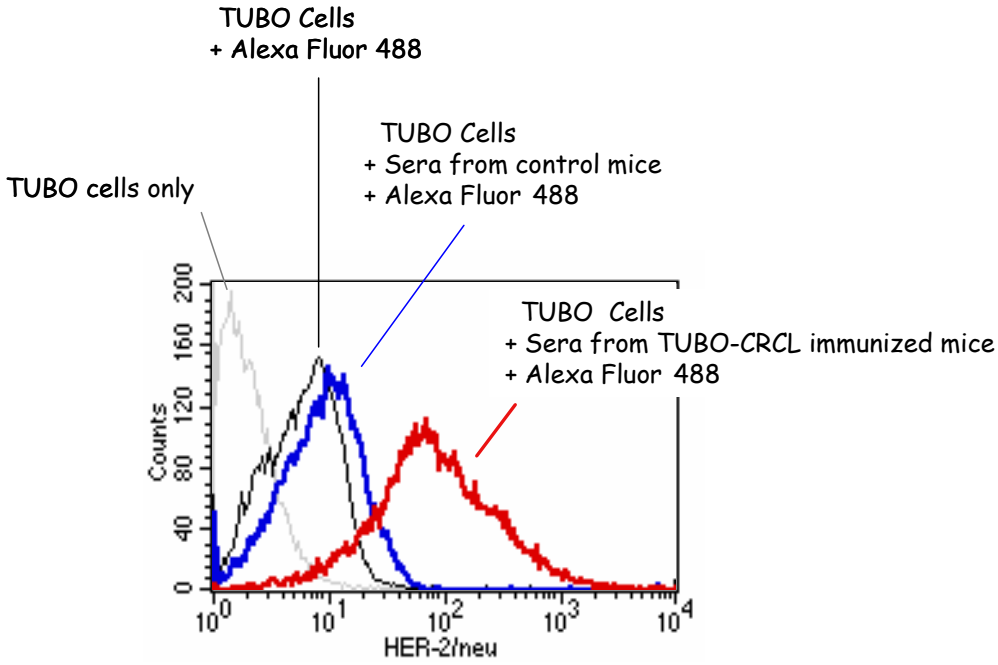

Figure 12 Ability of sera from TUBO tumor derived CRCL vaccinated mice to bind TUBO cells. A total of 2 x 10^5 TUBO cells were stained in a standard indirect immuno-fluorescence procedure with 50 µl of a 1:10 dilution in PBS-azide-BSA of normal or immune sera followed by a fluorescein-conjugated anti-mouse IgG. Grey line: TUBO cells only; Black line: cells stained with secondary antibody; blue line: cells stained with sera pool from control mice and secondary antibody; red line: cells stained with sera pool from TUBO tumor-derived CRCL vaccinated mice and secondary antibody. The ordinate represents the number of cells and the abscissa reports the fluorescence intensity in arbitrary logarithmic units.

