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IMMUNOLOGICAL CONSEQUENCES OF APOPTOSIS IN A TUMOR SYSTEM

by

Hanping Feng

A Dissertation Submitted to the Faculty of the

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In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

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

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Dissertation Director — Date 6/12/02

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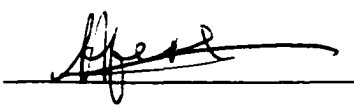
SIGNED: 

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ABSTRACT

Cell lines genetically deficient in caspase-8 have been shown to be resistant to Fas-induced apoptosis indicating that this pathway may be caspase-8-dependent. Some reports, however, have shown that Fas can induce cell death independent of caspase-8. In this study, we provide evidence for an alternative, caspase-8-independent, Fas death domain-mediated apoptotic pathway in the BCR-ABL positive leukemia cell line, 12B1-D1. Our data suggest that there is a novel, caspase-8-independent, Z-VAD-FMK inhibitable, apoptotic pathway in 12B1-D1 cells that targets mitochondria directly.

In attempting to develop effective anti-cancer immunotherapies the relative ability of apoptotic cells to induce an immune response remains an important but controversial consideration. Apoptotic tumor cells can theoretically be a suitable antigen source for stimulation of anti-tumor responses. HSPs can act as danger signals to the immune system. We, therefore, hypothesize that the immunogenicity of apoptotic cell may be enhanced if endogenous HSP expression is induced, or an exogenous source of HSPs is present. To induce endogenous HSPs expression, the engineered 12B1-D1 cells are heat stressed before the induction of apoptosis by AP20187. These stressed apoptotic 12B1-D1 cells express HSP60 and HSP72 on their surface. Vaccination of mice with stressed apoptotic 12B1-D1 cells, but not non-stressed ones, elicits a potent cell-mediated anti-tumor immunity that significantly retards tumor progression. We have further demonstrated that, stressed apoptotic cells had higher abilities to upregulate the co-

stimulatory molecules on the surface of DC, to stimulate DC to secrete proinflammatory cytokines, and to enhance their immunostimulatory functions. We further explored whether the immunogenicity of non-stressed apoptotic cell can be enhanced if an exogenous source of HSPs is present at the vaccination site. We used liver derived chaperone proteins co-injected with non-stressed apoptotic tumors to mice. Reproducibly this resulted in the generation of a durable and specific T-cell-mediated anti-tumor immunity. In summary, we have demonstrated that apoptotic tumor cells can be either immunogenic or non-immunogenic and DC may play a key role in determining the immunological consequences of apoptotic tumor cells. We have further demonstrated that normal tissue (liver) MCC may function as a danger signal for the immune system. These may provide new insights for combining immunotherapy with conventional therapies for treatment of cancers.

INTRODUCTION

Part I: the mechanisms of Fas death domain-mediated apoptosis in tumor cells

The mechanism of apoptosis has been under extensive investigation [1, 2]. Several apoptotic pathways have been elucidated [3-6]. Among them, Fas-mediated apoptotic pathway is the most well-studied in the present [7]. Fas (also known as CD95 or APO-1), a member of the tumor necrosis factor (TNF) receptor family, is a widely expressed cell death receptor that plays a critical role in the regulation of the immune system and tissue homeostasis [8, 9]. The intracellular tail of Fas, the Fas death domain (FasDD), is an approximately 80 amino acid protein motif that is critical for signaling apoptosis [7]. The activation of Fas by FasL or by agonistic antibody (Fas antibody) leads to trimerization of FasDD, which consequently recruits an adaptor molecule called FADD (Fas-associated protein with death domain) or MORT1, and caspase-8, forming the so-called death-inducing signal complex (DISC) [10]. Formation of the DISC leads to activation of caspase-8, an initiator of downstream apoptotic processes that include the activation of caspase-3, -6, and -7 and loss of mitochondrial transmembrane potential (MTP) [11].

Caspase-8, a member of a mammalian caspase family, has been demonstrated to play a key role in Fas-induced apoptosis [12-14]. Certain transgenic mice or cell lines deficient in caspase-8 have been shown to be resistant to Fas-induced apoptosis [15, 16],

suggesting that caspase-8 may be essential in Fas-mediated apoptosis. Recently, however, Yang *et al* showed that Fas could engage an apoptotic pathway independent of FADD and caspase-8 [17]. In addition, several reports have now shown that Fas signaling can trigger an alternative, caspase-8-independent necrotic cell death pathway [5, 18, 19]. Taken together, these results indicate that Fas-mediated cell death is much more complicated than originally thought.

Part II: the immunogenicity of apoptotic tumor cells

The deficiency of self/non-self paradigm has led to new hypotheses proposed by Janeway [20, 21] and Matzinger [22, 23] in that: the immune system is thought to respond to "danger" associated with infectious agents, with cell products released during tissue damage or with stress. According to this new paradigm, the occurrence of pathological or necrotic cell death in the organism is the 'danger signal' that initiates protective immune responses. In contrast to necrotic cell death, apoptosis is a physiological process, critical to development, tissue remodeling and cell turnover, and thought to be non-inflammatory and bland to the immune system, or even tolerogenic [24-26]. The intact membrane and exposure of surface molecules to specific receptors on phagocytes seem to allow the safe disposition of undesirable cellular remains [27]. This idea, however, has been challenged recently. Lopes and colleagues argued that phagocytosis of apoptotic cells plays a previously unrecognized role in regulating the nature of immune responses against pathogens [28]. Restifo proposed that the apoptotic death after viral infection can trigger powerful innate and adaptive immune responses though activating caspase-1 as well as the double-stranded-RNA-dependent protein kinase pathway and the RNaseL pathway [29]. Recent studies have documented that apoptotic cells induced by pathogens elicit potent immune responses [30-32]. These immune responses are associated with the induction of type I cytokines, such as interferon γ (IFN-γ) [30], and the generation of specific CTLs [32].

It is well accepted that antigen presenting cells [APCs] can efficiently acquire antigens from apoptotic tumor cells and present them to T cells [26, 33-35]. The immunological consequences of this remain controversial [28, 29]. Macrophages and dendritic cells (DCs) phagocytose apoptotic tumor cells through a receptor mediated pathway [33-35], tumor antigens can then gain access to the cytoplasm and be cross presented on the APC's MHC class I molecules [26, 33]. Reports addressing the immunological consequences following engulfment of apoptotic cells by DCs have been highly contradictory [32, 33, 36-38]. *In vitro* studies have demonstrated measurable cellular responses induced by apoptotic cells. However, a potent immune response was seldom generated *in vivo* [39-42]. When immature DCs endocytose apoptotic bodies they are not stimulated to mature, and consequently may present their processed antigens in the absence of adequate co-stimulation thereby inducing tolerance [39, 42, 43].

Tumor derived HSPs also called chaperone proteins, when used as vaccines, can induce protective immunity against their tumors of origin [44]. In our studies we found that vaccination with multiple HSPs/chaperone proteins enriched from tumor lysate by free solution isoelectric focusing (FS-IEF) provided specific anti-tumor immunity in various tumor models [45] (and manuscript submitted). Some important chaperone proteins such as gp96, HSP90, HSP70, and calreticulin enriched by this method tend to complex together [46]. We therefore refer to the FS-IEF derived preparation as multiple

chaperone complexes (MCC). We have previously reported that tumor-derived MCC have superior abilities to stimulate DCs when compared to purified individual HSPs such as HSP70 and gp96 (manuscript submitted), which have been widely used as tumor vaccines [47, 48].

Several mechanisms have been proposed to explain how these HSP complexes, which carry tumor derived peptides as part of their chaperoning functions, can elicit immune responses [49-51]. One possible hypothesis is that chaperone complexes not only supply antigens to the immune system but also danger signals [49-51]. These adjuvant effects/danger signals activate APCs such as DCs leading to more efficient processing and presentation of HSP chaperoned peptides [52, 53].

Apoptotic tumor cells can theoretically be a suitable antigen source for stimulation of anti-tumor responses. HSPs can act as danger signals to the immune system. We therefore, reasoned that the immunogenicity of apoptotic cell may be enhanced if endogenous HSP expression is induced, or an exogenous source of HSPs is present. This enhancement of immunogenicity of apoptotic tumor cells may partially be due to the immunostimulatory function of HSPs on DC.

CHAPTER I: EVIDENCE FOR A NOVEL, CASPASE 8-INDEPENDENT, FAS DEATH DOMAIN-MEDIATED APOPTOTIC PATHWAY

Hanping Feng, Yi Zeng, Michael W. Graner, Luke Whitesell, Emmanuel Katsanis

1.1 ABSTRACT

Cell lines genetically deficient in caspase-8 have been shown to be resistant to Fas-induced apoptosis in some cases indicating that the Fas/FasL apoptotic pathway may be caspase-8-dependent. Some reports, however, have shown that Fas can induce cell death independent of caspase-8. Here we provide evidence for an alternative, caspase-8-independent, Fas death domain- (FasDD) mediated apoptotic pathway in the *BCR-ABL* positive leukemia cell line, 12B1-D1. This cell line was stably transfected with a plasmid encoding a recombinant fusion protein that permits the oligomerization of FasDD upon exposure to synthetic, dimerizing agent AP20187 leading to rapid induction of apoptosis. 12B1-D1 cells express procaspase-3, 8, and 9, which are activated upon the dimerization of FasDD. Bid is cleaved and mitochondrial transmembrane potential is disrupted in this apoptotic process. All apoptotic events were completely blocked by the broad-spectrum caspase inhibitor Z-VAD-FMK, but not by up to 200μM of other peptide caspase inhibitors, such as an inhibitor of caspase-8 (Z-IETD-FMK) or caspase-3 (Z-DEVD-FMK). Since cyclosporin A (CsA) has been reported to inhibit the permeability transition pore of mitochondria, and block mitochondrial apoptotic events, we pre-treated 12B1-D1 cells with CsA before the activation of FasDD. We found that neither the

disruption of mitochondrial transmembrane potential nor the activation of caspases was blocked. However, all apoptotic events were completely blocked when 12B1-D1 cells were pre-treated with CsA and caspase-8 inhibitor followed by dimerization of FasDD. Our data therefore suggest that there is a novel, caspase-8-independent, Z-VAD-FMK inhibitable, apoptotic pathway in 12B1-D1 cells that targets mitochondria directly.

1.2 INTRODUCTION

Fas (also known as CD95 or APO-1), a member of the tumor necrosis factor (TNF) receptor family, is a widely expressed cell death receptor that plays a critical role in the regulation of the immune system and tissue homeostasis [8, 9]. Fas or Fas ligand (FasL) mutations in humans and mice cause a syndrome of massive lymphoproliferation and autoantibody production [8]. In addition, Fas-induced apoptosis is a major mechanism in cytotoxic T lymphocyte-mediated cytolysis [57].

The intracellular tail of Fas, the Fas death domain (FasDD), is an approximately 80 amino acid protein motif that is critical for signaling apoptosis [7]. The activation of Fas by FasL or by agonistic antibody (Fas antibody) leads to trimerization of FasDD, which consequently recruits an adaptor molecule called FADD (Fas-associated protein with death domain) or MORT1, and caspase-8, forming the so-called death-inducing signal complex (DISC) [10]. Formation of the DISC leads to activation of caspase-8, an

initiator of downstream apoptotic processes that include the activation of caspase-3, -6, and -7 and loss of mitochondrial transmembrane potential (MTP) [11].

Caspase-8, a member of a mammalian caspase family, has been demonstrated to play a key role in Fas-induced apoptosis [12-14]. Certain transgenic mice or cell lines deficient in caspase-8 have been shown to be resistant to Fas-induced apoptosis [15, 16], suggesting that caspase-8 may be essential in Fas-mediated apoptosis. It has been reported that there may be two alternative Fas signaling pathways [58]. In the so-called Fas type I cells, a relatively large amount of caspase-8 is recruited to DISC upon receptor cross-linking, resulting in the activation of caspase-8. The activated caspase-8 initiates a rapid apoptotic signal by directly activating downstream effector caspases through proteolytic cleavage, as well as by triggering mitochondrial damage that in turn activates a proteolytic cascade. In Fas type II cells, the relatively slowly activated caspase-8 mediates downstream apoptotic events mainly by inducing mitochondrial damage [58]. Recently, Yang *et al* showed that Fas could engage an apoptotic pathway independent of FADD and caspase-8 [17]. Fas activation induced Daxx to interact with apoptosis signal-regulating kinase 1 (ASK1). ASK1's activated kinase activity results in caspase-independent activation of JNK, leading to cell death [59, 60]. In addition, several reports have now shown that Fas signaling can trigger an alternative, caspase-8-independent necrotic cell death pathway [5, 18, 19]. Taken together, these results indicate that Fas-mediated cell death is much more complicated than originally thought.

In this study, using a *BCR-ABL* positive leukemia cell line 12B1-D1, we have demonstrated that a broad-spectrum peptide caspase inhibitor, Z-VAD-FMK (pan-caspase inhibitor) completely blocked FasDD-mediated cell death. Peptide caspase inhibitor Z-IETD-FMK (casp-8 inhibitor) or Z-DEVD-FMK (casp-3 inhibitor) could neither block the disruption of mitochondrial transmenbrane potential (MTP), nor chromosomal DNA fragmentation after activation of FasDD. However, all apoptotic events were completely blocked when 12B1-D1 cells were pretreated with cyclosporine-A (CsA) and casp-8 inhibitor followed by dimerization of FasDD. This suggests that FasDD triggers a novel caspase-8-independent apoptotic pathway in the 12B1-D1 leukemia cell line.

1.3 MATERIALS AND METHODS

Antibodies and Reagents: Anti-caspase-3 (clone 46) and anti-caspase-7 (clone 10-1-62) antibodies were purchased from BD PharMingen (Franklin Lakes, NJ). Rabbit anti-caspase-8 polyclonal antibody was from StressGen Biothechnologies (Victoria, BC Canada). Anti-caspase-9 antibody (clone 9CSP02) was from NeoMarkers (Fremont, CA). Goat anti-human/mouse BID antibody and anti-caspase-10 antibody (clone Mch 2) were purchased from R&D Systems (Minneapolis, MN). Cyclosporin A (CsA) was from Sigma. Peptide caspase inhibitors, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

(Z-VAD-FMK), benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK), benzyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethylketone (Z-YVAD-FMK), benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone (Z-VDVAD-FMK) and benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl-ketone (Z-IETD-FMK), benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (Z-LEHD-FMK), and benzyloxycarbonyl-Phe-Ala-fluoromethylketone (Z-FA-FMK), were all from R&D systems. 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) was from Molecular Probes (Eugene,OR).

Determination of Caspase Activities: Caspase activities from cytosolic extract were measured using a flurometric assay according to the manufacturer's instructions (R&D systems). In brief, 12B1-D1 cells were collected by centrifugation at 1,000 × g for 5 min at 4 °C. Cells were washed with ice-cold phosphate-buffered saline and resuspended in chilled lysis buffer. After 10 min on ice, the supernatant was collected following centrifugation at 10,000 × g and was assayed for protein content using the bicinchonic acid reagent (Pierce, Rockford, IL). For caspase activity measurements, cell extract (50 μg) was incubated at 37 °C in a reaction buffer (R&D systems) containing the substrates Asp-Glu-Val-Asp- amino-4-trifluoromethyl courmarin (DEVD-AFC), Ile-Glu-Thr-Asp-amino-4-trifluoromethyl courmarin (IETD-AFC), or Leu-glu-His-Asp-amino-4-trifluoromethyl courmarin (LEHD-AFC). After 1.5 to 2 hoursincubation at 37 °C, the

fluorescence intensity (excitation at 390 nm and emission at 510 nm) was measured using a microplate fluorometer (Labsystems).

Flow cytometry analysis—Phosphatidylserine (PS) exposure during apoptosis was evaluated by annexin V-FITC binding. Briefly, 12B1-D1 cells were washed with PBS and incubated in a solution of 0.5 mg/ml FITC-labeled annexin V and propidium iodide (PI) (Roche, Indianapolis, IN) in binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.4) at room temperature for 15 min. Cells were then washed, resuspended in 0.5 ml binding buffer and at least 10,000 cells per sample analyzed in a Becton Dickinson FACScan. To evaluate the disruption of mitochondrial transmembrane potential (MTP), the cationic lipophilic fluorochrome $DiOC_6(3)$ was used [61]. Cells were incubated with 40 nM $DiOC_6(3)$ for 15 min at 37°C. Cells were diluted with PBS to a final volume of 0.5 ml and analyzed by flow cytometry. Alternatively, MTP was measured using a DePsipher kit (R&D systems) following the manufacturer's instructions. Cells were analyzed by flow cytometry.

DNA fragmentation assay: To evaluate nucleosomal DNA fragmentation, total DNA from cells was extracted using an Apoptotic DNA ladder Kit (Roche, Indianapolis, IN). the presence of 200bp DNA laddering was evaluated by electrophoresis through a 1.5% agrose gel followed by ethidiom bromide staining and UV transillumination.

Immunoblotting: The cleavage of Bid and several caspases was detected by Western blotting as described previously [62]. Briefly, lysates containing 25 μg of protein were separated by electrophoresis through 15% SDS-PAGE gels and proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Equal loading was confirmed by Ponceau S staining of the membranes. Caspase-3, 7, 9, 10, and Bid were detected using relevant primary antibodies and alkaline phosphatase conjugated secondary antibody (Santa Cruz Biotechnology). Proteins were detected by color deposition of the alkaline phosphatase substrates NBT/BCIP (Roche, Indianapolis, IN).

1.4 RESULTS

Caspase activation and apoptosis induction of 12B1-D1 cells after dimerization of engineered FasDD: We have previously reported that the *BCR-ABL* positive cell line 12B1 [63] does not express Fas protein on its surface and consequently fails to undergo apoptosis in response to anti-Fas antibody [62]. Therefore, we transfected 12B1 cells with plasmid DNA encoding a fusion protein that consists of the extracellular domain of the human low affinity nerve growth factor receptor (NGFR), two copies of mutant FK506 binding proteins (FKBP), and the Fas death domain (FasDD) (kindly provided by Ariad Pharmaceuticals) (Figure I-1A). A stably transfected clone, 12B1-D1, was used for further study. Treatment of 12B1-D1 cells with the semi-synthetic FK506 derivative AP20187 resultsed in the rapid induction of apoptosis [62]. More than 80% of the cells became annexin V-FITC positive within 4 to 6 hours of 40 nM AP20187 treatment

(Figure I-1B). In addition, the chromosomal DNA was cleaved into 200 bp fragments, a typical feature of apoptosis, after 6 hours AP20187 treatment (Figure I-1C). The opening of mitochondrial pores is an early event of many types of apoptosis, leading to the depolarization of MTP. We used the potential-sensitive mitochondrial probe $DiOC_6(3)$ for the cytofluorometic determination of MTP during FasDD-induced apoptosis. Treatment of 12B1-D1 cells with AP20187 resulted in a marked decrease in the retention of $DiOC_6(3)$ within 3 hours and more than 70% of cells lost MTP within 5 hours (Figure I-1D).

To analyze caspase activities after AP20187 treatment, we used fluorochrome conjugated caspase specific peptide substrates LEHD-AFC, IETD-AFC, or DEVD-AFC for caspase-9, 8, or 3 respectively. All three caspases were activated within 30 min of AP20187 treatment, and reached maximum activity after 2 hours as judged by increasing fluorescence intensity (Figure I-2), resulting from the specific cleavage of substrates and release of the fluorochrome AFC. We also analyzed caspase activation during AP20187 induced apoptosis by Western blot (Figure I-3). Procaspase-3, a main effector caspase, began to be cleaved within 30 min of AP20187 treatment (Figure I-3A). Longer exposure of 12B1-D1 cells to AP20187 increased the intensity of a 17 kDa fragment (Figure I-3A). Another effector caspase, procaspase-7 (35kDa protein) was also cleaved within 30 min of AP20187 treatment (Figure I-3B). Pro-caspase 9 (46-48 kDa protein) was also cleaved within 30 min of AP20187 treatment generating a protein doublet at

35/37 kDa with a predominant 37 kDa fragment (Figure I-3C). Consistent with another report [15], we found that although caspase-10 was expressed in 12B1-D1 cells, it was not proteolytically cleaved (Figure I-3B).

