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SYMPOSIUM IN WRITING

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# Chaperone-rich cell lysates, immune activation and tumor vaccination

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**Abstract** We have utilized a free-solution-isoelectric focusing technique (FS-IEF) to obtain chaperone-rich cell lysates (CRCL) fractions from clarified tumor homogenates. The FS-IEF technique for enriching multiple chaperones from tumor lysate is relatively easy and rapid, yielding sufficient immunogenic material for clinical use. We have shown that tumor-derived CRCL carry antigenic peptides. Dendritic cells (DCs) uptake CRCL and cross-present the chaperoned peptides to T cells. Tumor-derived CRCL induce protective immune responses against a diverse range of murine tumor types in different genetic backgrounds. When compared to purified heat shock protein 70 (HSP70), single antigenic peptide or unfractionated lysate, CRCL have superior ability to activate/mature DCs and are able to induce potent, long lasting and tumor specific T-cell-mediated immunity. While CRCL vaccines were effective as stand-alone therapies, the enhanced immunogenicity arising from CRCL-pulsed DC as a vaccine indicates that CRCL could be the antigen source of choice for DC-based anti-cancer immunotherapies. The nature of CRCL's enhanced immunogenicity may lie in the broader antigenic peptide repertoire as well as the superior immune activation capacity of CRCL. Exongenous CRCL also supply danger signals in the context of apoptotic tumor cells and enhance the immunogenicity of apoptotic tumor cells, leading to tumor-specific T cell dependent long-term immunity. Moreover, CRCL based vaccines can be effectively combined with chemotherapy to treat cancer. Our findings indicate that CRCL have prominent adjuvant effects and are effective sources of tumor antigens for pulsing DCs. Tumor-derived CRCL are promising anti-cancer vaccines that warrant clinical research and development.

**Keywords** Chaperone/Heat shock proteins · Dendritic cells · Tumor · Vaccine

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## Introduction

The roles of chaperone proteins, often called heat shock proteins (HSPs) as mediators of the heat/stress response have been recognized and studied for nearly 20 years [47]. Arguably, the intersection of chaperone proteins and the immune response began in 1986 with the publications from Ullrich et al. [72]and Srivastava et al. [65] that described the purification of a protective immunizing activity from tumor lysates. The tumor associated antigens (TAAs) in those anti-cancer vaccines were identified as members of the HSP90 family, and since that time there has been a steady increase in the overall interest in chaperone protein involvement in the immune response. Since those ground-breaking publications, chaperone protein-based anti-cancer vaccines have progressed from an intriguing but essentially untenable phenomenon involving chemically induced carcinoma models in animals to a, cancer treatment strategy worthy of phase III clinical trials [48].

Subsequent studies demonstrated that chaperone proteins from other "families," i.e., HSP70 and calrectiulin, could also provide tumor-specific protection in animal tumor models [7, 31, 70]. As with the original works, the efficacy of the vaccine was inherently tied to the tumor from which the chaperones were purified. Vaccines prepared from one tumor type could not cross-protect against challenges from tumors of another type, and vice-versa. The immunological identity of the HSP vaccines appeared to rely on the demonstrated in vitro peptide-binding capacity of at least some of the chaperones [25] as well as the speculated "peptide antigen fingerprint" that presumably differed from tumor to tumor [14].

Mechanistically, several immunological criteria needed to be met for these vaccines, as any vaccine, to be effective:

- Antigens necessary to provide specific T cell activation
- Provision for cross-presentation of those antigens by antigen presenting cells (APCs), as the pathway is exogenous
- Activation of the APCs to effectively stimulate (and co-stimulate) T cells. This is usually provided by adjuvants, but chaperone proteins provide their own adjuvanticity (see below).

One additional feature of this immunogen/APC interaction that was postulated early on was the existence of specific receptors on APCs for chaperone proteins which might explain the unusually potent immunizing effect. Several of those receptors have been described (see below), although it remains an area of considerable controversy. These receptors are likely conduits for the intrinsic capacity to stimulate innate immune responses, regardless of the origin of those chaperones and their peptide cargo (see below). Thus, chaperones are at a nexus between the innate and adaptive immune responses, capable of providing both adjuvant and antigen as a unit.