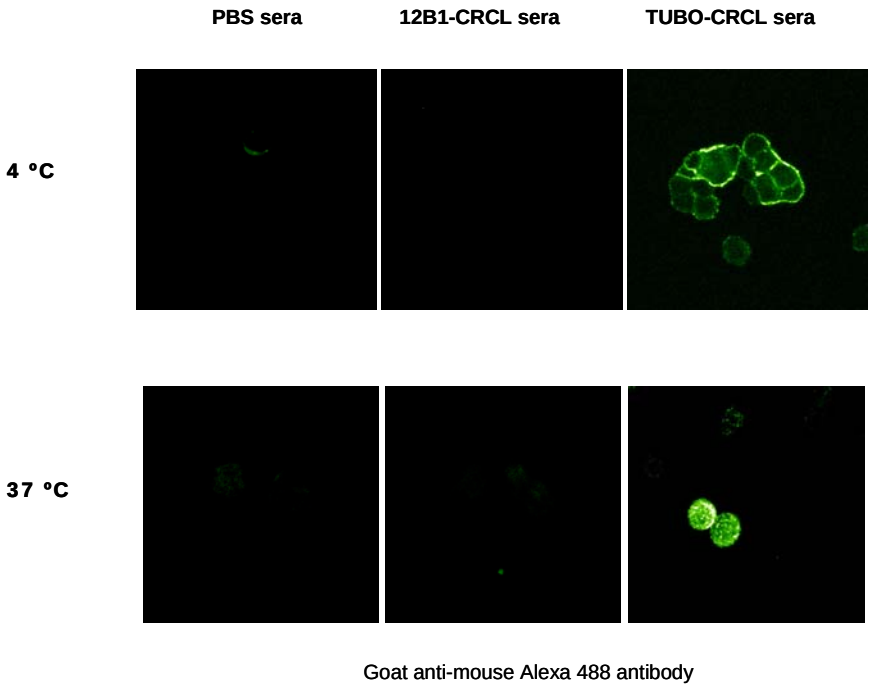

Figure 13 Down-modulation of cell membrane expression of HER2/neu. Confocal analysis of HER2/neu expression after a 3-hour incubation of TUBO cells at 4°C or at 37°C with sera from control or TUBO tumor-derived CRCL vaccinated mice. Membrane and cytoplasmic expression of HER2/neu TUBO cells was assessed by staining with Alexa Fluor 488-conjugated goat anti-mouse IgG. Representative data from two experiments are shown.

Serum from TUBO tumor-derived CRCL vaccinated mice facilitates ADCC

Antibody-dependent cellular cytotoxicity is one of the mechanisms by which antibodies act against tumors. In our HER2/neu positive breast cancer model, in addition to the direct down-regulation of HER2/neu molecule on the cell membrane, the antibodies may also affect tumor growth by ADCC. To address this issue, TUBO cells were incubated in the presence of sera from TUBO-CRCL vaccinated or from control mice and BALB/c mouse splenocytes. Approximately 30% cytotoxicity was detected with 1:5 sera from TUBO-CRCL vaccinated mice compared with about 5% killing with control sera (Figure 14). This result suggests that ADCC may consist of an additional mechanism by which TUBO-CRCL induced antibodies negatively modulate TUBO growth.

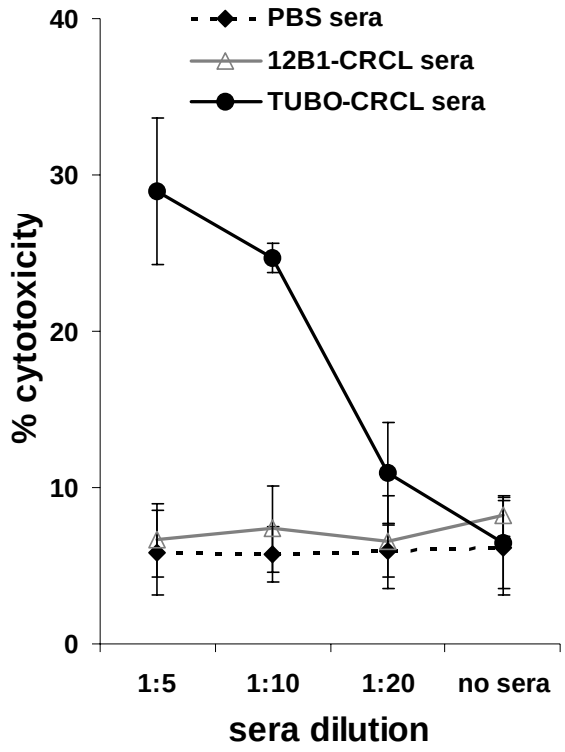

Figure 14 Antibody-dependent cellular cytotoxicity with sera from mice vaccinated with TUBO tumor-derived CRCL. TUBO cells were plated $1x10^4$ cells/well and allowed to attach overnight. Cultures were incubated for 2 hours at 4°C with sera from experimental mice at different dilutions. After washing, BALB/c spleen cells from normal mice were added at 50:1 E:T ratio and plates were incubated at 37°C overnight. Each point represents the mean ± SEM of triplicate sera sample from mice bled after the six times of vaccination. These data are representative of three experiments that were performed independently.