The effect of oligopeptide caspase inhibitors on caspase activation and FasDD mediated apoptosis: Irreversible oligopeptide caspase inhibitors have been used to study the role of different caspases in apoptosis. Z-IETD-FMK and Z-DEVD-FMK block caspase-8- and caspase-3-like proteases, respectively, whereas Z-VAD-FMK is a broad-range caspase inhibitor [64, 65]. To test whether these potent and selective inhibitors could block FasDD-induced cell death in 12B1-D1 cells, cells were pre-treated with 100 μM of either pan-caspase inhibitor, casp-3 inhibitor, or casp-8 inhibitor followed by exposure to AP20187. In agreement with other studies [66], the pan-caspase inhibitor completely prevented cells from undergoing apoptosis after activation of FasDD, as determined by the block of the PS externalization (Figure I-4A) and chromosomal DNA fragmentation (Figure I-4B). Moreover, cells completely excluded the DNA dye PI even after 13 hours of AP20187 treatment (Figure I-4C), suggesting that this pan-caspase inhibitor could completely block both FasDD induced apoptosis and necrosis. Surprisingly, we found that the casp-8 or casp-3 inhibitors could not prevent all major apoptotic events induced by dimerization of FasDD, such as PS externalization (Figure I-4A) and DNA fragmentation (Figure I-4B). As expected, the cells eventually developed secondary necrosis as determined by their inability to exclude PI (Figure I-

4C). Failure to block apoptotic cell death was not due to the incapacity of casp-3 inhibitor or casp-8 inhibitor to block caspase activities because neither caspase-3 nor 8-dependent- DEVD-AFC or IETD-AFC cleaving activity was detected in the lysate of 12B1-D1 cells that were pre-treated with either pan-caspase inhibitor, or caspase-3 or -8 inhibitor (Figure I-5). In addition, increasing concentrations of casp-8 inhibitor up to 200 μM did not alter its inability to block apoptosis, whereas pan-caspase inhibitor completely prevented apoptosis even at a substantial lower concentration (20 μM) (data not shown). Other peptide caspase inhibitors for caspase-1 (Z-WEHD-FMK), caspase-2 (Z-VDVAD-FMK), caspase 4 (Z-YVAD-FMK), caspase-6 (Z-VEID-FM), caspase-10 (Z-AEVD-FMK), and caspase-13 (Z-LEED-FMK), did not block the externalization of PS (Figure I-4A), which occurs early during the apoptotic process [67].

We examined the activation of caspase-3, a major effector caspase, after AP20187 treatment in the presence of pan-caspase inhibitor or casp-8 inhibitor (Figure I-6) using western blotting to detect the altered migration of the activated form of caspase-3. Casp-8 inhibitor may slightly delay the cleavage of the pro-form of caspase-3 but did not appear to block it. In contrast, the pan-caspase inhibitor completely blocked caspase-3 cleavage.

It has been reported that there are two types of cells in terms of Fas-mediated apoptosis [58], type I and type II cells. In the type I cells, a significant amount of caspase-8 is

rapidly activated after the ligation of Fas ligand and Fas, resulting in a strong signal which can bypass mitochondria and directly target effector caspases, such as caspase-3. We compared the total pro-caspase-8 expression of 12B1-D1 by western blotting to some other mouse tumor cell lines and found that the total pro-caspase-8 expression in 12B1-D1 was significant (date not shown). After dimerization of FasDD, the activity of caspase-8 increased dramatically within 30 min (Figure I-2). Caspase-3 was also rapidly activated (Figure I-2 and Figure I-3A). However, the majority of mitochondrial depolarization occurred relatively slowly (within 3 hours) (Figure I-1D). These data indicate that 12B1-D1 likely be type I cells. We then assessed the mitochondrial transmembrane potential disruption after activation of FasDD in the presence of casp-8 or casp-3 inhibitor. We found that neither the casp-8 inhibitor nor the casp-3 inhibitor at 100 μM blocked the depolarization of MTP (Figure I-7), even though caspase activity was completely blocked in the presence of the specific caspase inhibitor (Figure I-5). We then increased the concentration of caspase inhibitors up to 200 μM and confirmed that they still could not block the depolarization of MTP (data not shown).

Treatment of 12B1-D1 cells with casp-8 inhibitor and CsA reveals an alternative apoptotic death signaling pathway originating from Fas: After oligomerization of Fas death domain, an adaptor protein FADD/MORT is recruited, which in turn recruits pro-caspase-8 resulting in its activation [7, 10]. Caspase-8 induces cells to undergo apoptosis by either activating downstream caspases through proteolytic cleavage [58] or triggering

Bid cleavage to target mitochondria [66]. Our data documented that the dimerization of FasDD resulted in the depolarization of MPT even though caspase-8 activity was completely blocked (Figure I-5), suggesting that there is a pan-caspase inhibitor sensitive and casp-8 inhibitor insensitive protease, or group of proteases, activated by the dimerization of FasDD. This protease(s) may directly activate effector caspases or mitochondria, or both. It has been demonstrated that cyclosporine A (CsA) can block the depolarization of MTP, which in turn prevents the release of cytochrome C from mitochondria and even apoptotic cell death [68-71]. We tested the ability of CsA to protect 12B1-D1 cells from apoptosis induced by AP20187 treatment. Treatment of 12B1-D1 cells with several different concentrations of CsA could not prevent the externalization of PS and cell death induced by AP20187 treatment. In addition, treatment with CsA did not prevent the depolarization of MTP and DNA fragmentation (Figure I-9A and B, Figure I-9C). Interestingly, when the 12B1-D1 cells were pretreated with CsA in combination with casp-8 inhibitor followed by exposure to AP20187, the depolarization of MTP was completely blocked (Figure I-9 A), as evaluated by DiOC6(3) retention. Furthermore, pretreatment with CsA in combination with casp-8 inhibitor completely blocked DNA fragmentation (Figure I-9B). These data indicate that it is necessary to block both caspase-8 activity and mitochondrial damage in order to prevent apoptotic signaling initiated by FasDD oligomerization in 12B1-D1 cells.

The finding that CsA in combination with casp-8 inhibitor completely blocks 12B1-D1 apoptosis after dimerization of FasDD suggests that the proposed novel protease(s) targets mitochondria directly. It has been shown that activated caspase-8 rapidly cleaves Bid [66, 72], resulting in a truncated form of the molecule (tBid). Bid belongs to Bcl protein family [1] [72] and is normally localized in the cytosol. TBid is highly pro-apoptotic and targets mitochondria by inserting into their membrane, leading to disruption of transmembrane potential and release of cytochrome C [72]. To explore whether the novel protease(s) that we are proposing could cleave Bid and consequently target mitochondria, we examined the Bid cleavage in 12B1-D1 cells. Consistent with previous reports [66] [73], Bid was cleaved following dimerization of FasDD (Figure I-10). Bid started to be cleaved within 30 min of AP20187 treatment and a limited amount of Bid remained uncleaved after 2 hours (Figure I-10). As expected, the pan-caspase inhibitor blocked the cleavage of Bid completely. In the presence of casp-8 inhibitor, Bid cleavage was significantly inhibited even after 4 hours of AP20187 treatment, but not completely blocked (Figure I-10). This result suggests that other protease(s) may play a role in the cleavage of Bid [66] in the presence of casp-8 inhibitor especially, since casp-8 inhibitor did not block FasDD-mediated apoptosis (Figure I-4) and caspase-3 activation (Figure I-6). Furthermore, CsA pre-treatment exhibited no inhibitory effects on Bid cleavage (Figure I-10). However, the combination of CsA with casp-8 inhibitor completely blocked the cleaveage of Bid, suggesting that our proposed novel apoptotic pathway can bypass Bid and target the mitochondria directly (Figure I-11).

1.5 DISSUSION

Our findings indicate that FasDD-oligomerization can trigger a novel caspase-8-independent apoptotic pathway. This pathway is activated by FasDD, but is independent of Bid and the proteolytic activity of caspase-8. It appears to target mitochondria directly by a Z-VAD-FMK-inhibitable mechanism, suggesting the existence of a novel protease(s) that we are now attempting to identify.

Apoptosis is the main mechanism by which multicellular organisms eliminate unwanted cells to ensure proper development and to maintain cellular homeostasis [8, 9]. The execution of most if not all apoptosis requires caspase activation [2]. Caspase-8 is one apical initial caspase and has been thought to be essential in Fas-mediated apoptosis [15, 16]. Ligand binding-induced trimerization of death receptors results in recruitment of the receptor-specific adapter protein FADD, which then recruits caspase-8. Activated caspase-8 is known to propagate the apoptotic signal either by directly cleaving and activating downstream caspases (so called extrinsic pathway), or by cleaving the BH3-containing Bcl2-interacting protein Bid, which leads to the release of cytochrome c from mitochondria, triggering the intrinsic mitochondrial pathway [72]. After triggering the oligomerization of FasDD in 12B1-D1 cells by AP20187, caspase-8 is activated rapidly. Other effector caspases, such as caspase 3 and 7, are also activated within 30 min of AP20187 exposure, which may result from direct cleavage and activation of caspase-3.

While, activated caspase-8 can propagate apoptotic signals and initiate the extrinsic death receptor pathway, it has also been shown that the mitochondrion-mediated caspase-9 activation pathway (intrinsic pathway) amplifies Fas signaling through caspase-8-mediated cleavage of Bid and translocation into the mitochondria [66, 72]. In our study, we found that the pro-apoptotic Bcl-2 family protein Bid was cleaved, MTP was disrupted and caspase-9 was activated after the activation of FasDD. This indicates that the extrinsic and intrinsinc pathways of caspase activation are extensively interconnected in 12B1-D1 cells.

Irreversible oligopeptide caspase inhibitors have been used extensively to study the role of caspases in apoptosis. Z-IETD-FMK and Z-DEVD-FMK block caspase-8- and caspase-3-like proteases, respectively, whereas Z-VAD-FMK is a broad-range caspase inhibitor [64, 65]. We observed that neither casp-8 inhibitor nor casp-3 inhibitor prevented FasDD-mediated 12B1-D1 from undergoing apoptotic cell death, even at concentration of 200 μM; whereas only 20 μM pan-caspase inhibitor completely blocked cell death. Both casp-3 inhibitor and casp-8 inhibitor entered the cells effectively. Pretreatment of the cells with pan-caspase inhibitor, casp-3 inhibitor, or casp-8 inhibitor efficiently blocked cleavage of the fluorogenic substrate DEVD-AFC or IETD-AFC. A panel of other peptide caspase inhibitors, including Z-WEHD-FMK (caspase-1), Z-VDVAD-FMK (caspase-2), Z-YVAD-FMK (caspase-4), Z-VEID-FMK (caspase-6), Z-LEHD-FMK (caspase-9), Z-AEVD-FMK (caspase-10), or Z-LEED-FMK (caspase-13),

we also tested and did not block 12B1-D1 cells from undergoing apoptosis as determined by Annexin V-FITC/PI staining. Caspase-10 is a apical caspase [3] and can function independently of caspase-8 in initiating Fas- and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) -mediated apoptosis [74]. Consistent with another report [15], we found that although caspase-10 is expressed in 12B1-D1 cells, it is not proteolytically cleaved and activated, even after 2 hours of AP20187 exposure, a time point when caspase-8 activity reached its maximum as determined by cleavage of a fluorogenic peptide substrate. In addition, the caspase-10-inhibitor Z-AEVD-FMK did not block 12B1-D1 cells from undergoing apoptosis as judged by Annexin V-FITC/PI staining. Furthermore, the casp-8 inhibitor also has strong inhibitory activity for caspase-10 [65], since caspase-10 is highly homologous to caspase-8 [1, 75]. These findings indicate that caspase-10 is not involved in the novel apoptotic pathway that we are proposing.

Mitochondria play a critical role in mediating apoptotic signal transduction pathway [6]. Biochemical and structural changes of mitochondria in apoptosis include mitochondrial swelling, disruption of mitochondrial outer membrane, mitochondrial depolarization, and the release of cytochrome C [6]. It has been demonstrated that CsA is capable of blocking the depolarization of MTP, which in turn prevents cytochrome C from release and even apoptotic cell death [68-71]. FasDD dimerization by AP20187 resulted in the disruption of mitochondrial outer membrane and loss of transmembrane potential as determined both by DiOC6(3) retention and DePsipher exclusion. This effect could not

be blocked by pretreatment of 12B1-D1 cells with either casp-8 inhibitor or CsA. Growing evidence indicates that the extrinsic death receptor and intrinsinc mitochondrial apoptotic pathways are highly interconnected and Bid plays a major role in this connection [72]. Cytosolic Bid can be efficiently cleaved by activated caspase-8 and the truncated Bid (tBid) then translocates from cytosol to the mitochondria membrane, resulting in disruption of its outer membrane [66]. We found that Bid was rapidly cleaved after the activation of FasDD, even when cells were pretreated CsA. High levels of tBid may damage the mitochondrial outer membrane even if cells are pretreated with CsA. This may explain our finding that CsA pretreatment did not block the disruption of MTP. We noticed that pretreatment of 12B1-D1 cells with casp-8 inhibitor decreased the cleavage of Bid substantially but not completely after activation of FasDD. This finding raises the question again whether or not the casp-8 inhibitor completely blocked the proteolytic activity of caspase-8. Previous reports have shown that certain caspases other than caspase-8 have minor proteolytic activity for Bid [66]. Our data demonstrated that caspases were activated and cells underwent apoptotic death after dimerization of FasDD by AP20187 when the cells were pretreated with casp-8 inhibitor. Pretreatment of cells with CsA and casp-8 inhibitor, however, completely blocked apoptotic cell death, as well as caspases activation and Bid cleaveage, confirming that the proteolytic activity of caspase-8 was adequately inhibited by our casp-8 inhibitor since CsA had no inhibitory effect on Bid cleaveage. The fact that CsA in combination with casp-8 inhibitor completely blocked 12B1-D1 cells from undergoing apoptosis after dimerization of

FasDD indicates that the protease/pathway that we proposed targets the mitochondria directly.

Although knockout data indicate that caspase-8 may be required for apoptosis induced by the death receptor Fas [15, 16] in certain cases, other reports have shown that Fas can induce cell death independent of caspase-8. Recently, Yang et al showed that Fas can engage an apoptotic pathway independent of FADD and caspase 8 [17]. They found that Fas activation induced Daxx to interact with apoptosis signal-regulating kinase 1 (ASK1), leading to its activation and resulting in caspase-independent activation of JNK and cell death [59, 60]. This pathway, however, was not blocked by the broad-spectrum caspase inhibitor, Z-VAD-FMK, indicating that it is distinct from the pathway we are now proposing. More recently, several reports have shown that Fas signaling can trigger an alternative, caspase-8-independent necrotic cell death pathway, which is not blocked by the broad-spectrum caspase inhibitor, Z-VAD-FMK [5, 18] or even render the cells more sensitive to Fas-mediated cell death [19]. Our data show that the activation of FasDD resulted in the externalization of PS, disruption of MTP, and DNA fragmentation (into 200bp) when caspase-8 proteolytic activity was completely blocked, suggesting that cells underwent a cell death with typical apoptotic features. In summary, our findings indicate that that FasDD can trigger a novel caspase-8-independent apoptotic pathway. This pathway is activated by FasDD, is independent of Bid and the proteolytic activity of caspase-8, and targets mitochondria by a Z-VAD-FMK-inhibitable mechanism.

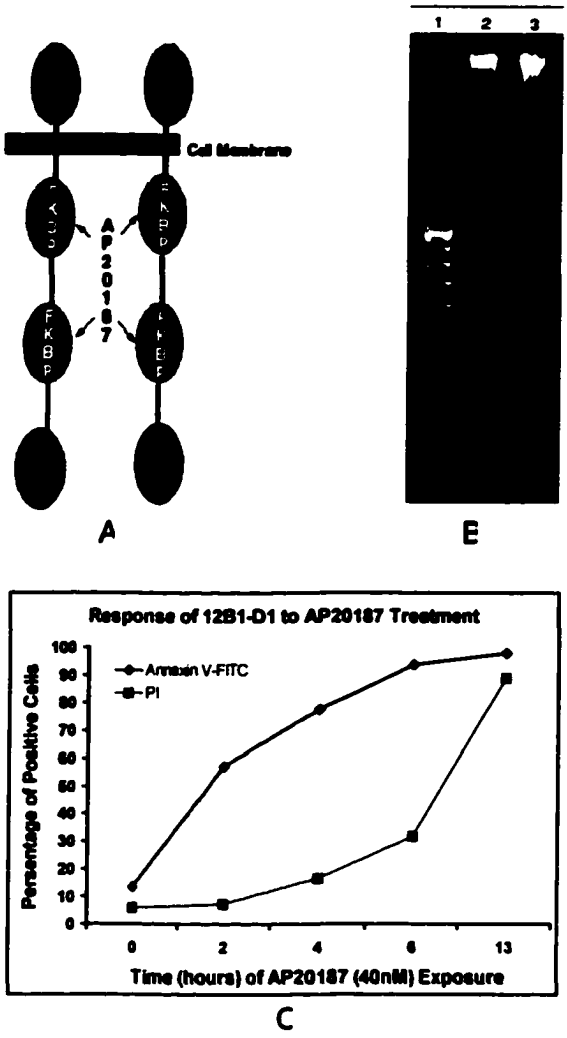
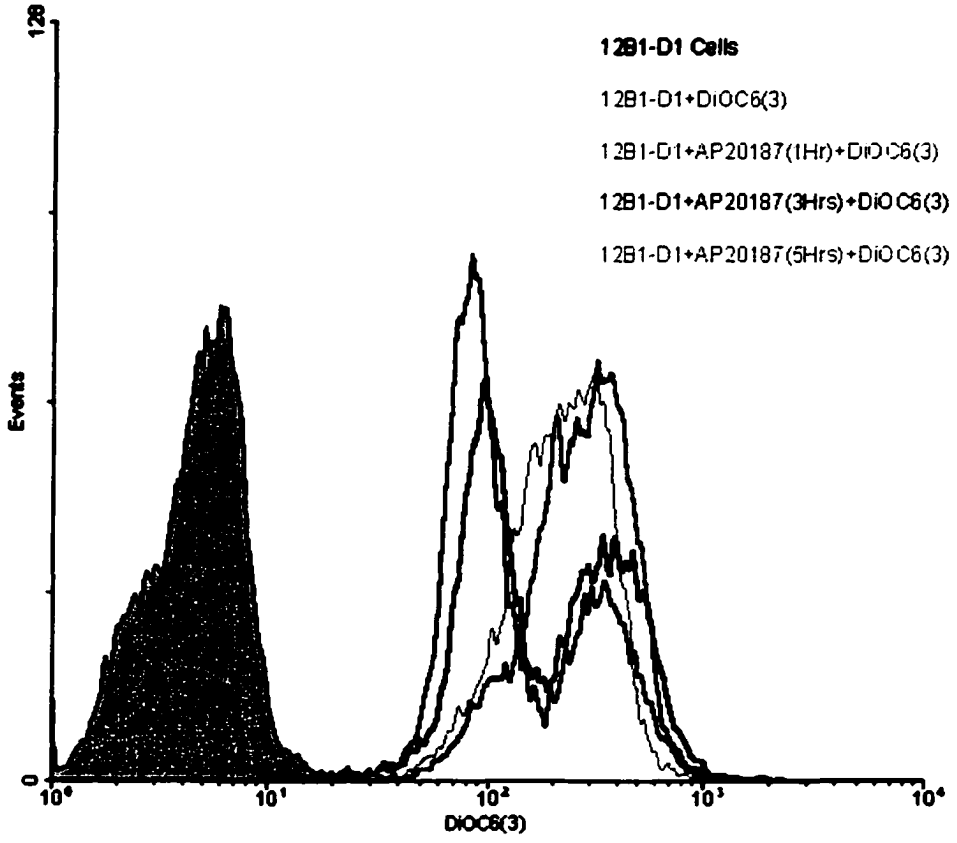


Figure I-1. Death construction and apoptosis induction of 12B1-D1 cells by AP20187 treatment. **(A)** Transmembrane fusion protein consisting of a low affinity nerve growth factor receptor (NGFR) accessible on the cell surface, two mutant FK506 binding protein (FKBP) domains and a Fas death domain (Fas DD) intracellularly. **(B)** 12B1-D1 cells were treated with 40 nM AP20187 for the indicated time, washed and then stained with Annexin V and PI. **(C)** DNA fragmentation analysis. Lane 1, 100-bp ladder; lane 2, DNA extracted from 12B1-D1 cells; lane 3, DNA extracted from 12B1-D1 that had been treated with AP20187 for 6 hours.