In a similar attempt to provide tumor-specific antigenicity as a form of immunotherapy, many groups have employed vaccines derived from dead or irradiated whole tumor cells, or tumor homogenate material, generated in any number of ways as necrotic or apoptotic lysates or forms of whole cell vaccines. The relative ease of production of this type of vaccine, as well as the idea that essentially all of the antigenic components of the tumor should be represented in the lysate (protein, peptide, carbohydrate, lipid, etc.), logically suggests that such preparations would be the perfect tumor-specific vaccine. However, compared to such whole tumor preparations, chaperone protein vaccinations have been demonstrated to be more effective [30, 32, 37, 71, 77, 78]. While there is considerable debate about the appropriate lysate format for vaccinations (i.e., necrotic versus apoptotic cell death), there is also some concern that most cell lysates are ineffective [40]. It is very likely that there are inhibitory substances present in tumor cell lysates that could actually suppress the anti-tumor immune response [33]. Thus, despite the relative ease of preparation of tumor cell lysates, and the expectation of high-level antigen representation, tumor cell lysates may not be the optimal antigen source for the generation of anti-tumor immunity.

Into this immunological arena we have brought novel anti-cancer vaccines that we call chaperone-rich cell lysates, or "CRCL." CRCL are generated via a free solution-isoelectric focusing technique (FS-IEF), using tumor-derived materials, which results in an enrichment for chaperone proteins rather than a purification of them. Thus, CRCL preserve what is hoped to be antigenic, while electrophoretically excluding at least some presumed, immunosuppressive agents. CRCL combine the relative simplicity of lysate preparations, along with high yield and large antigen repertoire of vaccine per unit amount of starting material. The primary feature of CRCL is the combination of chaperones, including HSP90, HSP70 family members, and the endoplasmic reticulum (ER) chaperones glucose regulated protein (GRP)94/glycoprotein (gp)96, and calreticulin. Each of those chaperones has been utilized individually as anti-tumor vaccines [31, 63]. In addition, CRCL contain HSP40, HSP60, GRP75, and GRP78, as identified by immunoblotting with specific antibodies [30]. All of these proteins are gathered into a single preparation by the unexpected clustering of the chaperones that occurred during the FS-IEF procedure [30]. The integrity of the chaperone "complex" was maintained as a high molecular weight entity over size-exclusion chromatography in 6 M urea, and this "complex" remains obdurate to further separation over a variety of chromatographic matrices (M. Graner and J. Davis, unpublished data). While there are a number of other unidentified proteins in CRCL, immunodepletion of HSP/HSC70, HSP90, GRP94, and calreticulin resulted in a significantly abrogated immune response [78; J. Davis et al., unpublished data], indicating that the chaperones were truly relevant to the vaccine effects.

Using this FS-IEF method we are able to generate approximately 1–2 mg of CRCL vaccine per gram of starting tissue in a timely fashion. Given the limitations of preparing tumor vaccines from autologous tumor in the clinical setting, FS-IEF is a relatively simple, rapid, and efficient procedure, allowing one to obtain 30–50 times as much vaccinating material from the same quantity of tumor as with conventionally purified HSPs. This makes the FS-IEF method of multiple chaperone complex enrichment desirable from a clinical standpoint in terms of high yield from a potentially limited tumor source, and with a rapid turn-around time from tumor harvest to treatment of the patient.

This review focuses on the immune system activation by chaperones, and by CRCL in particular. We also describe how CRCL-based vaccines might be applied with different immunotherapeutic strategies.

## Immune stimulating effects of CRCL

### Antigen specific effects of CRCL

#### *Cross-presentation of endogenous antigenic peptides carried by CRCL*

As mentioned above, chaperone proteins as "tumor antigens" have been postulated to be carriers of immunogenic moieties such as peptides [66]. Chaperone/peptide associations would thus reflect antigenicity in the currency of the cellular immune response; in essence, the chaperones take on the immumnological identity of the tissue in which they resided. This concept has become largely entwined with the antigenic properties of tumor-derived chaperones [63]. We reasoned that tumor-derived CRCL could chaperone antigenic peptides. Initially, the peptide content of the vaccine was implied by the specificity of the vaccine to protect immunized animals against tumor challenges when the vaccine was derived from the same tumor as used in the challenge. Vaccination of mice with CRCL from one tumor type, but challenge from a different tumor type, resulted in no protection for those animals [78, 79]. However, due to the isofocusing conditions used in the vaccine generation (i.e., 6 M urea), one could argue that the peptide repertoire of CRCL would be lost. Also, recent works have questioned both the ability of chaperones proteins to maintain interactions with peptides in vivo [58, 60] and the necessity of tumor-specific peptides for chaperone protein vaccine effects [3, 54]. We have further explored the peptide content of CRCL by acid-stripping of peptides followed by partitioning of low molecular weight compounds ($<3$ kDa) from the stripped proteins. The low molecular weight materials were separated via reverse-phase high pressure liquid chromatography (rpHPLC), and fractions were analyzed by mass spectrometry techniques. These studies demonstrated that a wide variety of peptides end up in the escort of CRCL chaperones, with several peptides derived from proteins that may have roles in cancer, including thymosin beta 4, glutathione transferase, and specifically identifiable tumor antigens such as peptides from triosphosphate isomerase 1 (M. Graner, unpublished data) and peptides derived from the BCR–ABL fusion junction in a chronic myelogenous leukemia (CML) models [80] (see below).