2.5 DISCUSSION

I mentioned previously that tumor-derived CRCL is a personalized vaccine carrying multiple original tumor antigens and epitopes. It has been demonstrated that CRCL vaccine is efficient in inducing T cell-dependent anti-tumor responses in various murine models by numerous published works (79, 80, 82) and further in humans by the part I of my work. In this part II study, we investigated whether our CRCL vaccine confers anti-tumor effect through antibody effects, which is the first study of the immuno-stimulatory effects of the HSP based vaccine on humoral immunity.

It is becoming clear that increasing anti-tumor immunity may require different approaches depending on the type of the cancer. Indeed, while some cancers are sensitive to cell-mediated immune responses, others are not but are readily eradicated by humoral mediated effects (32, 33, 147, 153). Although the detection of antibodies against tumor antigens has been described by a number of investigators in a variety of systems(154, 155), the role of antibodies in tumor immunity especially in cancer patients remains controversial. This uncertainty may reflect the fact that different tumor cell types or animal models require different mechanisms to invoke immunity against cancer. Therefore, it is important to choose an appropriate experimental model for the proposed study of humoral immunity.

Experimental animal models where antibodies play a role in tumor immunity will provide an enlightening evidence for the investigation.

We utilized a HER2/neu positive murine breast cancer model to test our hypothesis, considering the immunogenicity of this antigen and the importance of specific antibody immunity in the control of the tumor, TUBO. HER2/neu positive TUBO cells can grow in normal BALB/c mice and give rise to lobular carcinoma histologically similar to that seen in BALB-neuT-transgenic mice (32). Although TUBO expresses rat HER2/neu, it is 92% identical with human HER2/neu, and provides a feasible and reliable model system for HER2/neu study (156). In fact, most data to date on HER2/neu related vaccines have come from studies in genetically engineered animals expressing rat HER2/neu (157). Previous studies have shown that the TUBO tumor model can be used successfully to study HER2/neu related vaccination strategies (32, 147). For instance, humoral responses against TUBO tumor have been shown to be elicited by vaccines of HER2/neu ECD with antibody-cytokine fusion proteins (147, 148). In another study, plasmids encoding ECD^{HER2} and transmembrane domains protected normal BALB/c mice against a TUBO cell challenge, with induction of anti-HER2/neu antibody, but only a marginal CTL reactivity. Furthermore, the nonredundant role of antibody in the eradication of TUBO carcinomas has been

demonstrated by the fact that protection by this DNA vaccination was almost abrogated in B cell deficient mice (31, 32). Park et al. have documented that anti-HER2/neu serum not only protected mice from TUBO tumors *in vivo* but also inhibited growth of HER2/neu mammary tumor *in vitro*, suggesting a direct action of antibody on the tumor cells (11). Therefore, we consider TUBO as an ideal model for our study. Actually, we have demonstrated that tumor-derived CRCL could elicit humoral immunity. Additionally we found that HER2/neu internalization and ADCC might be two possible mechanisms by which CRCL mediates its potential anti-tumor effects. Therefore, CRCL is capable of eliciting the other very important arm of immunity, antibody production by B cells.

It has been found that IgG titer was amplified after the vaccination of TUBO tumor-derived CRCL. Anti-HER2/neu IgG2a, IgG3, and IgG1 were all enhanced by TUBO-CRCL vaccine. IgG2a (158) and IgG3 production has been reported to be associated with a T_H1 immune response, while IgG1 production is usually linked to a T_H2 immune response (147, 159). However, we have not yet clearly established any relationship between the isotype of the antibodies and their function. The anti-HER2/neu antibody titers did increase one week after the first vaccination, and reach a higher level one week after all the six vaccinations. In addition, within

three weeks, these high titers of antibody persisted in the host blood, suggesting that a long-term immunity had been elicited. Active vaccine strategies including HSP might be more effective in the treatment of patients with HER2/neu expressing cancers than passive infusion of monoclonal anti-HER2/neu antibodies. Indeed, passive antibodies may be cleared from the circulation, which may lessen their therapeutic potential (160, 161). Therefore, an effective vaccine targeting HER2/neu antibody production by the body's own B cells may provide a continuous supply of anti-HER2/neu antibodies.