C

Figure I-1. Death construction and apoptosis induction of 12B1-D1 cells by AP20187 treatment. **(D)** 12B1-D1 cells were treated with AP20187 for the indicated time, washed and then stained with mitochondrial probe DioC(6)3. The fluorescence intensity was measured using a microplate fluorometer.

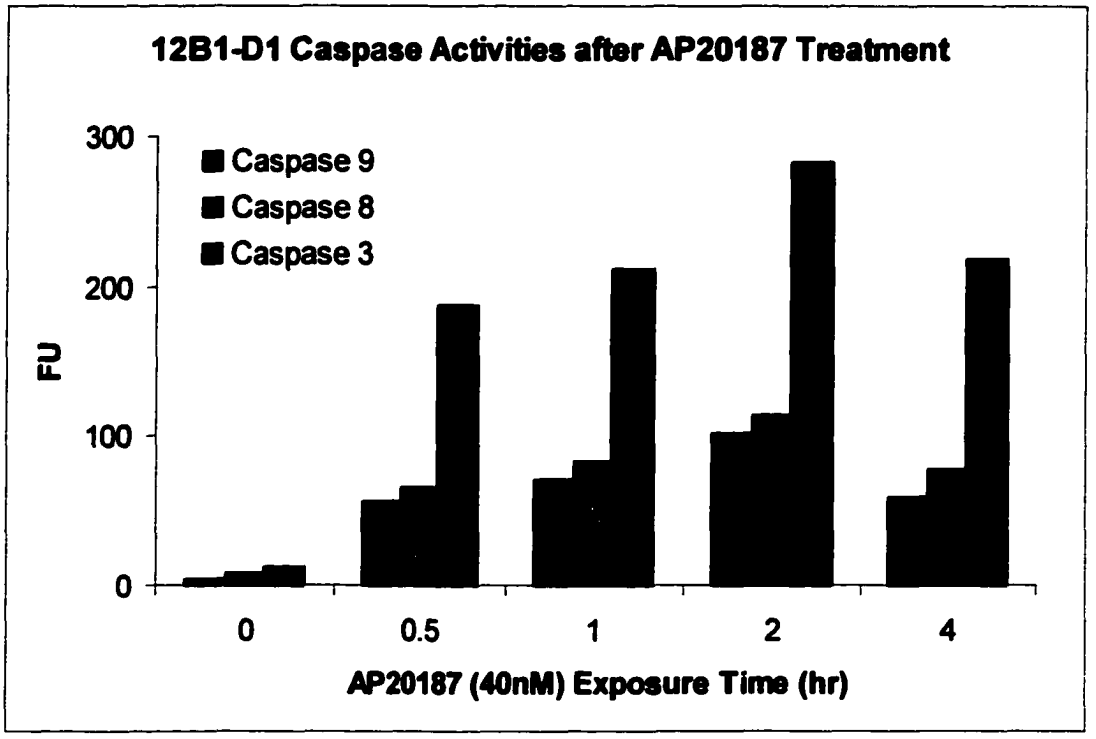


Figure I-2. 12B1-D1 caspase activities after AP20187 treatment. 12B1-D1 cells were treated with 40 nM AP20187 for indicated time, then washed and lysed. Cell extract was incubated at 37 °C for 1.5 to 2 hours in a reaction buffer containing the substrates DEVD-AFC), IETD-AFC, or LEHD-AFC. The fluorescence intensity was measured using a microplate fluorometer.

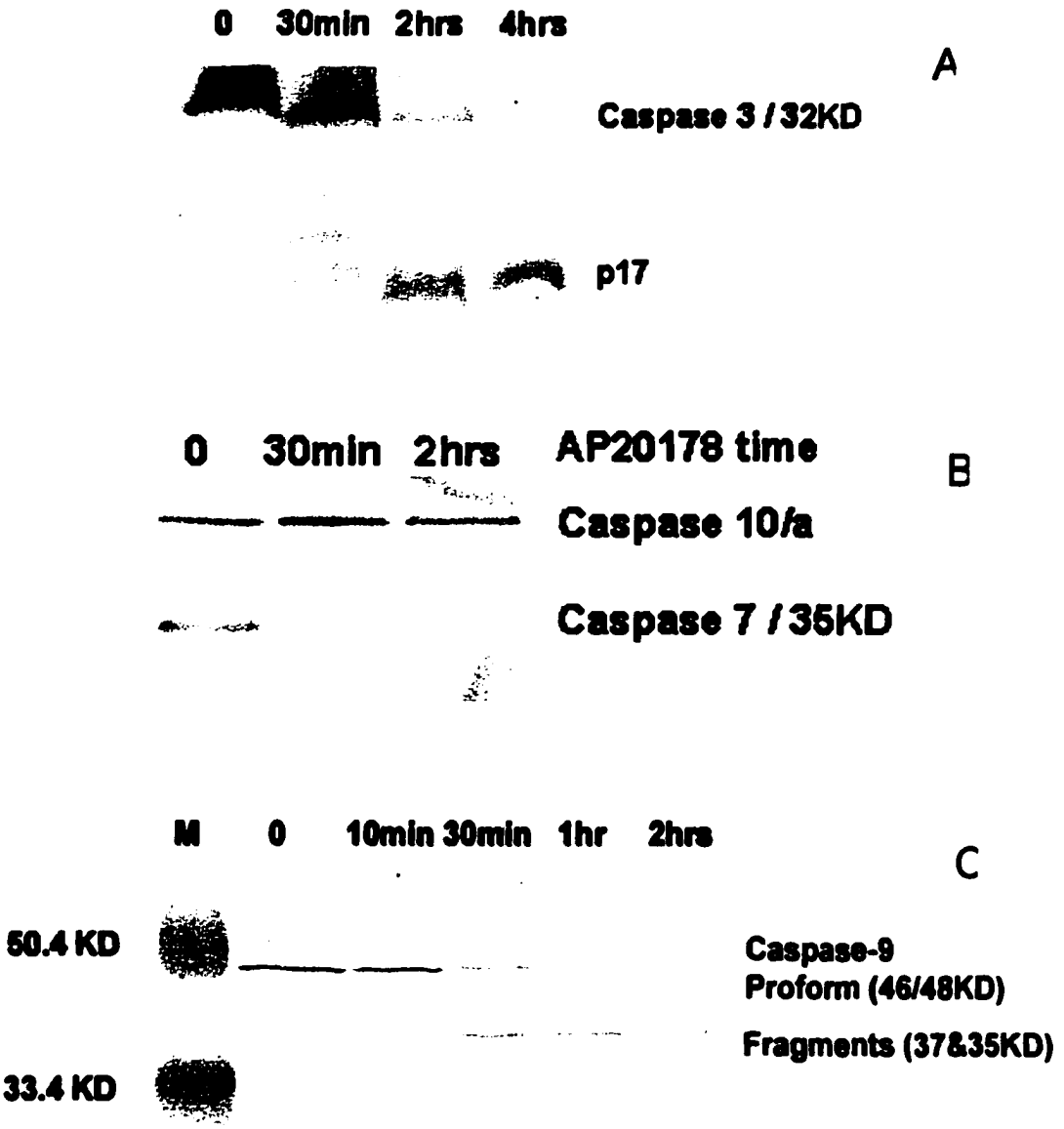
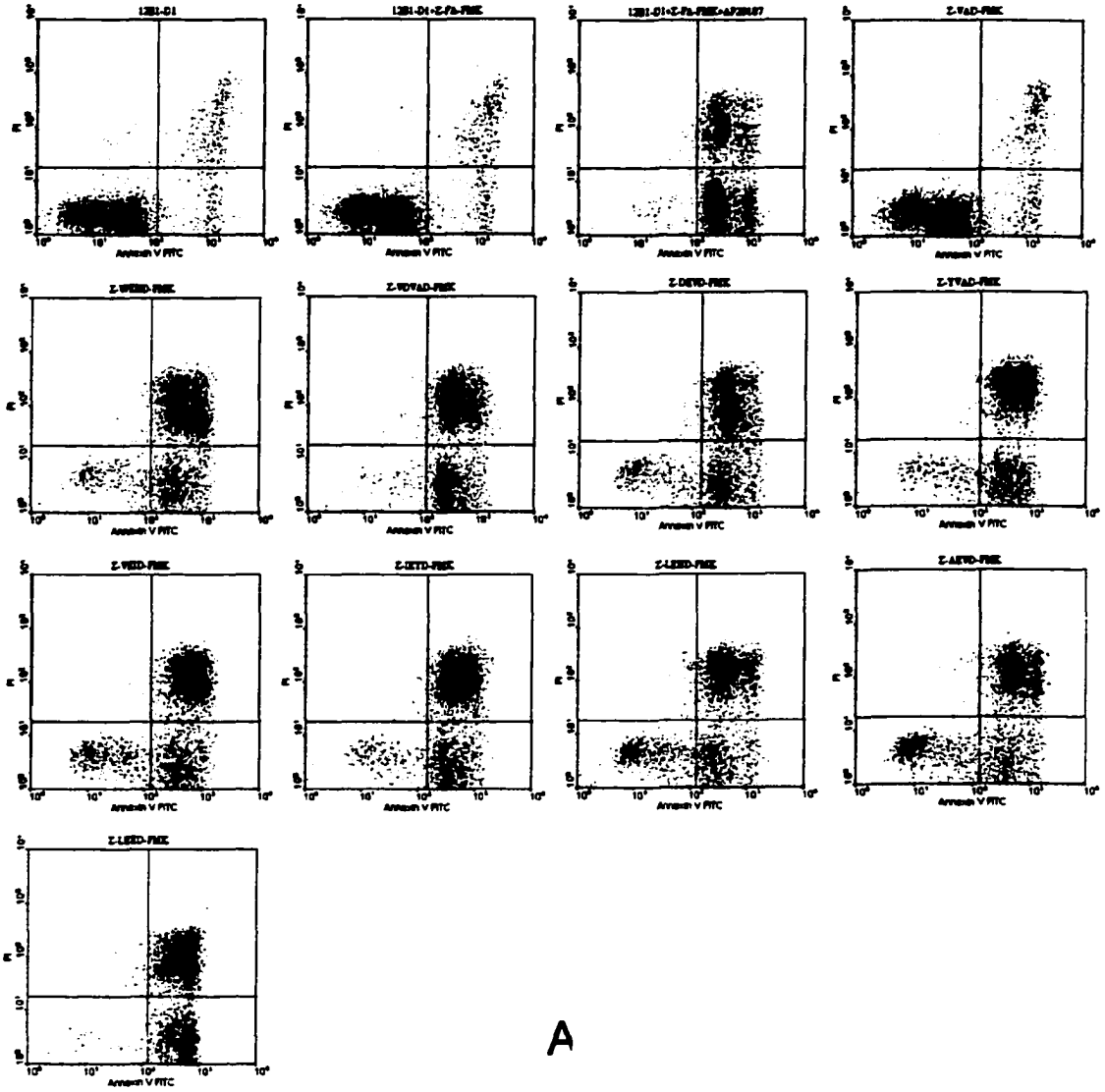
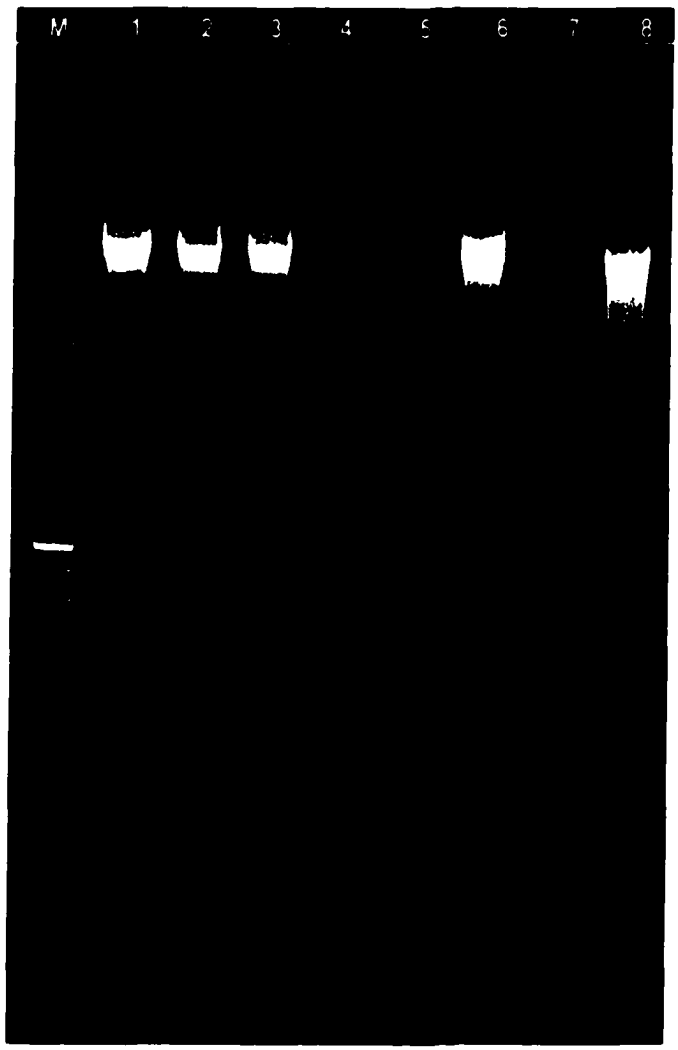


Figure I-3. 12B1-D1 caspase cleavage after AP20187 treatment. 12B1-D1 cells were treated with 40 nM AP20187 for the indicated time, then washed and lysed. Specific caspase cleavage was determined by western blotting using anti-caspase 3 **(A)**, anti-caspase 7 **(B)** and anti-caspase 9 **(C)** antibodies.



A

Figure I-4. Peptide-caspase inhibitors did not block 12B1-D1 cells to undergo apoptosis. (A) 12B1-D1 cells were pre-treated with indicated 100 μM peptide-caspase inhibitors for 30 min followed by 6 hrs of AP20187 treatment, then washed and stained with Annexin V and PI staining.



B

Figure I-4. Peptide-caspase inhibitors did not block 12B1-D1 cells to undergo apoptosis. (B) DNA fragmentation analysis. M, 100-bp ladder; DNA extracted from 12B1-D1 cells (lane 1), or 12B1-D1 cells that were pre-treated with DMSO (lane 2) or control caspase inhibitor Z-FA-FMK (lane 3); lane 4, DNA extracted from 12B1-D1 cells that had been treated with AP20187 for 6 hrs. DNA from cells that pre-treated with Z-FA-FMK (lane 5), pan-caspase inhibitor Z-VAD-FMK (lane 6), casp-8 inhibitor Z-IETD-FMK (lane 7), or casp-3 inhibitor Z-DEVD-FMK (lane 8) followed by 6 hrs of AP20187 treatment.

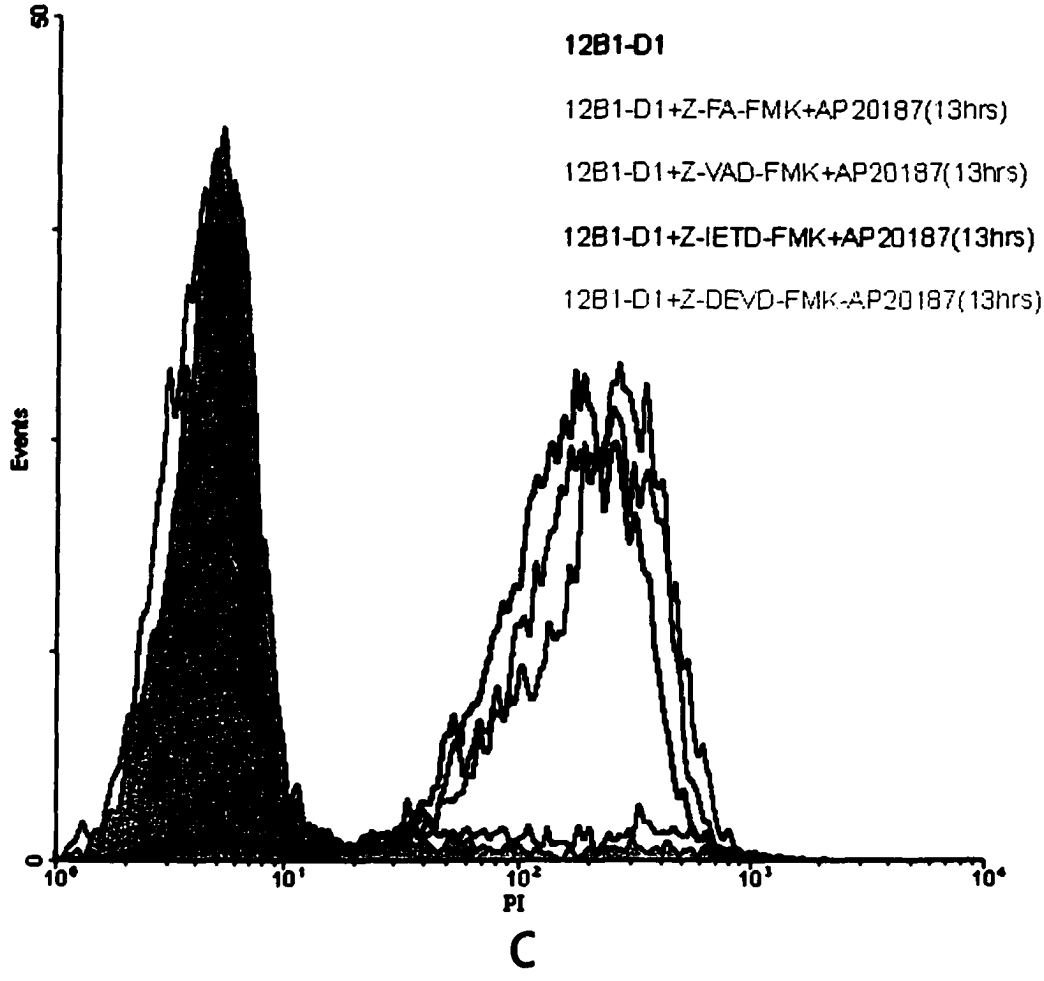
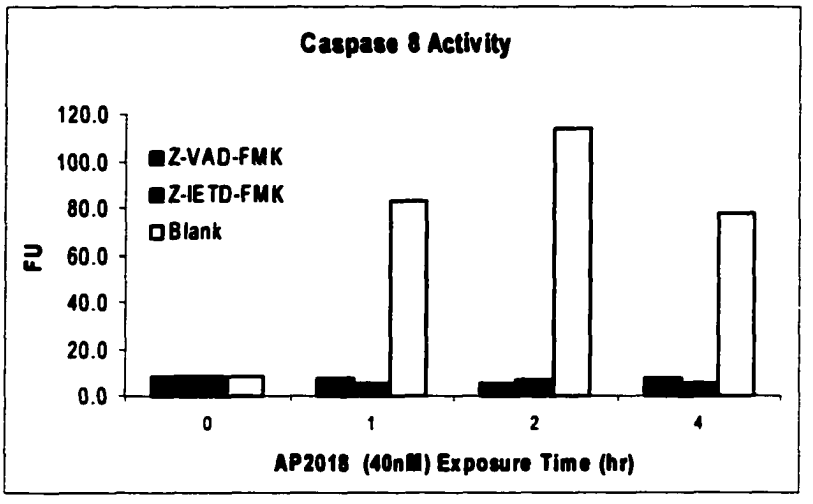
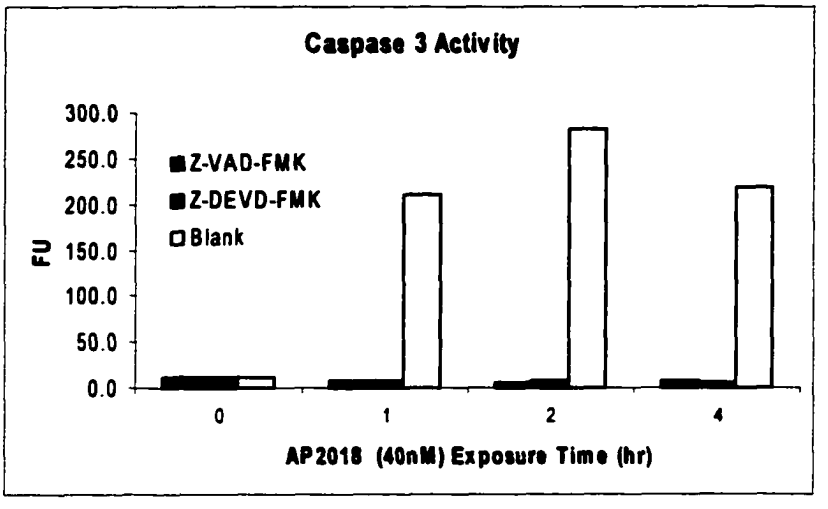


Figure I-4. Peptide-caspase inhibitors did not block 12B1-D1 cells to undergo apoptosis. (C) 12B1-D1 cells, with or without pretreatment of caspase inhibitors, were treated with 40 nM AP20187 for 13 hrs, washed and then stained with PI.