The elements of cross-presentation, cross-priming, and peptide antigens are closely linked, and well-illustrated by the antigenicity of CRCL as studied in aforementioned BCR-ABL$^+$ murine CML models [80]. CML is characterized by the presence of a protein called BCR–ABL p210, a fusion protein resulting from an in-frame chromosomal translocation that links the N-terminus of the BCR protein with the C-terminus of the c-ABL protein. Codon combination at the fusion junction results in the creation of a unique amino acid (lysine) not present at that position in either "parental" protein [46]; thus, a potential T cell epitope is generated. BCR–ABL peptides have been previously reported to be able to bind MHC-I and MHC-II molecules [16, 18].

Cross-presentation following immunization of vaccines containing peptide antigens is generally implied by the generation of antigen-specific T cell responses in vaccinated hosts. We have demonstrated tumor-specific and antigen-specific activity in murine splenocytes from animals that were vaccinated with tumor-derived CRCL. We have also shown that the immune responses generated were dependent on both CD4$^+$ T cells and CD8$^+$ T cells since the immunity was partly abrogated after depletion of either cellular population and was completely lost upon depletion of both populations [78, 80]. Murine leukemia cells 12B1 and 32Dp210 express BCR–ABL protein, but differ in MHC I haplotype. We reasoned that BCR–ABL fusion peptides are likely to be components of the antigenic repertoire of leukemia-derived CRCL, and tested whether we could identify cross-presented and cross-primed immuonreactivity against the peptide. Splenocytes from mice vaccinated with BCR–ABL$^+$ leukemia-derived CRCL secreted interferon-$\gamma$ (IFN-$\gamma$) when re-stimulated with a BCR–ABL peptide, GFKQSS $\underline{K}$AL ( $\underline{K}$ = the unique amino acid at the fusion junction), indicating that BCR–ABL peptides are chaperoned by leukemia-derived CRCL. Similarly, splenocytes from mice vaccinated with CRCL derived from 32Dp210 were able to respond to either GFKQSSKAL or CRCL from 12B1 (and vice versa), indicating that cross-priming over MHC restrictions is possible [80]. Moreover, BCR–ABL specific cytotoxic T lymphocytes (CTLs) were induced in vivo by immunization of mice with DCs loaded with leukemia-derived CRCL [80]. These findings clearly demonstrate that DCs process CRCL-chaperoned antigens and cross-present the escorted antigenic peptides T cells in vivo. BCR–ABL protein was undetectable by Western Blotting in 12B1 or 32Dp210 CRCL, but was present in the lysates from both tumors. This confirms that the BCR–ABL specificity of leukemia-derived CRCL immunization is not an artifact of BCR–ABL protein co-separating into fractions pooled for CRCL vaccine preparation, but likely due to BCR–ABL peptides chaperoned by CRCL.

In other experiments, mice were vaccinated with DCs, the most potent of APCs, which had been pulsed with GFKQSSKAL or with 12B1 CRCL. Splenocytes were harvested from those mice, and were restiumlated with rpHPLC fractions of peptides eluted from 12B1 leukemia-derived CRCL. The same overlapping rpHPLC peptide fractions derived from 12B1 CRCL and from "re-fractionated" (i.e., pure) GFKQSSKAL stimulated IFN-$\gamma$ production, suggesting the co-elution of BCR-ABL peptides in the peptide repertoire of 12B1 CRCL [80]. Moreover, other fractions from rpHPLC-separated 12B1 CRCL peptides stimulated IFN-$\gamma$ production of 12B1 CRCL primed splenocytes, but not GFKQSSKAL primed splenocytes. This suggests that there are additional (as yet unidentified) antigenic peptides in 12B1