Our current study also underlines the importance of B cells in tumor immunity. During an immune response B cells play multiple roles including acting as APCs to activate T cells (162, 163) and as plasma cells to produce antibodies. The present studies indicate that B cells play a crucial role in anti-tumor immunity in mice vaccinated with tumor-derived CRCL. However, unlike the depletion studies in which T cell or NK cells were depleted after induction of the immune response but prior to the tumor challenge (32, 33), B cells were absent during both the inductive and effector phases in our J_H knockout mice. Therefore, it is not possible to determine if B cells are required to induce tumor immunity as an APC or play an important role as immune effectors, or both. Since CRCL has been successfully shown to activate

other APC such as DC, it will be interesting to explore the APC function of the B cell in the vaccination of CRCL.

Although, anti-tumor immunity against HER2/neu expressing cancers correlates with increased levels of anti-HER2/neu antibodies, in particular, of the IgG2a isotype (32, 33, 147, 153, 164), it is still not clear what role anti-HER2/neu antibodies play in this immunity. It has been shown by other groups that HER2/neu immunization can induce antibodies that inhibit the *in vitro* growth of SK-BR-3 (147). However, we did not observe this effect with immune sera from TUBO tumor-derived CRCL vaccinated mice (data not shown). Another mechanism indicates that anti-HER2/neu antibody induces a functional block of HER2/neu receptor function (165) by down-regulating its expression on the cell membrane (165, 166). The decreased expression could impede the ability of HER2/neu receptor to form homo- or hetero-dimers (166, 167), and block its ability to bind ligands (168). In our study, the sera from TUBO tumor-derived CRCL vaccinated mice, when incubated with TUBO cells, could bind to the membrane and induced down-regulation of HER2/neu from the membrane and its cytoplasmic internalization. Reduced HER2/neu expression may be sufficient for the reversal of the transformed phenotype into a more normal one (165, 166). This result suggested that the HER2/neu

down-regulation here may reduce carcinogenic progression. This effect appears to mostly depend on an antibody response to a growth factor receptor whose down-regulation slows preneoplastic cell proliferation and tumor development, and the inhibition mechanism is different from immunological destruction of the malignant cells. Usually, in vaccinated mice, leukocytes present at the tumor growth site also play an important regulatory role in cytotoxicity (79, 80). In fact, ADCC in the presence of vaccinated sera has been demonstrated, implying another of antibody-dependent mechanisms.

We did not detect any direct killing by $CD8^+$ T cell through the *in vitro* Cr release assay (Figure 13). However, we cannot rule out the possibility that TUBO-CRCL does elicit functional CTLs by our current data. Even though CTL activity can be demonstrated by Cr release assays in other tumor models that we have examined, cytolytic activity was not seen against TUBO. It is unknown whether we can generate potent effector cells from splenocytes by using 5-day stimulation with TUBO-CRCL. Considering these aspects, we may need to stimulate the responding cells from the spleen several more times, augmenting specific T cell activity. Alternatively, we can isolate the $CD8^+$ T cell and perform the same assay. Secondly, as mentioned in immunosuppression discussion, tumor cells could escape the efficient

immune surveillance by non-immunogenic tumour cell variants, such as by the low expression of MHC-I molecules (125). To prove this, the target TUBO cells can be pre-treated before Cr release assay with IFN-γ, which can amplify the epitope processing and presentation with MHC-I of the cell (169). If all of the optimizations do not work, we can utilize the $CD8^+$ T cell depletion mice to confirm the effect of CTL, since the anti-tumor effect of TUBO-CRCL will be lost in depletion mice compared to wild type mice if the effect is dependent on $CD8^+$ T cells.