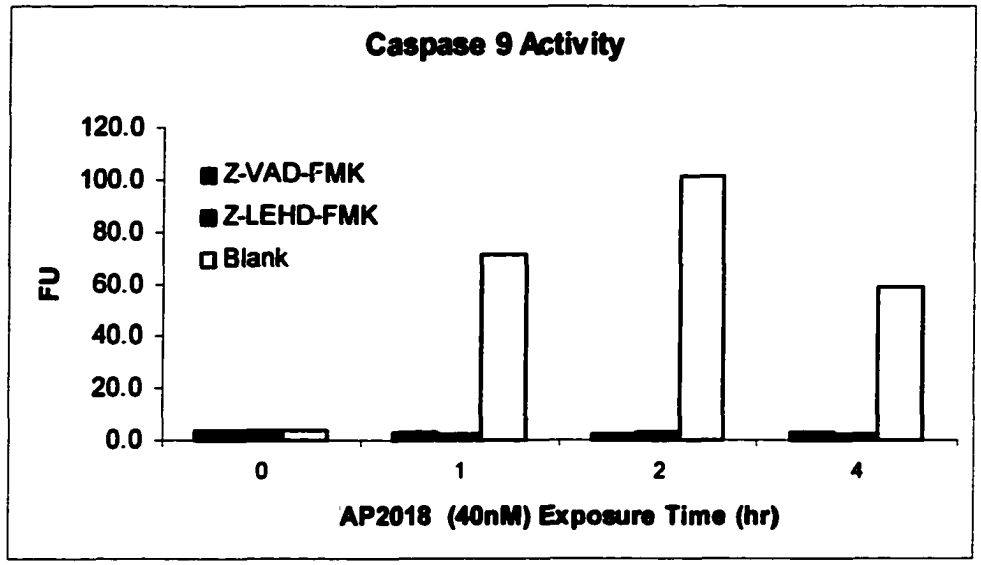


A



B

Figure I-5. 12B1-D1 caspase activities in the presence of caspase inhibitors. 12B1-D1 cells, with or without pretreatment of indicated caspase inhibitors, were treated with 40 nM AP20187 for the indicated time, then washed and lysed. Cell extract was incubated at 37 °C for 1.5 to 2 hours in a reaction buffer containing the substrates IETD-AFC **(A)**, DEVD-AFC **(B)**



C

Figure I-5. 12B1-D1 caspase activities in the presence of caspase inhibitors. 12B1-D1 cells, with or without pretreatment of indicated caspase inhibitors, were treated with 40 nM AP20187 for the indicated time, then washed and lysed. Cell extract was incubated at 37 °C for 1.5 to 2 hours in a reaction buffer containing the substrates LEHD-AFC (**C**). The fluorescence intensity was measured using a microplate fluorometer.

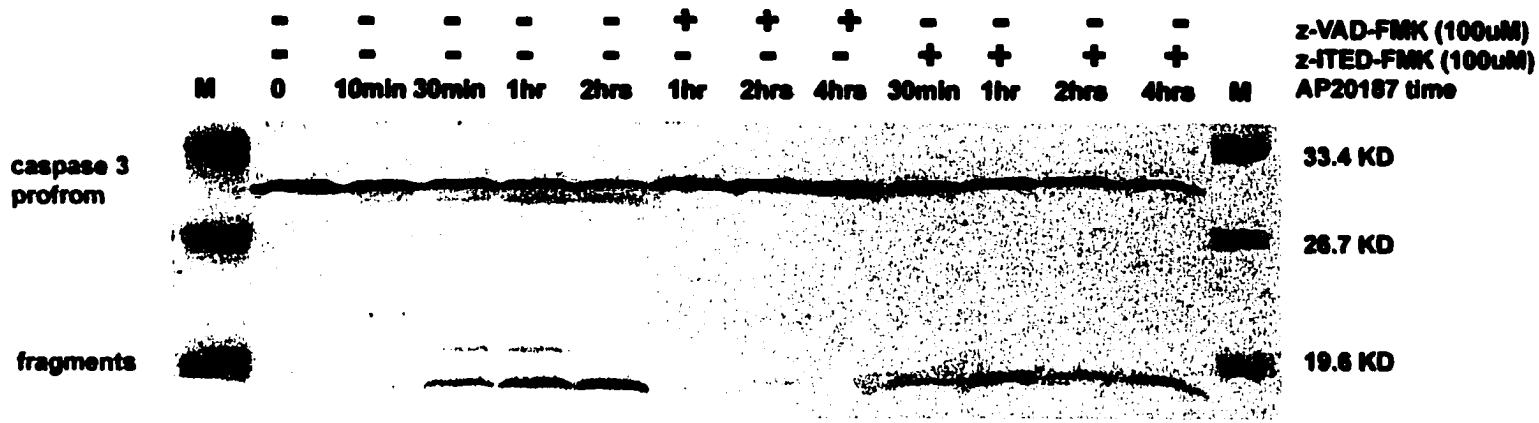


Figure I-6. 12B1-D1 caspase 3 cleavage in the presence of caspase inhibitors. 12B1-D1 cells, with or without pretreatment of indicated caspase inhibitors, were treated with 40 nM AP20187 for indicated time, then washed and lysed. Caspase-3 cleavage was determined by western blotting using anti-caspase 3 antibody.

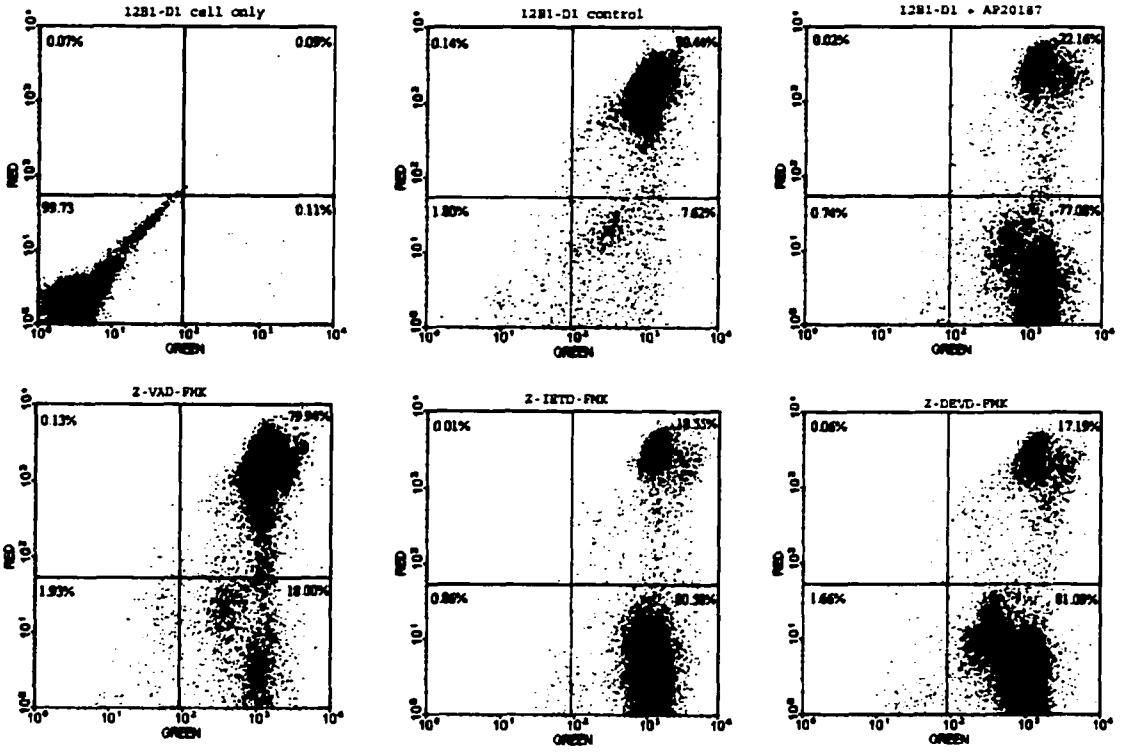


Figure I-7. Loss of MTP of 12B1-D1 cells after AP20187 treatment in the presence of caspase-3 or -8 inhibitor. 12B1-D1 cells, with or without pretreatment of indicated caspase inhibitors, were treated with 40 nM AP20187 for indicated time, then washed and stained with DePsipher following manufacturer's instructions.

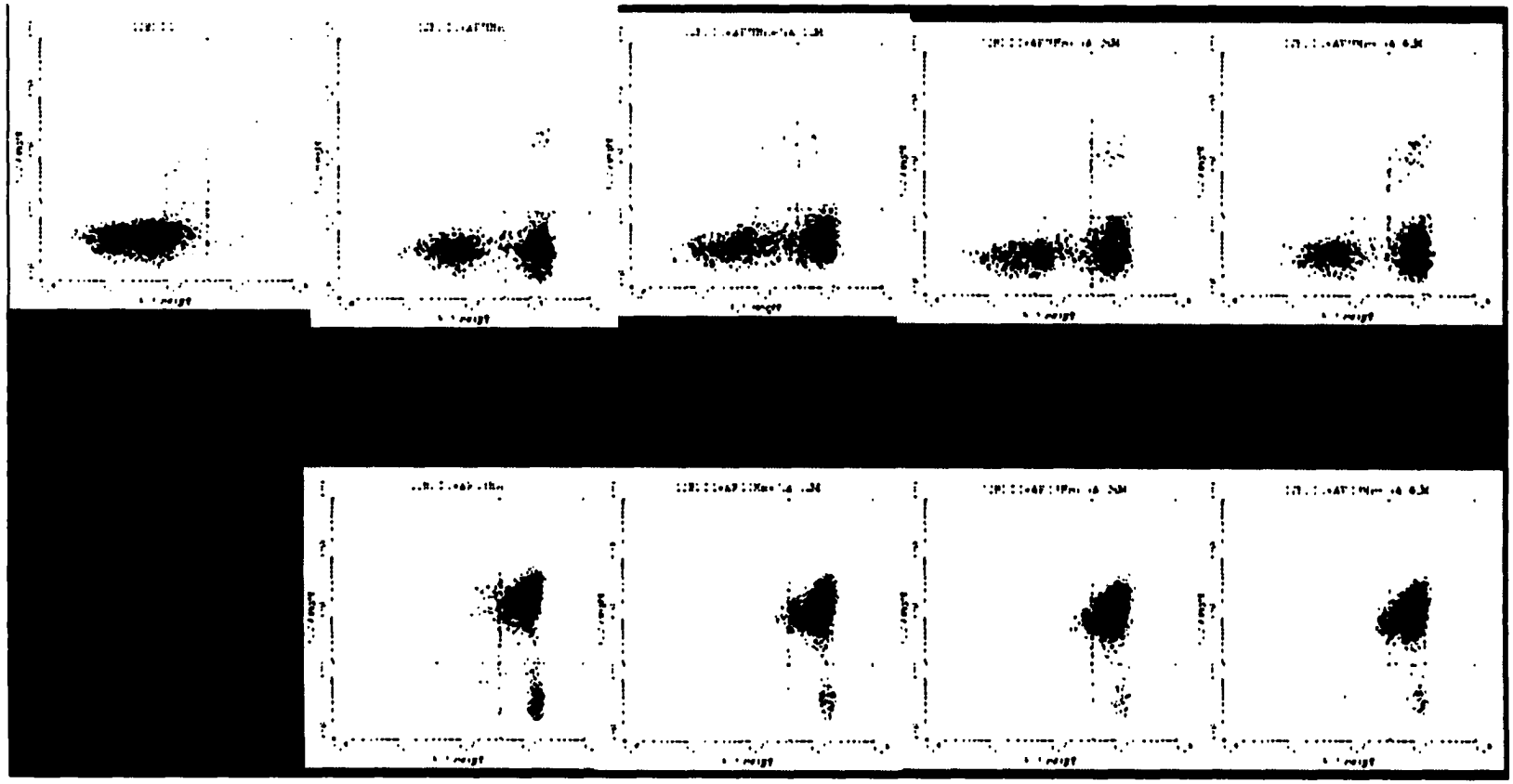
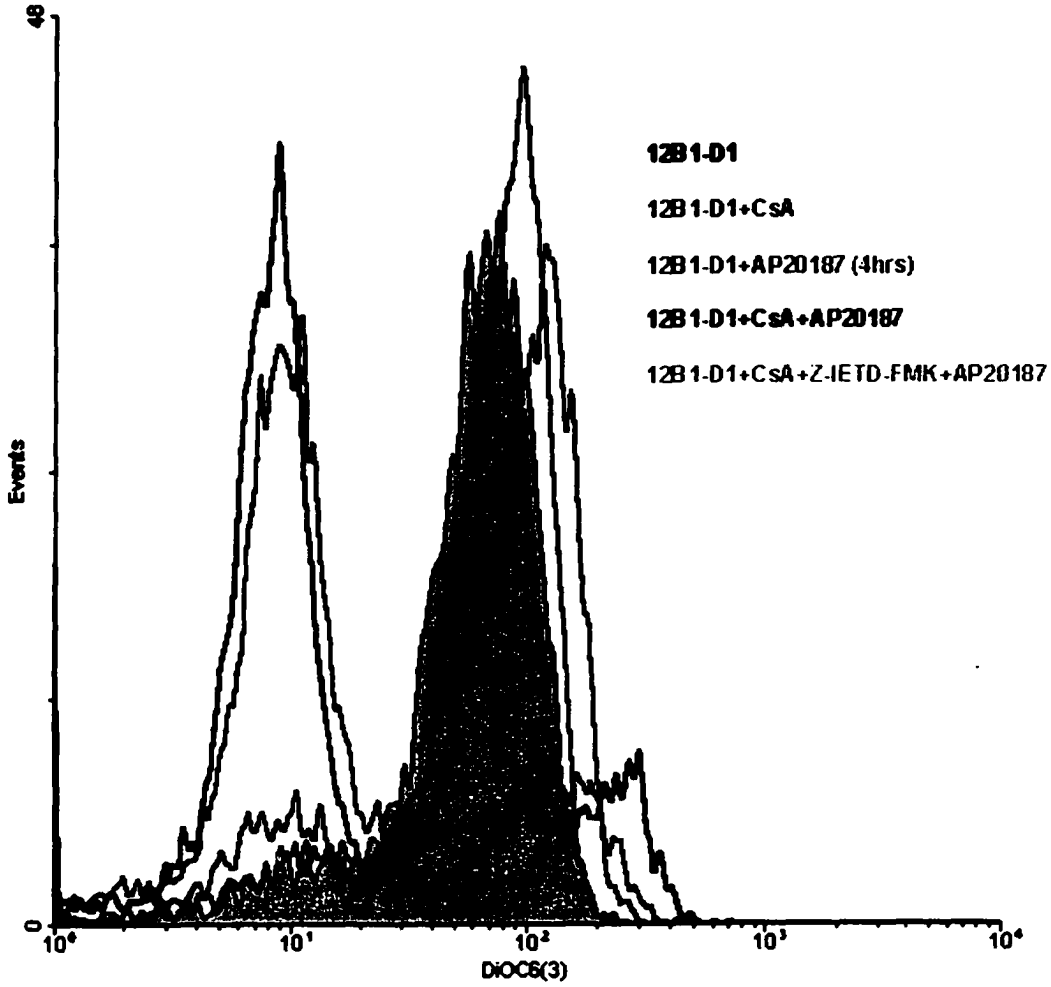
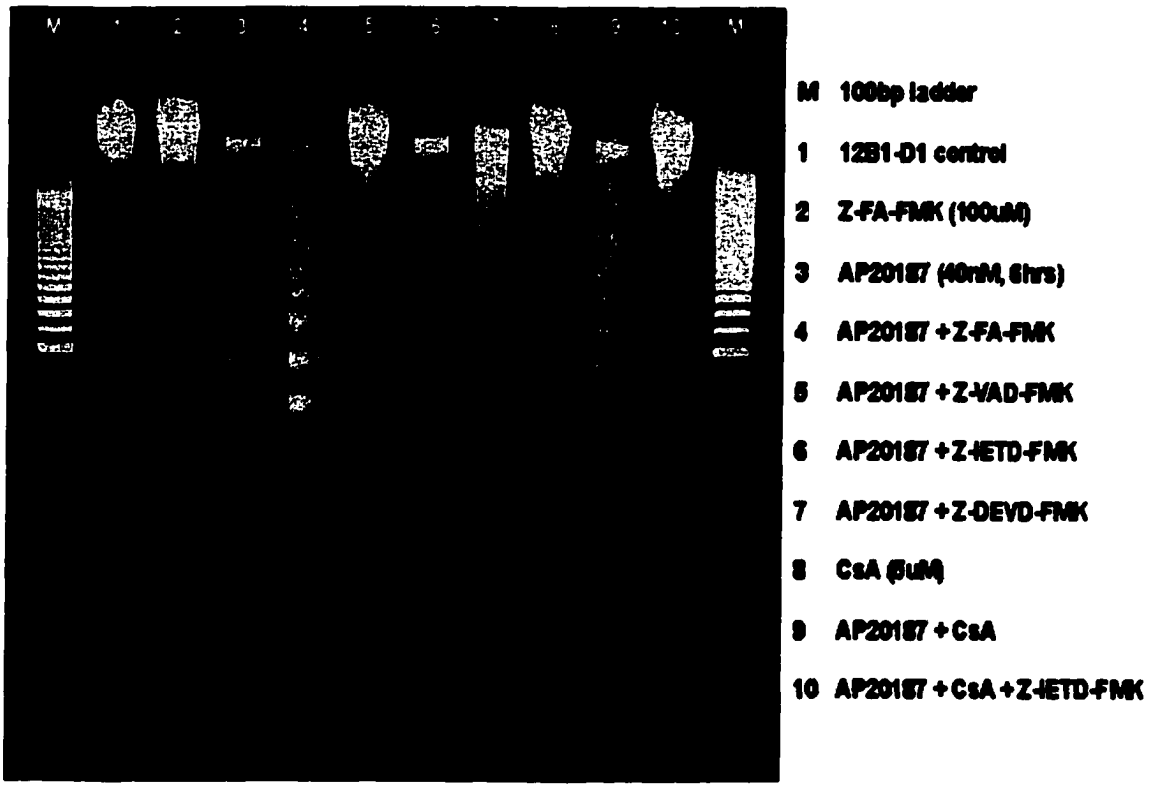


Figure I-8. Apoptotic death of 12B1-D1 cells after AP20187 treatment in the presence of CsA. 12B1-D1 cells, with or without pretreatment of different concentrations of CsA, were treated with 40 nM AP20187 for indicated time, then washed and stained with Annexin V and PI.