CRCL. Potential candidate antigens chaperoned by CRCL may include Wilms tumor antigen (WT-1) [36], minor histocompatibility antigens [52], and protease 3 antigen (PR1) [51], which have been documented to be potential CML tumor associated antigens. 12B1 cells express WT-1 protein as demonstrated by Western blotting (Y. Zeng, unpublished data), and other peptide candidate antigens have been mentioned above. Analyses are ongoing in our laboratory to identify the antigenic components of CRCL chaperoned peptide repertoire.

In addition to the endogenous peptide content of CRCL, studies in our laboratory have also shown that DCs take up CRCL that have been "embedded" with exogenous peptide, and those DCs can present the exogenous CRCL-associated-peptides on their cell surfaces, leading to specific T cell stimulation (Kislin et al., manuscript in preparation).

Thus, we have demonstrated that there are numerous peptides confined within CRCL, and that some of those peptides are antigenic and may be cross-presented by DCs to T cells. While recent reports have implied that chaperone adjuvant effects are of primary importance in generating anti-tumor immunity following chaperone protein immunization [4], the specificity of CRCL immunization draws attention to CRCL's peptide repertoire. Due to the complicated admixture of CRCL's multiple chaperone complexes, we do not have clear evidence as to the nature of the peptide-chaperone protein interactions within CRCL, nor do we necessarily believe that such interactions must occur readily in vivo and prior to cell lysis [43, 50]. Nonetheless, the presence and immunogenicity of tumor-derived peptides and the cross-presentability of those peptides undoubtedly contribute to CRCL vaccine efficacy.

### *CRCL activate DCs and elicit specific T cell responses*

DCs are professional antigen presenting cells known to be critical activators of T cell responses. Pulsing DCs with tumor antigens, such as peptides, tumor lysate, or apoptotic tumor cells has been demonstrated to generate tumor-specific protective immunity [23, 34, 43]. Although the mechanisms are not completely clear, an increasing body of data suggests that DCs take up chaperone-peptide complexes through specific receptors and re-present the peptides on MHC-I molecules [12]. Certain chaperones appear to interact with professional APCs via specific receptors, although that area of research is emerging as somewhat contentious. Over a decade ago, Srivastava [67] predicted the existence of such receptors on APCs as a conduit for exogenous peptide delivery into the antigen processing pathway. Subsequently, Binder et al. [13] reported that CD91 (also known as the $\alpha$ 2 macroglobulin receptor and the low-density lipoprotein receptor–related protein) was a macrophage receptor for GRP94/gp96. Ensuing work by Basu et al. [6] indicated that on both macrophage and DCs, CD91 was a common receptor for GRP94/gp96, HSP70, HSP90, and calreticulin. Among the plethora of substances bound by the Toll-like receptors 2 and 4, HSP60 is present [55], as is GRP94/gp96 [73]. HSP60 has been shown to bind to monocyte CD14 [42]. HSP70 has been described as a chaperone cytokine, or "chaperokine" [2] that also utilized CD14 as a "co-receptor", and LOX-1 [20] and CD-40 [8] are reported as cell surface receptors for HSP70. Confounding things further, Berwin et al. [10] provided recent evidence in macrophage cells that GRP94/gp96 can mediate specific immunity independent of CD91, eventually identifying scavenger receptor A as a receptor for GRP94/gp96 and calreticulin [11]. Scavenger receptor CD36 had previously been demonstrated to be a receptor for GRP94/gp96 [56], and the large chaperones GRP170 and HSP110 appear to interact with macrophages via scavenger receptors (J. Subjeck, personal communication). Preliminary data shows that CRCL enhance the immunostimulatory ability of DCs derived from TLR-4 competent C3H/HeN mice but not from TLR-4 deficient C3H/HeJ mice in mixed lymphocyte reactions (MLRs), suggesting that TLR-4 receptor may be involved in the activation of DCs by CRCL (S. Thompson, unpublished data). Whether or not CD91 or the scavenger receptors are involved in the uptake of CRCL by DCs is currently under investigation in our laboratory.