2.6 ACKNOWLEDGEMENTS

We are grateful to Dr. Penichet for providing us the ECD^{HER2} protein for the ELISA test of the antibody and Barb Carolus for her valuable assistance during the confocal experiment.

CONCLUSION

The work presented here is part of an ongoing effort to better understand the immune responses elicited by tumor-derived CRCL and its potential for further clinical application. As we all know, methods of generating anticancer immunity must focus on the initiation and maintenance of an immune response that can stimulate tumor-specific T lymphocytes and B cells. Chaperone-rich cell lysate isolated from tumors is possible to present multiple epitopes including naturally processed T and B cell specific epitopes. This is especially important in HER2/neu positive cancers as both cellular and humoral immunity appear to play critical roles. Therefore, we chose the HER2/neu positive tumor as our model expecting this multi-epitope based HSP vaccine, CRCL, would be capable of eliciting both innate and adaptive immune responses and assist the host against HER2/neu positive cancers.

HER2/neu is over-expressed in various cancers such as breast, ovary, colon, prostrate and bladder (6). As an oncoprotein, its over-expression provides both growth and survival advantages for the tumor and is consequently associated with poor prognosis (7, 8). The selective expression of HER2/neu and its role in carcinogenesis makes HER2/neu an ideal candidate for immunotherapy (170).

Preclinical and clinical trials have shown the effectiveness of HER2/neu related immunological strategies such as peptides and humanized monoclonal antibody (5, 62, 139, 141). However, none of these approaches has improved the outcome of metastatic HER2/neu cancers. The major disadvantages with peptide vaccination are the magnitude and durability of responses along with the limitations of MHC restriction (5, 62, 146). The main problems with monoclonal antibody therapy have been the development of toxicity and resistance (36, 52). Thus, more innovative approaches to treat HER2/neu positive cancers such as CRCL deserve to be intensely investigated and developed.

Although numerous studies have confirmed that tumor-derived CRCL could induce T cell responses against the original tumor, those experiments are all based on murine tumor models. Therefore, the immunological T cell responses to CRCL vaccine are worthy of further definition in humans. For this reason, we performed the first part of the study to test the immuno-stimulatory effects of CRCL on HER2/neu positive human ovarian cancer. The significance of this system is to substantiate in humans that CRCL could deliver a broader repertoire of tumor-specific antigens such as HER2/neu and WT1. Those results have proved the efficacy of obtaining CRCL from human tumors and generating T cell related immunologic responses *in vitro*.

Therefore CRCL vaccine deserves further exploration in cancer patients in the clinic.

As mentioned in the background, the humoral response induce by CRCL vaccines has not been sufficiently addressed. Hence, I investigated antibody responses elicited by HER2/neu positive tumor-derived CRCL, in a murine model in the second part of my study. The finding is unique and suggests that CRCL could induce tumor specific antibodies in vaccinated mice, validating that in addition to T cell epitopes CRCL can also present B cell epitopes to the immune system. Another significant observation is the characterization of the antibody responses, as limited information was known for CRCL/HSP vaccines about the mechanism of HER2/neu specific antibodies.

Conclusively, as a polyvalent vaccine, CRCL can chaperone both T cell and B cell epitopes. Accordingly, both cellular and humoral immunity can be elicited by CRCL vaccine. My studies about the anti-tumor effect of CRCL on a HER2/neu positive cancer confirm the T cell efficacy generated by tumor-derived CRCL in humans. On the other hand, HER2/neu specific humoral responses elicited by tumor-derived CRCL were demonstrated for the first time, and the mechanisms of how it works were elucidated. Altogether, the successful completion of this work advances our knowledge on the utility of CRCL vaccine, and provides a foundation that could lead to

improved therapy for HER2/neu positive cancer, for which ineffective and expensive therapy is the current option.

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