A

Figure I-9. Apoptotic death of 12B1-D1 cells was blocked by the treatment of casp-8 inhibitor and CsA. 12B1-D1 cells, with or without pretreatment caspase inhibitors or CsA, were treated with 40 nM AP20187 for indicated time, then washed and stained with mitochondrial probe DioC(6)3 **(A)**



B

Figure I-9. Apoptotic death of 12B1-D1 cells was blocked by the treatment of casp-8 inhibitor and CsA. 12B1-D1 cells, with or without pretreatment caspase inhibitors or CsA, were treated with 40 nM AP20187 for indicated time, then extracted DNA for DNA fragmentation assay **(B).** M, 100-bp ladder; DNA extracted from 12B1-D1 cells (lane 1), or 12B1-D1 cells that pre-treated with control caspase inhibitor Z-FA-FMK (lane 2); lane 3, DNA extracted from 12B1-D1 cells that had been treated with AP20187 for 6 hour. DNA from cells that pre-treated with Z-FA-FMK (lane 4), pan-caspase inhibitor Z-VAD-FMK (lane 5), casp-8 inhibitor Z-IETD-FMK (lane 6), or casp-3 inhibitor Z-DEVD-FMK (lane 7) followed by 6 hrs of AP20187 treatment; 12B1-D1 cells were treated with CsA (lane 8), or CsA followed by AP20187 treatment (lane 9), or CsA together with casp-8 inhibitor followed by AP20187 treatment (lane 10).

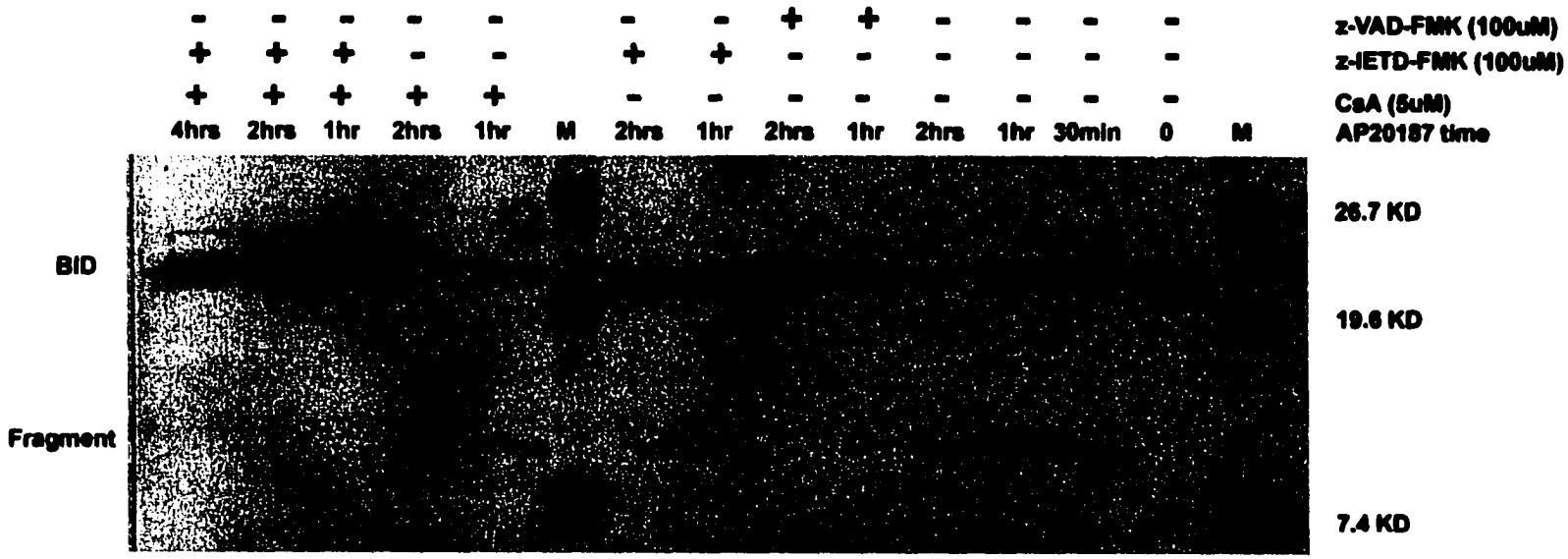


Figure I-10. 12B1-D1 Bid cleavage in the presence of CsA or caspase inhibitors. 12B1-D1 cells, with or without pretreatment of CsA or indicated caspase inhibitors, were treated with 40 nM AP20187 for indicated time, then washed and lysed. Bid cleavage was determined by western blotting using anti-Bid antibody.

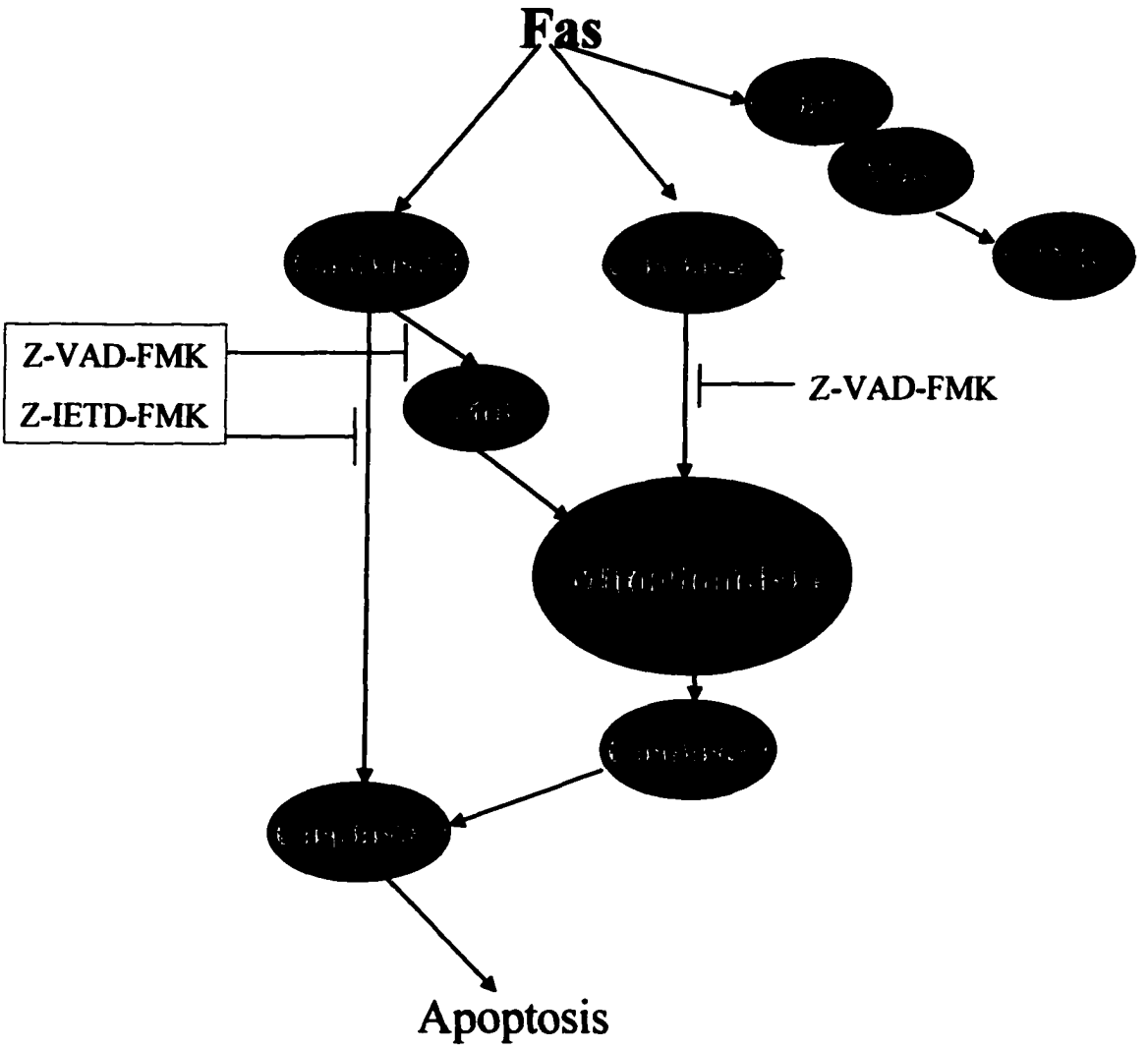


Figure I-11. Our findings indicate that FasDD-oligomerization can trigger a novel caspase-8-independent apoptotic pathway. This pathway is activated by FasDD, but is independent of Bid and the proteolytic activity of caspase-8. It appears to target mitochondria directly by a Z-VAD-FMK-inhibitable mechanism.

CHAPTER II: STRESSED APOPTOTIC TUMOR CELLS EXPRESS HEAT CHOCK PROTEINS AND ELICIT TUMOR-SPECIFIC IMMUNITY

Hanping Feng, Yi Zeng, Luke Whitesell, and Emmanuel Katsanis

2.1 ABSTRACT

In attempting to develop effective anti-cancer immunotherapies the relative ability of apoptotic cells to induce an immune response remains an important but controversial consideration. We utilized a novel gene transfer approach by which rapid induction of pure apoptosis can be selectively achieved in a transfected tumor cell population following exposure to a semisynthetic dimerizing ligand, AP20187. Inoculation of BALB/c mice with apoptotic and viable 12B1.D1 cells, at a 12 to 1 ratio subcutaneously, led to early tumor growth. Heat stress upregulated expression of membrane heat shock proteins (HSP72 and HSP60) on apoptotic 12B1-D1 cells, and stressed apoptotic cells were capable of generating a T cell mediated specific anti-tumor response. Pulsing of stressed apoptotic leukemia cells onto syngeneic dendritic cells (DCs) resulted largely in rejection of co-injected viable 12B1-D1 cells. Mice rejecting the primary 12B1.D1 inoculum were immune to the same but not to a different leukemia challenge. Our findings indicate that tumor immunogenicity is dependent on whether cells are stressed prior to apoptosis induction and suggest that the immune system is capable of distinguishing between stressed and non-stressed cells undergoing programmed cell death.

2.2 INTRODUCTION

The mechanism by which a cell dies determines whether or not an immune response will be generated [22, 76]. Recent studies have documented that apoptotic tumor cells have low immunogenicity *in vivo* while those that die by necrosis can generate an anti-tumor response [77]. This may be due in part to the high expression of inducible HSP70 found in necrotic but not apoptotic cells [77]. Since apoptotic tumor cells apparently do not increase the expression of HSPs during their demise, we hypothesized that stressing the cells prior to apoptosis may re-create the danger signal to which antigen presenting cells are primed to respond.

To test whether apoptotic tumor cells can become immunogenic following stress, we utilized an approach by which we selectively induced programmed cell death following heat shock. The bcr-abl positive and Fas negative murine leukemia cell line, 12B1, was stably transfected with cDNA encoding a recombinant protein that consists of two mutant FK506 binding protein (FKBP) domains and the Fas death domain (Fas DD) [54-56]. Using this novel system we were able to trigger classical apoptotic death in stably transfected leukemia clones with the use of an FKBP specific dimerizing drug, AP20187. The 12B1-D1 clone was selected for further study based on its sensitivity to apoptosis following incubation with AP20187. 12B1-D1 cells were found to have low basal surface expression of HSP72 and HSP60, which could be significantly increased following heat exposure. Tumor development following injection of viable 12B1-D1

mixed with heat-stressed and AP20187-treated leukemia cells into syngeneic BALB/c mice was significantly delayed when compared to co-injection of live 12B1-D1 cells with non-stressed apoptotic leukemia cells. The data presented herein clearly demonstrate that the immunogenicity of apoptotic leukemia cells is relatively poor but can be significantly increased by application of stress stimuli such as induction of HSPs on the surface of apoptotic tumor cells.

2.3 MATERIALS AND METHODS

Bcr-abl positive leukemia cell line: 12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the human *bcr-abl* (b_3a_2) fusion gene and expresses the p210 *bcr-abl* protein [78]. This is an aggressive leukemia with the LD_{100} being 100 cells following tail vein injection [63]. The 12B1 cell line was kindly provided by Dr. Wei Chen (Cleveland Clinic, Cleveland, OH).

Transfection of 12B1 cells with plasmid DNA encoding a mutant FK506 binding protein (FKBP): The cDNA encoding for a recombinant protein that consists of the extracellular domain of the low affinity nerve growth factor receptor (NGFR) fused to two mutant FKBP domains and the Fas death domain was kindly provided by Ariad Pharmaceuticals, Cambridge, MA. This mutant FKBP has reduced affinity for the natural ligand (FK506) and high affinity for synthetic ones [54, 55]. The fusion protein gene was excised from the original cloning vehicle and ligated into the expression vector

pcDNA containing a Zeocin resistance gene (Clontech, Palo Alto, CA). The construct was sequenced to verify ligation fidelity, transformed into E.coli, and plasmid DNA was extracted using a Qiagen Maxiprep kit (Qiagen Valencia, CA). Plasmid DNA was then transfected into 12B1 bcr-abl positive leukemia cells via electroporation. Transfected cells were grown in selective media to obtain stable transfectants, which were then plated in serial dilution to < 1 cell per well. Multiple stable clones were obtained by selection and analyzed by flow cytometry to confirm surface expression of NGFR.

Flow cytometry: 12B1 leukemia cells were washed in phosphate buffered saline (PBS) containing 2% heat inactivated fetal bovine serum and 0.1% sodium azide (Sigma Chemical Co, St. Louis, MO). 2 x 10^5 cells were placed in each well of 96-well U-bottomed microtiter plates. Surface expression of specific antigens was determined by incubating with saturating amounts of monoclonal antibodies for 30 minutes at 4° C. Antibodies used included purified anti-mouse Fas-FITC conjugated (CD95) (clone Jo2, hamster IgG, PharMingen, San Diego CA), purified anti-HSP72 (clone C92F3A-5, mouse IgG_1, StressGen, Victoria, B.C., Canada), purified anti-HSP60 (clone LK-1, mouse IgG_1, StressGen), and anti-NGFR tissue culture supernatant (clone 200-3-G6-4, mouse IgG_1, ATCC, Rockville, MD). The cells were then washed three times in PBS containing 2% heat inactivated fetal bovine serum and 0.1% sodium azide. Secondary antibody used was PE-conjugated affiniPure F(ab')2 fragment goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). After a 30 min incubation the cells were

washed three times and fixed with PBS containing 1% paraformaldehyde (Polysciences, Warrington, PA). Ten thousand cells were analyzed using a Becton Dickinson FACScan.

Immunoblotting: The presence of HSP72 and HSP60 protein in the 12B1-D1 cells was confirmed by Western blotting. Following SDS-PAGE, the gels were electroblotted to nitrocellulose using an Idea Scientific electroblotter (Minneapolis, MN). Gels were transferred in 25 mM Tris, 200 mM glycine, and 20% methanol for one hour at 25 V, stained with Ponceau Red to verify adequate protein transfer and destained in TBST (50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.4). Blots were blocked in 5% non-fat dried milk in TBST for 20-60 min, followed by 3 x 5 min rinses in TBST. The protein of interest was identified using the monoclonal antibodies anti-HSP72 (clone C92F3A-5, mouse IgG_1, StressGen) and purified anti-HSP60 (clone LK-1, mouse IgG_1, StressGen). Primary antibody solutions were prepared in blocking solution, and blots were incubated in primary antibody for 1 hr at room temperature or 12 hr at 4°C, followed by 3 x 5 min rinses in TBST. Alkaline phosphatase conjugated goat anti-mouse secondary antibody was applied for 1 hr at room temperature or 12 hr at 4°C. Immunoreactive signals were detected by color deposition of the alkaline phosphatase substrates NBT/BCIP (Roche, Indianapolis, IN).

Mice: Six to ten week old female BALB/c ($H\text{-}2^d$) mice (Harlan Sprague Dawley, Indianapolis, IN) and C.B-17 *scid/scid* (University of Arizona animal breeding facility)

were used for the experiments. The animals were housed in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Generation of bone marrow derived dendritic cells: BALB/c mouse bone marrow DCs were generated using a slightly modified protocol from that previously described [79]. Bone marrow was harvested from femurs and tibiae and filtered through a nytex screen. Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in AIM V medium (therapeutic grade, GIBCO BRL, Gaithersburg, MD) which contains L-glutamine, human serum albumin, 50 μg/ml streptomycin sulfate and 10 □g/ml gentamicin sulfate. Murine GM-CSF (10 ng/ml, kindly provided by Immunex, Seattle. WA) and IL-4 (10 ng/ml, Peprotech, Rocky Hill, NJ) were added to the culture. After four days, the non- and loosely-adherent cells were harvested, layered onto a metrizamide gradient (14.5% metrizamide solution, Sigma), and centrifuged. The enriched DCs were then washed and used for the *in vivo* experiments.

Cell viability assessment using MTT assay: Parental 12B1 and transfected 12B1-D1 clones were plated in 96 well flat-bottomed plates (50,000/well) in the presence of increasing concentrations of AP20187 for 24 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, stock solution 5 mg/ml) at 10 □l per well was added for an additional 4 hours. The supernatant was aspirated and the formazan crystals

solubilized in DMSO followed by determination of optical densities at 560nm and 690nm using a microtiter plate reader.

Frequency estimation of surviving 12B1-D1 cells following treatment with dimerizer: To estimate the sensitivity of 12B1-D1 to AP20187, heat- or non-heat-stressed cells were studied. Limiting dilution was performed with 12B1-D1 cells added to 96 well plates at 30 replicates with 6 dilutions of 1000 down to 4 cells/well. Individual wells were considered negative if there was no cell growth after 2 weeks. The 95% confidence intervals (CI) of the frequencies and chi^2 estimates of probability were calculated. *P* values >0.05 are statistically significant and confirm that the data follow "single hit" kinetics. The Poisson equation was used [80] to calculate the frequency of surviving cells.