Chaperone proteins, regardless of their tissue source, have been reported to activate APCs as part of an innate immune response, where the chaperones act essentially as pro-inflammatory cytokines [59, 64]. This spurring of innate immune responses leads to activation of APCs, which are then capable of effectively prompting T cells into action by direct stimulation and by cytokine secretion. We have shown that CRCL upregulate the expression of MHC class II molecules, enhance co-stimulatory molecule expression of CD40, and CD80/CD86 (B7.1/B7.2), increase interleukin (IL)-12 production, and promote immmunostimulatory function (e.g., improved stimulators in MLRs) of DCs in vitro. CRCL confer this superior activation of DC when compared to HSP70 or lysates [78]. Essentially, CRCL serve as their own adjuvants. The enrichment of CRCL for chaperone proteins, or perhaps the appropriate combinations of chaperones, may play a role in this increased activation of DC, possibly via multiple receptor complexing. In addition to HSP70, HSP90, GRP94/gp96, and CRT, other chaperone protein members are also present in the CRCL fractions (as mentioned above) as well as numerous other unidentified proteins [30]. These additional proteins may certainly have a part in CRCL's immune stimulus. As noted in the Introduction, immunodepletion of GRP94/gp96, HSP90, HSP70, and calreticulin from CRCL significantly reduces—but does not completely abrogate—the effectiveness of CRCL-pulsed DCs to stimulate T cells to release IFN-$\gamma$ (J. Davis et al., unpublished data). It is therefore possible that other proteins in the CRCL may contribute to the superior immune activation capacity of CRCL. Identifying the role of each of these proteins is critical

and may help in the discovery of additional immunogenic chaperone proteins. Thus, chaperones appear at the nexus of the innate and adaptive immune responses, providing adjuvant-like stimulus to professional APCs, and delivering antigenic peptides at the same time. The end result is a more potent immunizing agent.

Although most of our data so far support the activation of DCs, and T cells by CRCL, the effects of CRCL on immune responses may not be restricted to these cell populations. The immune system is composed of complicated interactions among different cell populations. The cross talk between NK and DCs has been reported. DCs directly trigger NK cell functions through cell–cell contact between DCs and NK cells as well as through the IL-12 secreted by DCs [24, 68]. Moreover, interaction between NK cells and macrophages has been shown to be important in controlling pathogen infections [27]. Activated NK cells prime macrophages though cell contact and soluble mediators such as IFN-$\gamma$ [45]. Since CRCL stimulate IL-12 secretion from DCs, we therefore reason that CRCL may activate NK cells through DCs; moreover, the activated NK cells produce IFN-$\gamma$ resulting in macrophage activation. Preliminary data in our laboratory indicate that CRCL enhance IFN-$\gamma$ production of NK cells directly or in the presence of immature DCs (Y. Zeng, unpublished data). More detailed studies are ongoing to identify the effects of CRCL on both innate and adaptive immune responses.

Since it has been reported that HSPs may share some common pathways with lipopolysaccharide (LPS) [63], it is important to exclude the possibility that the innate and adaptive immune activation effects of CRCL are due to LPS contamination. Endotoxin level of CRCL is less than 0.01 EU/μg as examined by LAL assay [78]. In addition, IFN-$\gamma$ production of NK cells as well as tumor necrosis factor (TNF) production of DCs following CRCL stimulation was not blocked by pretreatment of cells with polymyxin B, a LPS antagonist (Y. Zeng, unpublished data). Furthermore, no evident tumor specific immunoprotection was induced by vaccination of mice with CRCL derived from normal tissue or from other types of tumor [22, 78]. These data support the probability that immune activation effects of CRCL are not artifacts of LPS contamination.

## Adjuvant effects of CRCL

### *CRCL provide danger signals to APCs in the presence of apoptotic tumor cells*

The relative ability of necrotic versus apoptotic cells to induce an immune response remains an important but controversial consideration in attempts to develop effective anti-cancer vaccines. We have previously reported that heat stress induces HSP expression on the surface of apoptotic tumor cells and concurrently increases their immunogenicity [23]. Stressed apoptotic tumor cells were more effective in upregulating co-stimulatory molecules (CD40, CD80, and CD86), in stimulating IL-12 secretion, and in enhancing the immunostimulatory functions of DCs. Immunization of mice with stressed apoptotic tumor cells induced $T_H$ 1 profile of cytokine secretion and specific CTLs in vivo [21]. HSPs have been reported to supply adjuvant effects/danger signals to activate APCs, such as DCs, leading to more efficient processing and presentation of HSP chaperoned peptides [75]. Our findings indicate that stressed apoptotic tumor cells are capable of providing the necessary danger signals, likely through increased surface expression of HSPs, resulting in activation/maturation of DCs, and ultimately the generation of potent antitumor T-cell responses. It is therefore plausible to reason that the immunogenicity of non-stressed apoptotic cells (which do not express HSPs on their surface) may also be enhanced if an exogenous source of HSPs is present at the vaccination site. This hypothesis was confirmed when co-injection of normal syngeneic liver-derived CRCL, (devoid of tumor specific antigenic peptides) with non-stressed apoptotic tumor cells resulted in the generation of durable and specific T-cell-mediated anti-tumor immunity [22]. CRCL provided better adjuvant effects than normal tissue-derived HSP70. Non-stressed apoptotic cells alone or when combined with liver lysate were ineffective vaccines.

Cell-mediated immunity, which is particularly important against tumors, is characterized by production of type I cytokines, activation of macrophages, and induction of CTLs, which are important mediators of ant-tumor immunity [15]. Several reports have shown that professional APCs can acquire antigens from apoptotic bodies and cross prime CTLs in vitro [1, 9]. However, some reports have shown that apoptotic cells are associated with induction of type II immune suppressive cytokines, such as tumor growth factor-$\beta$ (TGF-$\beta$) and IL-10 [74], and evidence that those apoptotic cells prime CTLs in vivo remains limited. In our studies, we found that vaccination with CRCL adjuvant plus apoptotic tumor cells induced IL-2 and IFN-$\gamma$ production. This indicates that CRCL steer the immune system toward a TH1 type response, which is critical in suppressing tumors [5]. Vaccination with CRCL adjuvant plus apoptotic tumor cells also induced potent and specific CTLs that lysed tumor cells in vitro. The antitumor immunity was partially abolished when either $CD8^+$ T cells or $CD4^+$ T cells were depleted, indicating that CTLs played an important role in tumor killing in vivo and that $CD4^+$ T cell help was also required [22].

The implications of chaperone protein modulation, either exogenously or pharmacologically, may have profound effects on anti-cancer therapies. Currently, chemotherapy and radiotherapy remain the main treatment modalities for many cancers. Most of these therapies are thought to induce tumor cells to undergo apoptosis [39]. These apoptotic tumor cells are attractive tumor antigen sources, not only in (poly)peptide antigens, but they are also rich sources of lipids and carbohydrates that may have immunogenic effects.

However, without proper danger signals, these antigen sources are largely ignored by the immune system, or may even induce tolerance [74]. CRCL, enriched from normal or tumor tissues, provide potent adjuvant effects for enhancement of the immunogenicity of apoptotic tumor cells that have been shown to induce potent, long lasting anti-tumor immunity in animal models. These findings, together with the superior adjuvant effects, confer significant advantages to CRCL-based vaccine strategies in terms of clinical applications.

### *CRCL-pulsed DC vaccines can be effectively combined with chemotherapy*

It is widely accepted by immunologists/oncologists that immunotherapy will likely be used as adjuvant therapy in the management of cancer. Patients who achieve remission after conventional therapy may receive immunotherapy in different forms. The effects of tumor-derived CRCL based vaccines were therefore tested in combination with the chemotherapy drugs imatinib mesylate and cyclophosphamide in murine models.

Imatinib mesylate has become the drug of choice against CML in chronic phase. However, it is much less effective against CML in accelerated phase or during blastic transformation [61]. Its effectiveness has also been tempered by the increasing number of cases where drug resistance develops [19]. These situations have lead to drug combination approaches to augment the activity of imatinib via alternative targeting of either the p210 proteins or other important downstream signal transduction molecules [35]. However, there have been few attempts to combine imatinib with immunotherapy [41], and there are no published articles on utilization of vaccine therapy in conjunction with imatinib either in human trials or in animal models. On the other hand, imatinib mesylate is generally considered to be non-myelosuppressive, and it has even been reported to enhance the antigen-presenting capacities of DCs, suggesting that imatinib may even be useful in the immunotherapy of cancer [76]. Using the 12B1 murine CML model, we have shown that the combination of imatinib with cellular vaccines of DCs pulsed with tumor-derived CRCL yields tumor-specific T cells, and potent therapeutic anti-tumor activity resulting in tumor-free survival in a high percentage of mice. It should be noted that 12B1 is an extremely aggressive tumor that more accurately resembles CML in blast crisis rather than true chronic phase, and thus is quite refractory to imatinib.