Confirmation of apoptotic cell death in dimerizer treated 12B1-D1 cells: To confirm that 12B1-D1 cells were in fact dying by apoptosis, we performed Annexin V-FITC and Propidum Iodide staining using the Annexin-V-FLUOS staining kit (Roche, Indianapolis, IN) followed by flow cytometric analysis of cells. DNA fragmentation analysis was done using the apoptotic DNA ladder kit from Roche. Electron microscopy was also performed to confirm specific apoptotic morphology.

***In vivo* tumor growth experiments:** Non-stressed 12B1-D1 cells or those incubated at 42°C in a water bath for 1 hour were treated with 40 nM AP20187 for 6 hours, then washed three times in PBS. In experiments with DCs, the 12B1-D1 cells were incubated with the DCs for 6 hrs in the presence of AP20187. 5×10^5 viable 12B1-D1 cells, determined by trypan blue exclusion, were inoculated s.c. into the right groin of BALB/c or SCID mice. In experiments with DCs the ratio of viable tumor cells to DCs was 1:1. Tumor size was measured every other day with calipers once tumors became palpable. Tumor volume was calculated using the formula $length \times width^2 \times \pi/6$. Differences in mean tumor volume between groups was compared using unpaired t test. In rechallenge experiments 2×10^4 live 12B1-D1 cells (LD_{100}) were injected into the right groin 40 days following the first challenge while 10^6 A20 leukemia cells (LD_{100}) [45, 81] were injected into the left groin.

2.4 RESULTS

Transfected 12B1 cells expressing mutant FK506 binding protein and the Fas death domain undergo apoptosis following exposure to AP20187: We used a bcr-abl positive leukemia, 12B1, to investigate whether or not apoptotic leukemia cells can elicit an immune response if induced to express heat shock proteins. 12B1 cells do not express endogenous Fas on their surface (Figure II-1A) and consequently do not undergo apoptosis following incubation with anti-Fas antibody (Figure II-1B). We therefore established a system in order to induce selective apoptosis of 12B1. The cDNA encoding

for a fusion protein consisting of the extracellular domain of the low affinity NGFR fused to two mutant FKBP domains and the Fas DD was transfected into the 12B1 leukemia line (Figure II-2B). Multiple stable clones were obtained by zeocin antibiotic selection and analyzed by flow cytometry to confirm surface expression of NGFR (Figure II-2B). Zeocin-resistant, NGFR-expressing clones were studied further for *in vitro* sensitivity to the dimerizer, AP20187. Using MTT assays we demonstrated that transfected clones (12B1-Dx) were sensitive to increasing concentrations of AP20187 while the parental line (12B1) was not (Figure II-2C). The clone with the highest expression of NGFR, 12B1-D1, was also one of the most susceptible to AP20187. To verify that 12B1-D1 cells were dying by apoptosis, we performed flow cytometry on cells stained with FITC conjugated Annexin V and propidum iodide (Figure II-3A). We also performed electron microscopy (Figure II-3b) and DNA fragmentation analyses (Figure II-3C). All of these assays confirmed that 12B1-D1 cells were undergoing cell death with the classical features of apoptosis. We should note that heat stress prior to AP20187 treatment of cells did not alter the features of apoptosis as determined by annexin staining (data not shown) and DNA fragmentation analysis (Figure II-3C). Limiting dilution analyses were done to assess the sensitivity of 12B1-D1 to AP20187. Using the Poisson equation [80, 82] we calculated that the frequency of surviving cells, when AP20187 was continuously present in the assay, was approximately 1 in 400 cells (Figure II-4). For the *in vivo* experiments (decribed below) heat and non-heat stressed 12B1-D1 cells were exposed to the drug for only 6 hours; limiting dilution assays indicated that this short exposure resulted in 1 of 13

and 1 of 12 cells respectively surviving (Figure II-4). These assays therefore confirmed that a heat shock of 42°C for 1 hr did not modify the sensitivity of 12B1-D1 to AP20187.

Stressed apoptotic tumor cells express HSP72 and HSP60 on their cell surface and these cells elicit an anti-tumor response. *In vivo* experiments were performed to test the immunogenicity (or lack thereof) of apoptotic 12B1-D1 cells. Subcutaneous inoculation of 5 x 10^5 AP20187-treated 12B1-D1 cells into the groin of BALB/c mice invariably led to early tumor growth (by day 10). This indicated that despite injection of greater than 90% apoptotic cells, a host response potent enough to result in rejection of the remaining viable tumor cells (~ 4 x 10^4 live cells based on Figure II-4) did not take place. Silent apoptotic death induced by AP20187 therefore appeared to fail to alert the immune system to danger. We reasoned that stressing the tumor cells prior to triggering apoptosis might induce HSPs, hence supplying danger signals, which may be critical for the generation of tumor specific immunity. 12B1-D1 cells were consequently heat-stressed prior to apoptosis induction. This resulted in upregulation of membrane HSP72 and HSP60 as determined by flow cytometry (Figure II-5A) and total cell HSP72 as assessed by Western blotting (5B). Total HSP60, which is abundantly expressed even under control conditions, did not appear to be increased.

In vivo experiments were then performed to evaluate whether stress prior to apoptosis induction of 12B1-D1 cells would promote an anti-leukemia immune response. Non-

heat-stressed and heat-stressed 12B1-D1 cells were exposed to AP20187 for 6 hours prior to their injection into BALB/c mice. Figure II-6A demonstrates that tumors developed substantially slower in animals receiving viable cells mixed with heat shocked, dimerizer-treated 12B1-D1 cells when compared to non-heat treated cells exposed to dimerizer only ($p<0.01$). The fact that all animals received comparable numbers of live cells ($\sim 4 \times 10^4$) but tumor growth was delayed in those receiving heat-stressed cells suggested that a significant host response was induced in this group. Heat- or non-stressed AP20187-exposed cells were then injected into SCID mice in order to assess whether this response was T cell mediated. In contrast to what was found in immunocompetent BALB/c mice, heat stress prior to apoptosis induction did not retard tumor growth in immunodeficient mice (Figure II-6B) indicating that the host response was, in fact, T cell dependent.

To determine whether the delayed tumor development may have been the result of heat shock alone and not related to apoptosis per se, we performed additional experiments in which BALB/c mice were injected with 12B1-D1 cells that were exposed to heat but not to AP20187. Heat-stressed 12B1-D1 cells when injected into BALB/c mice grew at the same rate as non-stressed cells (data not shown) suggesting that the combination of stress and apoptosis was necessary for generation of the anti-tumor response. It is however, possible that with 5×10^5 cells, any evolving immune response was overwhelmed by the large number of live tumor cells injected. Similar experiments were therefore repeated using the LD_{100} (2×10^4 cells s.c.). This cell dose was previously determined by *in vivo*

dose titration experiments. Comparable results were obtained (Figure II-6C) confirming that both apoptosis and heat stress were required for an anti-tumor response to take place. Two heat-treated groups were included in these experiments. In one group, 12B1-D1 + HS (A), the cells were injected immediately following the 1hr heat shock. The other group, 12B1-D1 + HS (B), (heat shock followed by incubation at 37°C for 6 hr) was included to control for the 6 hour delay in injection of heat-treated cells while they were incubating with AP20187 (Figures 6A and 6B). Failure to induce protection by this group argues against the possibility that simply resting the heat stressed cells for six hours may have contributed to the delay in tumor growth seen in the experiments depicted in Figure II-6A.

We used short time exposure of 12B1-D1 cells to AP20187 to avoid so-called secondary necrosis. Annexin V-FITC/PI staining confirmed that the majority of cells became Annexin V-FITC positive but PI negative (indicating early apoptosis) with a 6 hour exposure to AP20187 (Figure II-3A). To exclude the possibility that the delayed tumor development observed was the result of secondary necrotic 12B1-D1 cells from which cytoplasmic components were released, we performed the following experiments. Heat-stressed or non-stressed AP20187 treated 12B1-D1 were frozen and thawed for 6 cycles to yield a necrotic lysate. Co-injection of lysates with live 12B1-D1 cells did not retard tumor growth compared with mice injected with an equal number of live 12B1-D1 cells

only (Figure II-6D), suggesting that tumor growth delay in Figure II-6A was not due to release of cytoplasmic components.

Pulsing of dendritic cells with stressed apoptotic tumor cells results in the generation of tumor specific immunity. Finally we examined whether dendritic cells (DCs) incubated *ex vivo* with stressed and non-stressed apoptotic cells could induce rejection of co-injected viable leukemia cells, and if such DCs could generate long lasting specific immunity. As in previous experiments, s.c. inoculation of non-stressed dimerizer treated 12B1-D1 cells led to early tumor growth in all mice injected. DCs were incubated with non-heat stressed 12B1-D1 cells in a 1:1 ratio for 6 hours in the presence of AP20187 prior to injection into BALB/c mice. AP20187 is not toxic to normal cells allowing the co-incubation with DC. The delay in tumor growth seen in this group when compared to non-stressed AP20187 treated 12B1-D1 cells (without DCs) suggested that some protection was generated by pulsing DC with apoptotic 12B1-D1 cells (Figure II-7, panel A). Perhaps expression of HSP72 and HSP60 by about 30% of the AP20187 treated 12B1-D1 cells (Figure II-5, panel 4) contributed to maturation/stimulation of DCs. Although injection of DCs incubated with non-stressed, dimerizer-treated 12B1-D1 cells delayed tumor growth, all the mice eventually developed tumors. In contrast, injection of DCs pulsed with heat-stressed dimerizer-treated 12B1-D1 cells, of which 90% expressed HSP72 and HSP60 (Figure II-5, panel 5), led to a significant delay in tumor growth with complete rejection of tumors in about two thirds of the challenged

mice (Figure II-7, panel A). Rechallenge of surviving mice 40 days later with live 12B1-D1 cells (2 x 10^4 =LD_{100}) failed to generate tumors in all mice (Figure II-7, panel B). However, all of the naïve mice that received the same number of 12B1-D1 cells developed s.c. tumors. Moreover, all the immune and naïve mice that were challenged with an LD_{100} (=10^6 cells) injection of different syngeneic leukemia (A20) in the opposite groin developed s.c. tumors, which confirmed the specificity of the anti-tumor response (Figure II-7, panel C).

2.5 DISCUSSION

In this study we have demonstrated that apoptotic tumor cells can be either immunogenic or non-immunogenic. Heat stress prior to apotosis induction increases cell surface expression of HSP72 and HSP60. These upregulated HSPs may play a role in converting non-immunogenic apoptotic tumor cells into immunogenic ones. Apoptosis, or programmed cells death, is important in normal development and in maintaining physiological homeostasis. *In vivo*, apoptotic cells are rapidly phagocytosed by antigen presenting cells (APCs). Macrophages and dendritic cells, express pattern recognition receptors (PRRs), which specifically bind to apoptotic cell associated molecular patterns (ACAMPs) and mediate the efficient phagocytosis of apoptotic bodies [83]. Exogenous antigens acquired from apoptotic cells can gain access to the cytoplasm and be cross-presented on MHC class I molecules [26]. The fact that professional APCs are involved in phagocytosis, processing, and presentation of apoptotic cell derived antigens suggests

that there may be immunological consequences of apoptosis. Antibody-crosslinking of scavenger receptor CD36 leads to IL-10 secretion [25], whereas $\alpha_v\beta_3$ integrin ligation promotes TGF-β production by macrophages [84]. DCs apparently use a distinct $\alpha_v\beta_5$ receptor to phagocytose apoptotic cells [33]. Reports addressing the immunological consequences following engulfment of apoptotic cells by DCs have been highly contradictory [33, 36, 38]. *In vitro* studies have demonstrated measurable cellular responses induced by apoptotic cells. However, a potent immune response was seldom generated *in vivo* [39, 40, 42]. When immature DCs endocytose apoptotic bodies they are not stimulated to mature, and consequently may present their processed antigens in the absence of adequate co-stimulation thereby inducing tolerance [39, 42, 43]. This appears to be an important protective physiologic process since normal cell turnover must not induce autoimmune responses. Similarly, cancer cells undergoing apoptosis typically do not elicit immune responses against their own antigens.

We utilized a novel gene transfer approach by which rapid induction of pure apoptosis downstream of the Fas/FasL apoptotic pathway can be selectively achieved in a transfected tumor cell population. This can be accomplished without any toxicity to other cells following exposure to AP20187. This system allowed us to perform both *in vitro* and *in vivo* studies. To avoid so-called secondary necrosis, a short six hour exposure of 12B1-D1 cells to AP20187 was used. This resulted in a greater than 90% apoptotic cell population with the remaining cells being clonogenic as determined by limiting dilution

assay and viable. This cell mixture was used as a concurrent vaccine-challenge preparation. Mice injected with non-stressed apoptotic and viable 12B1-D1 cells cell mixture subcutaneously developed early growth of tumor. These tumors developed at the same rate as tumors following injection of an equal number of viable cells only. This indicated that a measurable immune response induced by non-stressed apoptotic cells did not take place. "Silent" apoptotic death induced by AP20187 therefore apparently failed to alert the immune system to danger.

APCs can process and present antigens acquired from apoptotic cells, but an active immune response is not induced, which may be due to lack of danger signals [22]. Heat shock protein induction following stress can provide the danger signals required for generation of a more effective immune responses [51]. Recent studies have indicated that tumor immunogenicity is associated with upregulation of the highly inducible HSP70 isoform, HSP72 [77, 85]. Inducible HSP70 can assume dual roles as a chaperone and a cytokine [86] to activate monocytes and upregulate the expression of pro-inflammatory cytokines. Exogenous HSP60 has also been shown to stimulate macrophages to express IL-12 and IL-15 and rapidly release TNF-α [87]. HSP60 appears to signal through CD14 and Toll-like receptors, thus sharing the same pathways as LPS [88, 89]. HSPs may directly mature DCs [49], or act indirectly by stimulating macrophages [87] and CTLs [90] to secrete pro-inflammatory cytokines. The induction of HSPs expression may

therefore provide the danger signals required to convert non-immunogenic apoptotic tumor cells into immunogenic ones.

We reasoned that heat stressing the tumor cells prior to triggering apoptosis might induce HSPs, hence supplying danger signals, which may be critical for the generation of tumor specific immunity. Heat stress treatment (42°C, 1 hour) per se did not induce 12B1-D1 cells to undergo apoptosis, and heat stress prior to AP20187 treatment did not alter the features of apoptosis and had no effects on the sensitivities of 12B1-D1 cells to AP20187 treatment. We found that heat stress resulted in upregulation of membrane HSP72 and HSP60 as determined by flow cytometry and total cell HSP72 as assessed by Western blotting. Co-injection of mice with heat stressed apoptotic and viable cells resulted in a substantial delay in tumor development compared with mice co-injected with non-stressed apoptotic 12B1-D1 cells and the same number of viable cells. This was due to a T-cell mediated immune response induced by stressed apoptotic 12B1-D1 cells since this effect was not seen in SCID mice. It is not yet clear whether HSPs have to be on the surface of apoptotic cells or whether an increase of total cell levels is sufficient for this response to take place. Current studies in our laboratory are addressing this issue.

Previous studies have shown that surface expression of inducible HSP72 by tumor cells increases their susceptibility to lysis by NK cells [91-93]. Among the cell lines tested by these investigators was the NK sensitive, bcr-abl positive human leukemia line K562. If

NK activity was critical in our model system, one would have expected delayed growth of heat-stressed non-apoptotic 12B1-D1 cells due to increased killing *in vivo* by BALB/c or SCID mouse NK cells. However this, was not evident from our *in vivo* studies, suggesting that there may be differences between HSP72-expressing cell lines with respect to their sensitivities to NK mediated killing.

Lysis or necrosis of cells leads to release of intracellular danger stimuli [24]. To exclude the possibility that the delayed tumor growth we observed was the result of necrotic 12B1-D1 cells from which cytoplasmic components were released, heat stressed or non-stressed viable plus apoptotic cells were lysed. The lysates were then co-injected with 2 x 10^4 live 12B1D.1 cells. Interestingly, co-injection of lysates with viable tumor cells did not retard tumor growth when compared with mice injected with an equal number of viable cells only. This was consistent with our previous studies using a B cell leukemia/lymphoma model [46]. Even heat treatment prior to lysing the cells did not influence tumor growth. It therefore appears that the combination of efficient phagocytosis of stressed apoptotic cells coupled with increased HSP levels generated effective immunity against this tumor.

Antigens acquired by DCs from apoptotic cells can induce MHC restricted CTLs *in vitro* [33]. The chemokine receptor-7 (CCR-7) is upregulated when DCs are co-incubated with apoptotic tumor cells promoting DC migration to regional lymph nodes [94] and

potentially generating tumor specific immune responses [40]. However, whether or not apoptotic cells will induce potent T cell responses *in vivo* may ultimately depend on the local cellular and cytokine milieu. Proinflammatory cytokines such as IL-12, IFN-γ, or TNF-α, promote T cell responses while anti-inflammatory cytokines such as IL-10 and TGF-β suppress them. Recent studies have documented that macrophages ingesting apoptotic bodies inhibit proinflammatory cytokine production through an autocrine/paracrine mechanism [95]. The presence of macrophages phagocytosing apoptotic cells may therefore have a negative effect on DC mediated anti-tumor T cell immunity. We examined whether DCs incubated *ex-vivo* with stressed and non-stressed apoptotic cells could induce rejection of co-injected viable leukemia cells and generate long lasting specific immunity. Injection of syngeneic DCs that had been pulsed with stressed apoptotic leukemia cells resulted largely in rejection of co-injected viable leukemia cells. Mice rejecting the primary 12B1-D1 inoculum were immune to the same but not to a different leukemia challenge, confirming the long term and specific anti-tumor immunity.

In summary, our studies conclusively demonstrate that apoptotic tumor cells can be either immunogenic or non-immunogenic *in vivo*. Antigen presenting cells such as macrophages and DCs can efficiently endocytose apoptotic cells whose antigens can gain access to the cytoplasm and be cross-presented on MHC class I molecules. During physiologic processes such as normal development and tissue homeostasis however, an

active immune response is usually not induced due to the lack of danger signals. Heat stress prior to apoptosis induction appears to result in substantial expression of membrane HSPs on tumor cells. HSP72 and HSP60 on the surface of apoptotic cells may provide the necessary danger signals to macrophages and DCs to induce the secretion of proinflammatory cytokines, maturation of DCs and ultimately generation of potent anti-tumor T cell responses. Necrotic cell death therefore is not a prerequisite for the development of an immune response; apoptosis under stress conditions can also elicit T cell responses *in vivo*. These findings may have implications for the development of anti-cancer vaccines using stressed apoptotic tumor cells.