At the level of immune cell mechanisms, mice receiving combination immuno- and chemotherapy were found to have higher splenic IFN-$\gamma$ production and increased CTL activity when compared with those receiving imatinib or DC/CRCL vaccine alone. Although imatinib has been shown to induce apoptosis in $BCR{-}ABL^{+}$ leukemia cells both in vitro and in vivo [28], imatinib treatment results in minimal or no upregulation of cell surface HSP70 or HSP60 (Y. Zeng et al., unpublished data). The lack of HSPs, in other words, danger signals, may partly explain the lack of measurable immune responses in mice treated with imatinib monotherapy. It is conceivable, however, that imatinib-induced apoptosis of 12B1 releases tumor antigens, and when given in combination with CRCL that possesse both antigenic as well as adjuvant effects, the end result is an enhanced anti-tumor immune response. While the search for better drug combinations to pair with imatinib continues [44], we suggest that immunotherapy be given a higher priority in that endeavor. CRCL vaccines, especially when used as immunogens for pulsing DCs, may represent a novel form of immunotherapy ideally suited for augmenting the targeted imatinib chemotherapy.

CRCL vaccines (sans dendritic cells) have also been combined with cyclophosphamide to treat pre-existing tumors in mice. We have shown that the combination therapy significantly delayed both B16 melanoma and 12B1 leukemia development in mice (M. Graner, unpublished data). Although the mechanisms of cyclophosphamide in cancer treatment remain unclear, there are reports showing that cyclophosphamide may deplete $CD4^{+}$ $CD25^{+}$ T regulatory cells in vivo [26]. As regulatory T cells may be one of the major barriers against cancer vaccine immunotherapy, these results highlight the fine line between autoimmunity and cancer vaccine efficacy. Studies exploring the effects of depleting T regulatory cells and CRCL vaccination are currently under investigation in our laboratory.

## Discussion

### Application of CRCL in immmunotherapy

#### *Comparison of CRCL to other immunotherapy methods*

As cancer immunotherapy continues to play a larger role as an oncology treatment modality, vaccines against tumors will generate more interest because of the exquisite specificity of the immune response and the generally mild side effects. Chaperone protein-based vaccines have begun to draw attention due to their efficacy in animal models, leading up to several ongoing phase III clinical trials. Such vaccines must meet the same criteria that all vaccines must meet, such as possessing an adequate and presentable antigen supply, and some form of adjuvanticity to stimulate priming of T cells. The multi-chaperone vaccine CRCL meet or exceed these requirements, particularly when compared with other more commonplace vaccine strategies, such as the use of individual tumor-derived chaperone protein, known antigenic peptides, irradiated whole tumor cells, or tumor cell lysates.

CRCL from a wide variety of tumor types have been shown to be able to elicit specific protective and therapeutic immunity against those multiple tumor types [32].

We have compared the immunizing effect of CRCL to tumor-derived HSP70 and GRP94/gp96, the two most heavily studied single-chaperone vaccines (and those currently in clinical trials), in both a prophylactic setting and against pre-existing tumors. We have shown that CRCL vaccines are at least as effective as—and generally more effective—than HSP70 and GRP94/gp96 in a variety of functional assays, including tumor rejection experiments [78].

The efficacy of antigenic peptide-based immunotherapy, such as BCR–ABL peptide, has been widely studied [57]. However, the lack of DC activation by peptides, followed by disappointing immune responses by single peptide vaccines, has been a concern recognized by cancer immunotherapists [17]. The translocation resulting in the *bcr–abl* fusion gene is the primary mutation that leads to malignant transformation; however, secondary mutations often occur [38]. Therefore, leukemic cells can easily escape immune responses generated by single peptide vaccination. The immunogenicity of DCs loaded with CRCL vaccine was therefore compared with that of DCs loaded with single antigenic peptide. Vaccination with DCs loaded with CRCL led to significantly higher survival rates, confirming the superiority of CRCL immunization. CRCL may circumvent the problems associated with single peptide vaccines by providing both large antigen repertoires as well as adjuvant effects that stimulate APCs.