2.6 ACKNOWLEDGEMENTS

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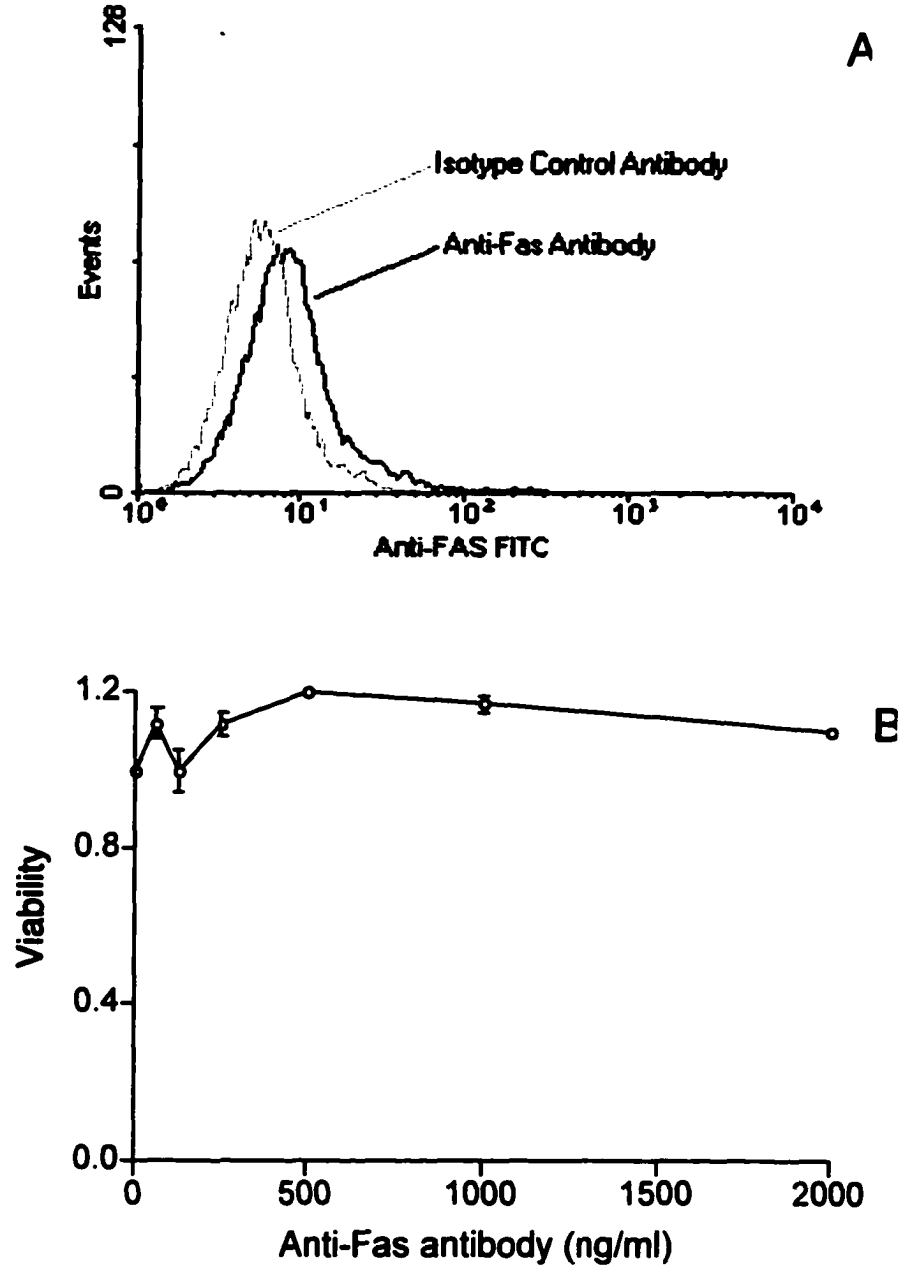
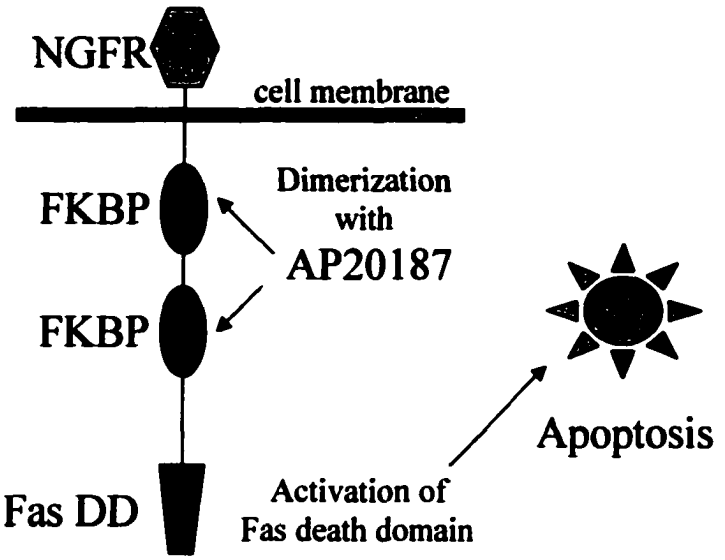


Figure II-1: A. 12B1 cells do not express Fas on their surface. 12B1 cells were incubated with purified anti-mouse Fas-FITC conjugated antibody, washed and analysed by flow cytometry. **B.** Failure of increasing concentrations of anti-mouse Fas antibody to induce apoptosis in 12B1 cells *in vitro* as evaluated by MTT assay after 24 hours.



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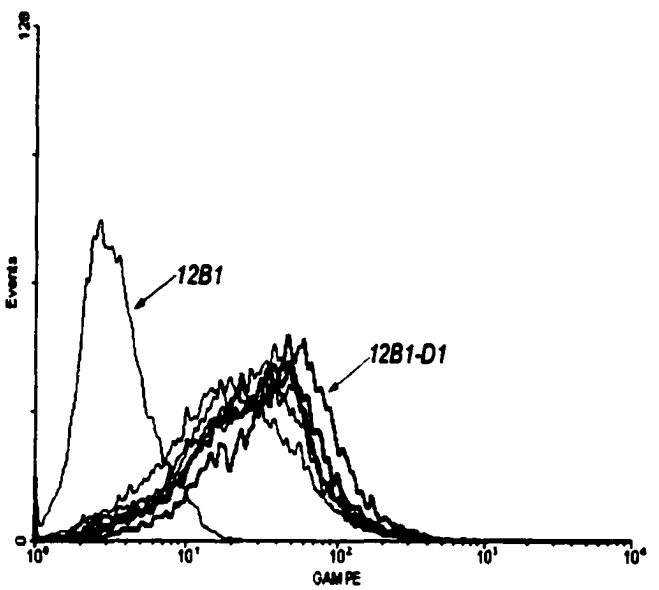


B

Figure II-2: (A). Transmembrane fusion protein consisting of a low affinity nerve growth factor receptor (NGFR) accessible on the cell surface, two mutant FK506 binding protein (FKBP) domains an a Fas death domain (Fas DD) intracellularly. **(B).** Expression of surface NGFR by stably transfected 12B1 clones. The parental line 12B1 has no expression while clone 12B1-D1 has high levels of NGFR on its surface.

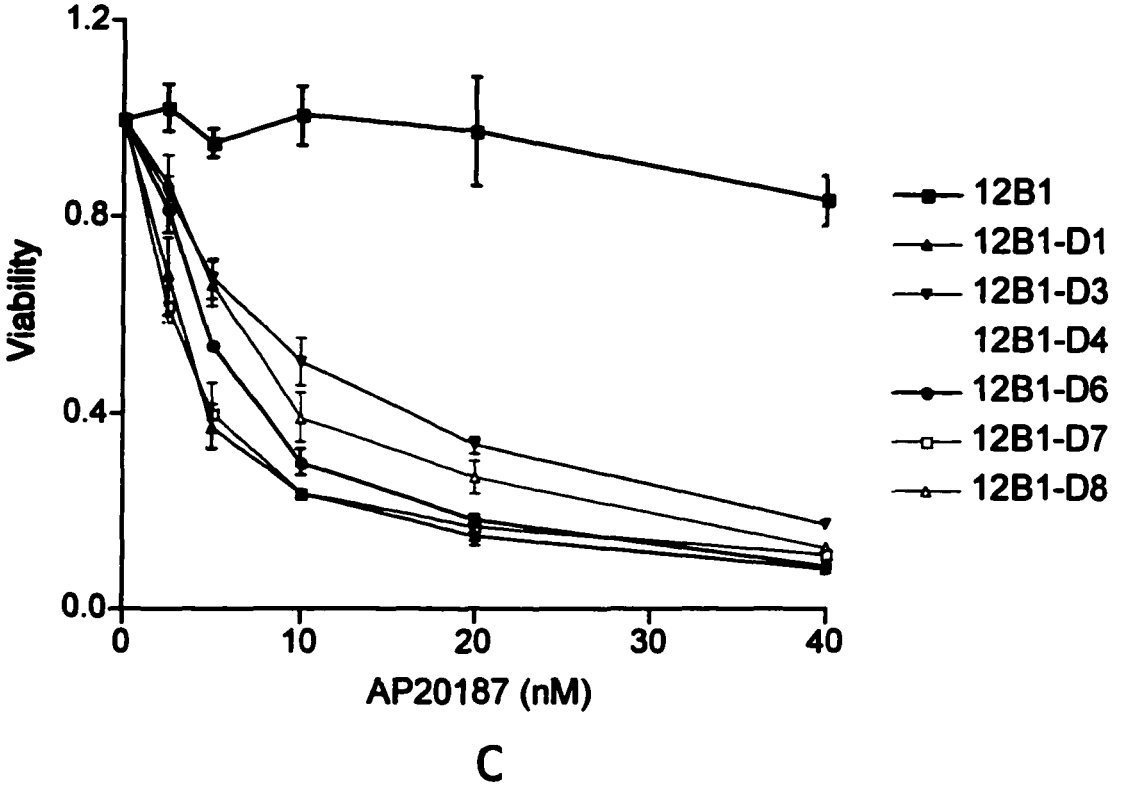
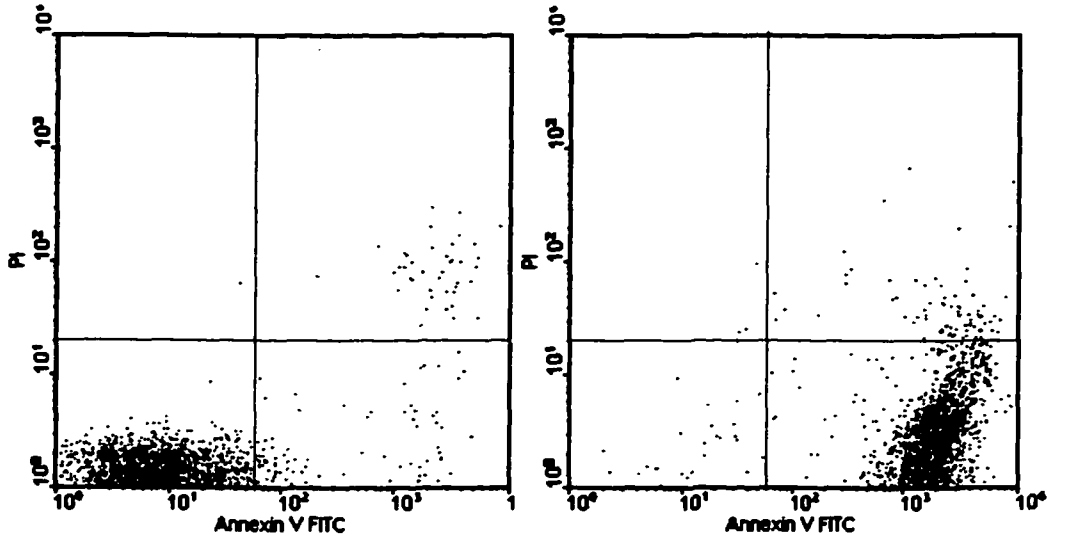


Figure II-2 (C). Sensitivity of transfected 12B1 clones to increasing concentrations of AP20187. Parental 12B1 cells were found to be resistant to AP20187 while transfected clones such as 12B1-D1 were sensitive. Evaluated by MTT assay after 24 hours.



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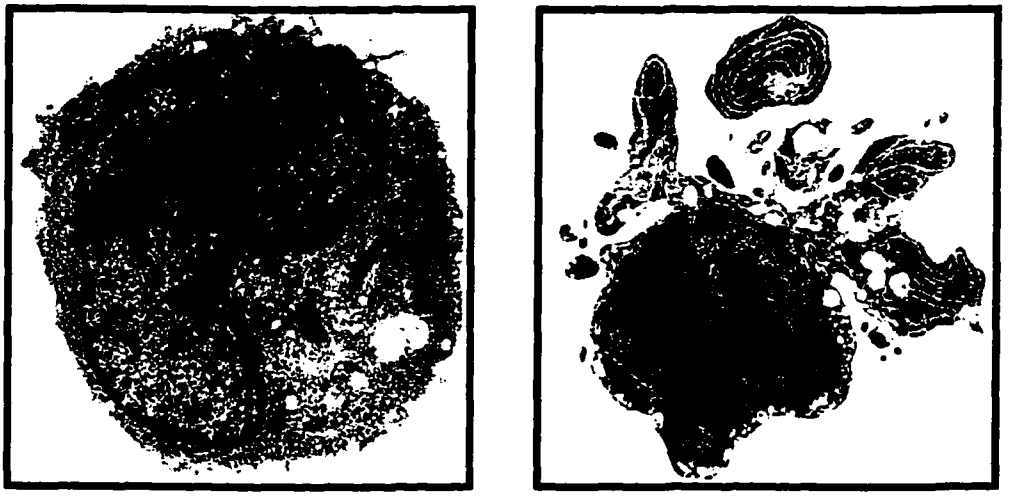
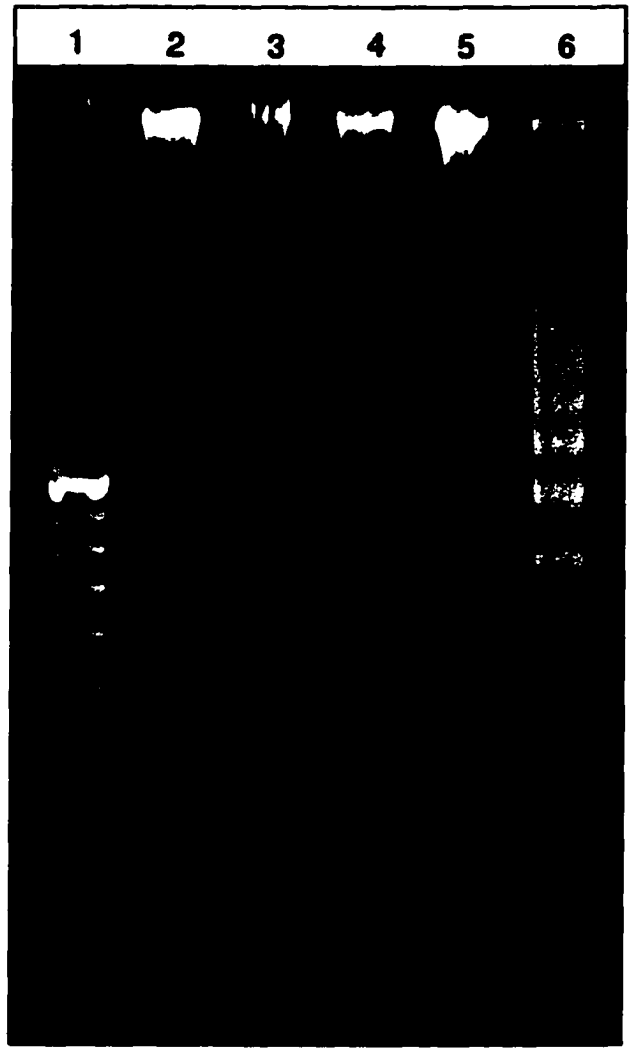


B

Figure II-3: 12B1-D1 cells exposed to AP20187 undergo apotosis (A). The 12B1-D1 clone was cultured in the presence of 40 nM of AP20187 and induction of apoptosis was assessed by Annexin V and propidium iodide staining at the indicated times. **(B).** Electron micrographs of 12B1-D1 cells exposed to AP20187 at 0, 6hr (6000X)



C

Figure II-3: 12B1-D1 cells exposed to AP20187 undergo apoptosis (C). DNA fragmentation analysis. Lane 1, 100bp ladder; Lane 2, DNA extracted from 12B1-D1; Lane 3, 12B1-D1 + HS (A), cells were heat shocked at 42° C for 1hr; Lane 4, 12B1-D1 + HS (B), cells were heat shocked at 42° C for 1hr and then incubated for 6hr at 37° C; Lane 5, 12B1-D1 + AP20187 for 6 hr; Lane 6, 12B1-D1 + HS for 1hr + AP20187 for 6 hr.

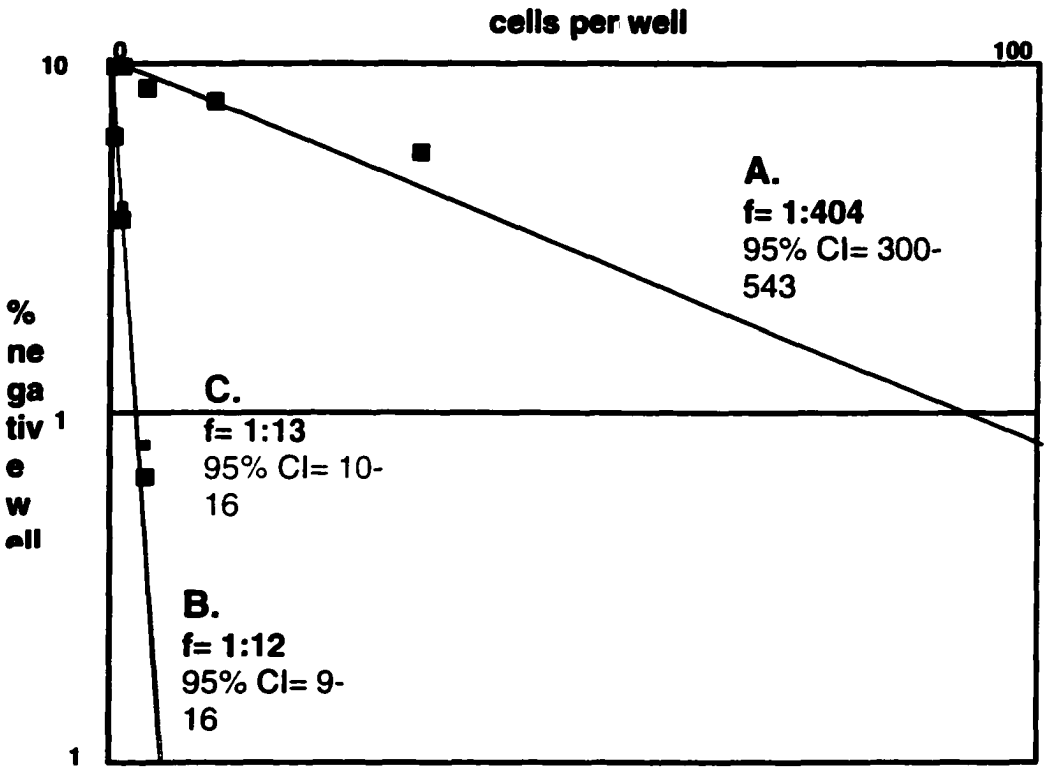
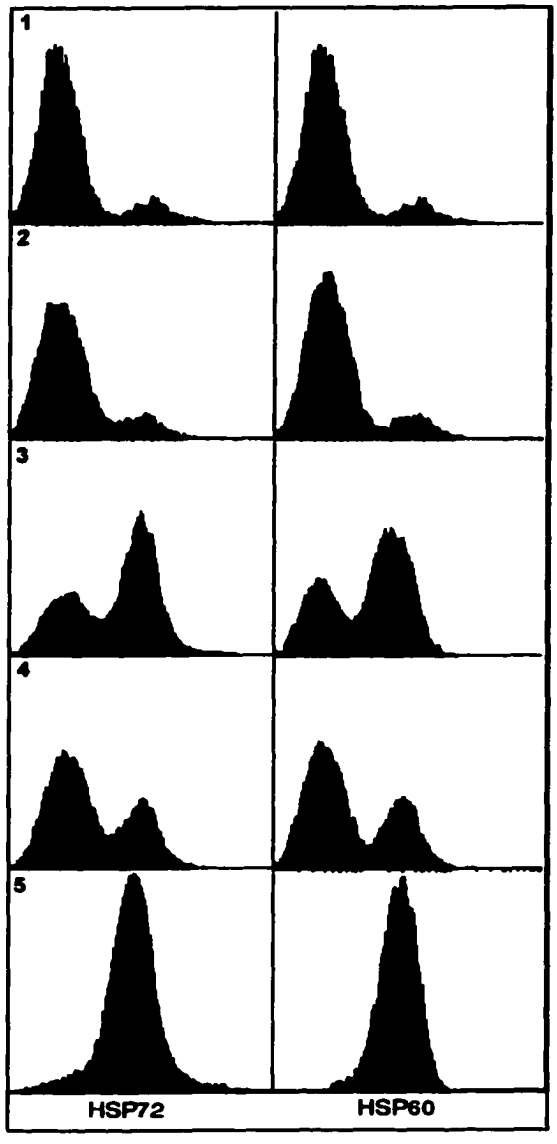
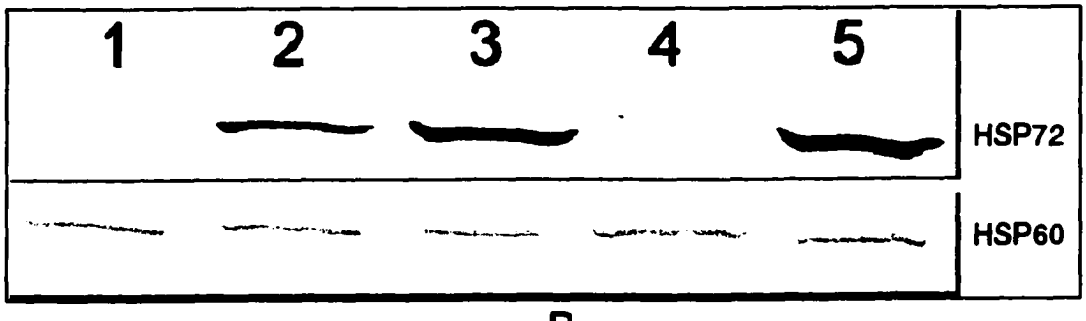


Figure II-4: Frequency estimate of 12B1-D1 cells surviving treatment with AP20187. Limiting dilution was performed with **A.** 12B1-D1 cells plated in the presence of 40nM of AP20187 or **B.** the cells were previously incubated for 6 hr with 40nM of AP20187, washed and then added to 96 well plates or **C.** the cells were previously heat shocked at 42° C for 1hr then incubated for 6 hr with 40nM of AP20187, washed and then seeded into 96 well plates . Individual wells were considered negative if there was no cell growth after 2 weeks. The 95% confidence intervals (CI) of the frequencies and chi2 estimates of probability were calculated. *P* values >0.05 are statistically significant and confirm that the data follow "single hit" kinetics.