Tumor lysate has been used as a source of antigen for loading DCs in preclinical and clinical studies [53]. However, in the numerous tumor models we have studied, such as 12B1 leukemia, A20 and BDL-2 lymphoma, B16 melanoma, neuro-2a neuroblastoma, Sa1 fibrosarcoma, and 4T1 mammary carcinoma, tumor lysate did not stimulate a measurable immune response when used alone, while some protection was achieved when lysates were loaded onto DCs. Moreover, when incubated with DCs, unfractionated tumor lysate did not change the DC phenotype or enhance their immunostimulatory function in MLRs. In addition, liver lysate failed to provide danger signals to apoptotic tumor cells and did not induce anti-tumor immunity in vivo (H. Feng et al., unpublished data). It is possible that subtle differences in the preparation of lysate and DCs may contribute to the differential outcomes between our studies and those of others. The higher concentrations of HSPs in CRCL may be a more important factor that leads to the superior immunogenicity of CRCL to lsyate. It has been shown that the local concentrations of danger signals released from dying cells may be important [62, 69]. Tumor immunogenicity is associated with increased expression of HSP 70 when tumor cells are undergoing necrotic death [49]. Tumors that were genetically modified to express HSP, or where exogeneous HSP70 was provided during tumor cell killing, decreased the immunosuppressive cytokine IL-10 expression [29]. Furthermore, lysate from primary cells contains less HSPs than their transformed counterparts and fails to mature DCs [62]. CRCL, which contain at least a 20-fold of enrichment of major HSPs [30, 31], appear to provide a higher concentration of local danger signals.

## Advantages of CRCL vaccines

We believe that we have provided compelling evidence for the consideration of tumor-derived, FS-IEF-generated CRCL vaccines as a useful immunotherapy that warrants further clinical investigation for several reasons. First, the FS-IEF technique for enriching multiple chaperones from tumor lysate is relatively easy and rapid, yielding sufficient immunogenic material for clinical use in a multiple vaccination setting. As mentioned previously, we are able to obtain 1–2 mg of CRCL per gram of tumor, some 30–50 times more material than we can obtain from individual chaperone purifications.The clinical utility of high CRCL yields from small amounts of tumor with short turn-around time is obvious, making CRCL a viable union of the best qualities of unfractionated lysates and purified chaperones. Second, the effectiveness of CRCL as anti-cancer vaccines does not appear to be limited to certain tumor types; it has been effective against numerous murine tumor types that differ in rodent genetic strain, histological origin, tumorgenicity, and metastatic potential. In short, we have not yet utilized a tumor model whereby CRCL vaccination did not prove effective. These successes support the generalized application of this type of vaccine strategy to patients with different types of cancers. Moreover, when compared with purified HSP70, single antigenic peptide or unfractionated lysate, CRCL have superior abilities to activate/mature DCs, and can induce potent, long-lasting, tumor specific T-cell-mediated immunity. Finally, while CRCL vaccines were effective as stand-alone therapies, the enhanced immunogenicity arising from CRCL-pulsed DCs as a vaccine indicates that CRCL could be the antigen source of choice for DC-based anti-cancer immunotherapies, particularly in light of the lack of potent immune responses engendered by tumor lysate-pulsed DCs in the models we have used.

## Ultimate evaluation of CRCL as vaccines

However, one has to recognize that the transplantable animal tumor models are seldom perfect mirrors of human malignancies, where immune tolerance, advanced immunosuppression, and genetic instability make any therapeutic attempts difficult at best. Although the translation of promising animal model data into the clinical setting may be a daunting task, we have been able to replicate some of the findings obtained from animal experiments using immune cells from healthy donors as well as from cancer patients. CRCL from cancer patients' tumors activated allogeneic DCs as indicated by the higher amounts of IL-12 secretion and stronger immunostimulatory functions of DCs. More

importantly, we have generated specific T cell responses in vitro by stimulating peripheral blood mononuclear cells (PBMCs) with tumor-derived CRCL. These CTLs displayed specific IFN-$\gamma$ secretion and tumor cell killing (G. Li et al., unpublished data). These findings, in combination with the data from murine studies, further support the use of CRCL vaccines in human cancer immunotherapy.

Thus, as tumor-derived chaperone protein vaccines make their way into the clinic, we would suggest that this "second-generation" chaperone-based vaccine is a good candidate for clinical development. From their research origins as stress-response molecules, to one of their current dimensions as immune-response molecules, chaperone proteins might provide the link between the cell biology of tumors and the immunology necessary to eradicate them.

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