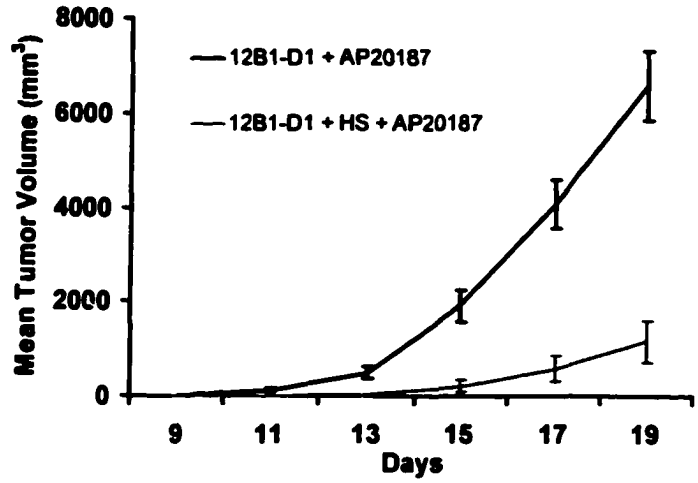


A

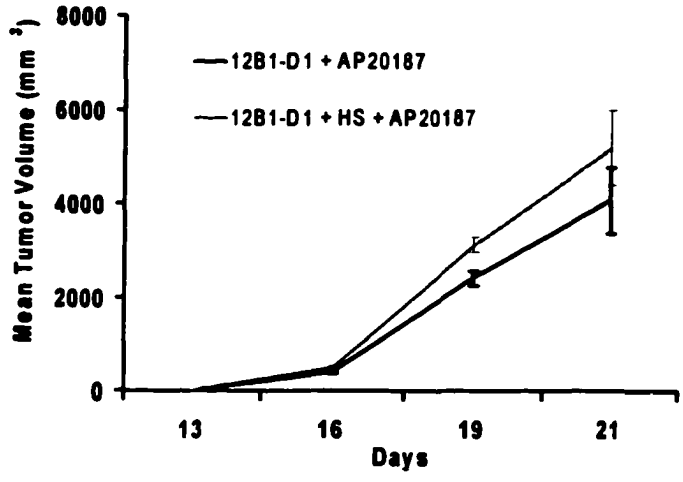
Figure II-5: **A.** Surface expression of HSP72 and HSP60 on 12B1-D1 cells as determined by flow cytometry. Panel 1. 12B1-D1 cells were incubated with isotype control antibody followed by PE-conjugated goat anti-mouse IgG and analysed by flow cytometry. Panels 2, 3, 4, 5. 12B1-D1 cells incubated with anti-HSP72 antibody (left) and anti-HSP60 antibody (right) followed by PE-conjugated goat anti-mouse IgG. Panel 2. Untreated 12B1-D1 Panel 3. 12B1-D1 cells were heat shocked for 1 hr at 42° C and then incubated for 6 hr at 37° C Panel 4. 12B1-D1 cells were incubated for 6 hr with 40nM of AP20187. Panel 5. 12B1-D1 cells were heat shocked for 1 hr at 42° C then incubated for 6 hr with 40nM of AP20187. **B.** Immunoblot of 12B1-D1 cell lysates. Lane 1, untreated 12B1-D1; Lane 2, 12B1-D1 + HS (A), cells were heat shocked at 42° C for 1hr; Lane 3, 12B1-D1 + HS (B), cells were heat shocked at 42° C for 1hr and then incubated for 6hr at 37° C; Lane 4, 12B1-D1 + AP20187 for 6 hr; Lane 5, 12B1-D1 + HS for 1hr + AP20187 for 6 hr.



B

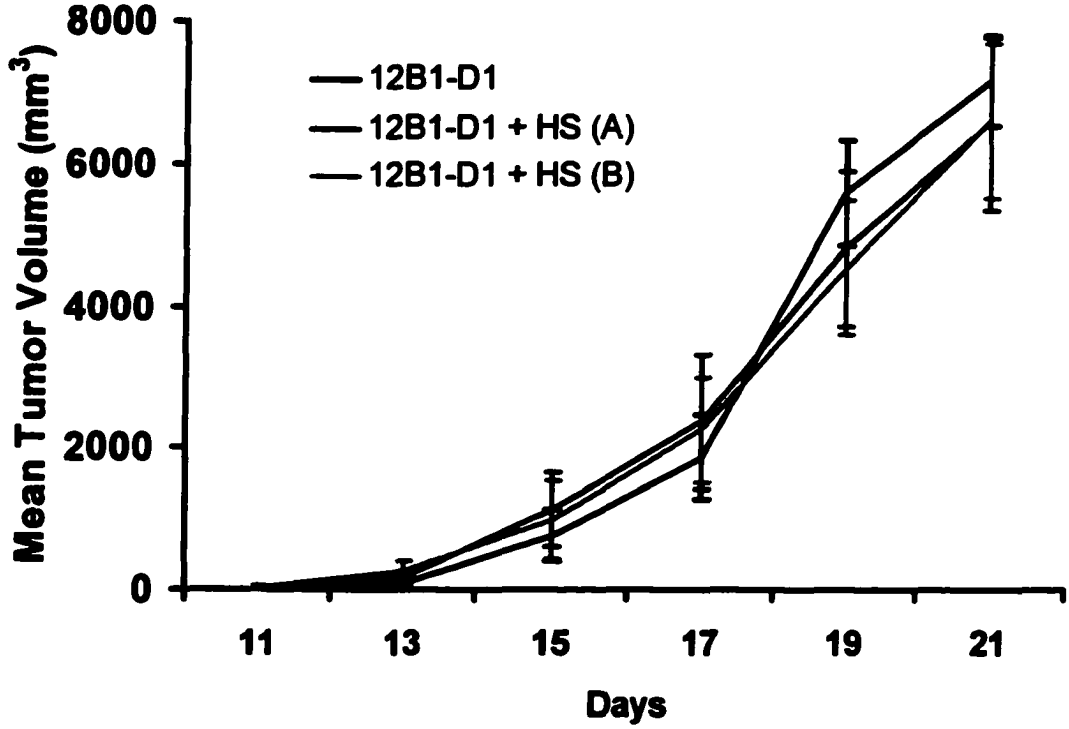


A



B

Figure II-6: **Effects of heat shock and apoptosis induction in anti-tumor responses.** **(A).** 12B1-D1 cells (5 x 10^5) were incubated for 6 hr with 40nM of AP20187 or heat shocked for 1 hr at 42° C (HS), incubated with AP20187 for 6 hr and then injected s.c. into BALB/c mice. Mice were followed for tumor development. Shown are pooled data from 3 experiments (n=20 mice per group); $p < 0.01$ from day 13 onwards. **(B).** 12B1-D1 cells were treated as in A and injected into SCID mice (n=8 mice per group); p=n.s.



C

Figure II-6: Effects of heat shock and apoptosis induction in anti-tumor responses. (C). 12B1-D1 (2 x 104) cells were untreated or heat shocked for 1 hr at 42° C and immediately injected into BALB/c mice, 12B1-D1 + HS (A); or heat shocked for 1 hr at 42° C, incubated at 37° C for 6 hr and then injected 12B1-D1 + HS (B). (n= 8 mice per group) p= ns.

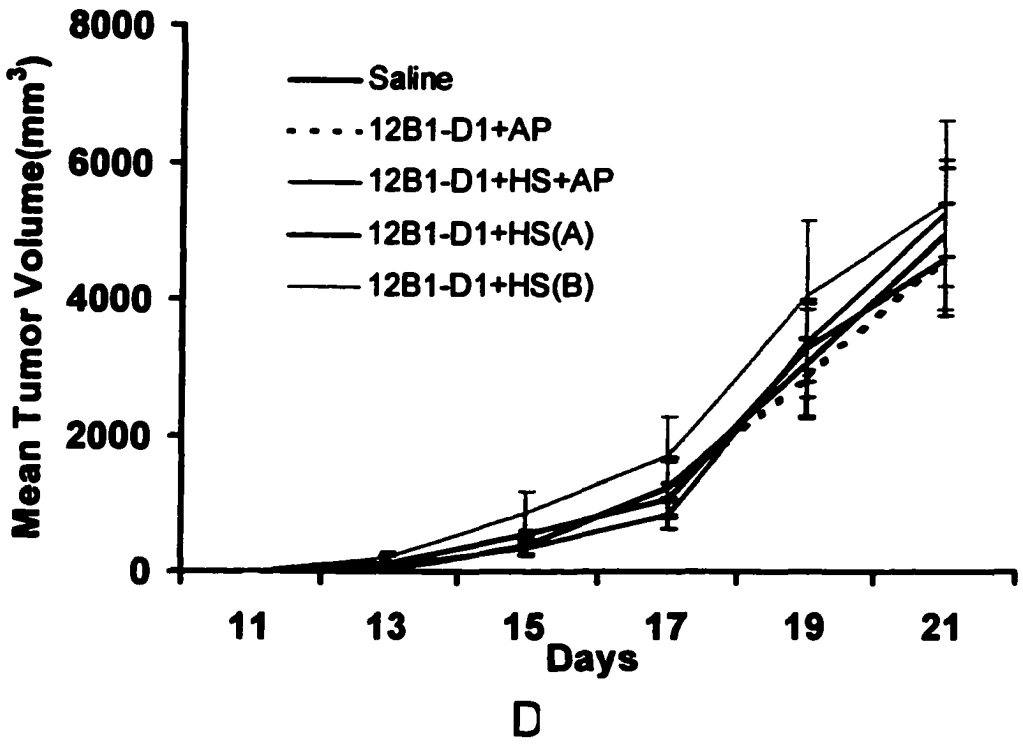
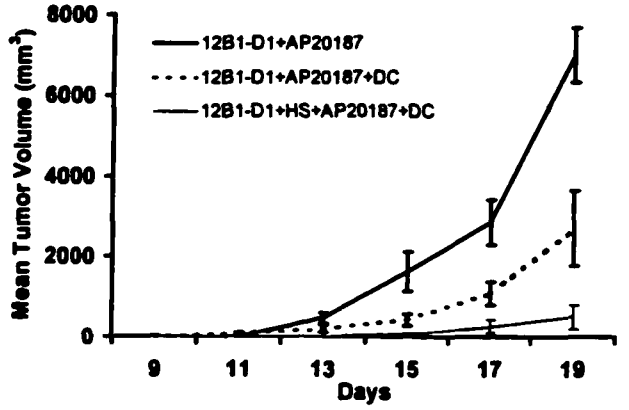
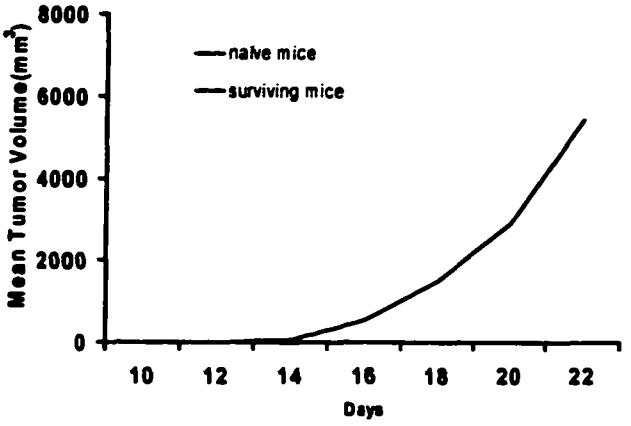


Figure II-6: Effects of heat shock and apoptosis induction in anti-tumor responses. (D). Heat-stressed or non-stressed AP20187 treated 12B1-D1 (5 x 10^5) were frozen and thawed for 6 cycles to yield a necrotic lysate and co-injected with 2 x 10^4 live 12B1-D1 cells. (n= 8 mice per group) p= ns. Shown in panels B, C, and D are representative data from a total of 6 experiments performed.



A



B

Figure II-7: **DCs pulsed with heat-stressed apoptotic cells induce a tumor-specific immune response. (A).** 12B1-D1 cells (5 x 10^5) were incubated for 6 hr with 40nM of AP20187 with or without an equal number of DCs and 12B1-D1 cells were heat shocked for 1 hr at 42° C then incubated with AP20187 and DCs for 6 hr. The cell mixtures were washed and injected s.c. into BALB/c mice. Mice were followed for tumor development. (n=8 mice per group). $p < 0.01$ (for group 12B1-D1 + AP20187 versus 12B1-D1 + HS + AP20187 + DC from day 13 onwards) **(B).** Naïve and surviving BALB/c mice from A. were rechallenged 40 days later with 10^6 A20 cells in the right groin.

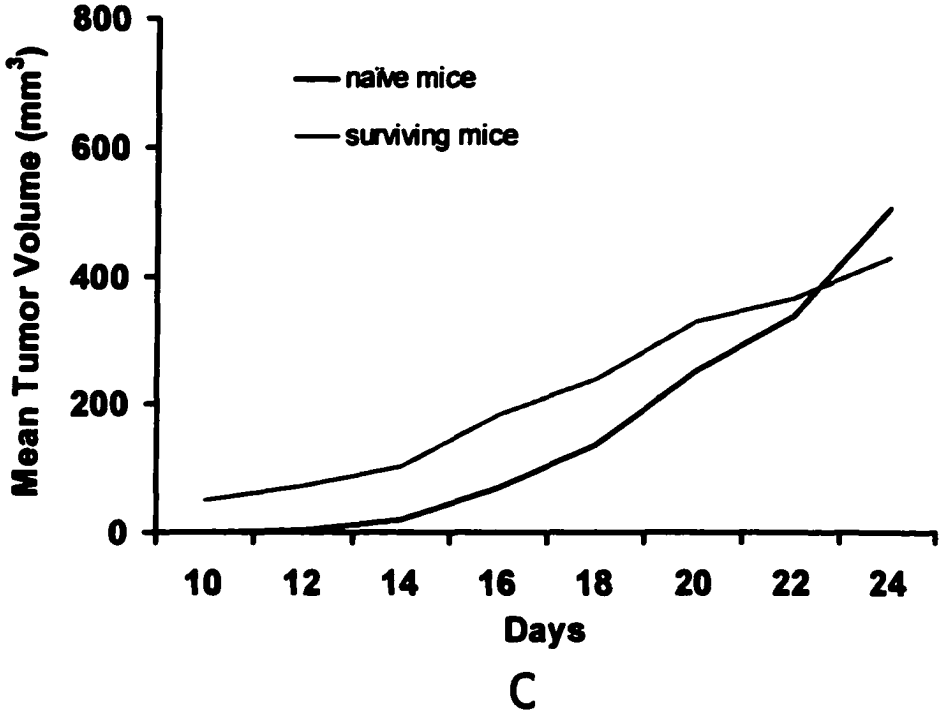


Figure II-7: DCs pulsed with heat-stressed apoptotic cells induce a tumor-specific immune response. (C). The same mice were challenged with 2 x 10^4 12B1-D1 cells in the left groin (n= 5 surviving and 8 naïve mice). Shown are representative data from a total of 3 experiments performed.

CHAPTER III: STRESSED APOPTOTIC TUMOR CELLS EXPRESS HEAT CHOCK PROTEINS AND ELICIT TUMOR-SPECIFIC IMMUNITY

Hanping Feng*, Yi Zeng*, Michael Graner and Emmanuel Katsanis

3.1 ABSTRACT

We have previously reported that stressed apoptotic tumor cells are more immunogenic in vivo than non-stressed ones. Using confocal microscopy we have confirmed our previous observation that heat-stressed apoptotic 12B1-D1 leukemia cells (*BCR-ABL*$^+$) express HSP60 and HSP72 on their surface. To explore how the immune system distinguishes stressed from non-stressed apoptotic tumor cells, we analyzed the responses of dendritic cells to these two types of apoptotic cells. We found that non-stressed and heat-stressed apoptotic 12B1-D1 cells were taken up by dendritic cells in a comparable fashion. However, when stressed apoptotic 12B1-D1 cells were co-incubated with immature dendritic cells for 24 hours, this resulted in greater upregulation of co-stimulatory molecules (CD40, CD80, and CD86) on the surface of dendritic cells. Moreover, stressed apoptotic 12B1-D1 cells were more effective in stimulating dendritic cells to secrete IL-12, and in enhancing their immunostimulatory functions in mixed leukocyte reactions. Furthermore, we demonstrated that immunization of mice with stressed apoptotic 12B1-D1 cells induced the secretion of Th1 profile of cytokines by spleen cells. Splenocytes from mice immunized with stressed apoptotic cells, but not

non-stressed ones, were capable of lysing 12B1-D1 and the parental 12B1 line, but not a B cell leukemia line, A20. Our data 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 dendritic cells, and ultimately the generation of potent antitumor T-cell responses.

3.2 INTRODUCTION

The deficiency of self/non-self paradigm has led to new hypotheses proposed by Janeway [20, 21] and Matzinger [22, 23] in that: the immune system is thought to respond to "danger" associated with infections or stress. According to this paradigm, the occurrence of pathological or necrotic cell death, with cell products released during tissue damage, is a "danger signal" that may initiate protective immune responses. In contrast to necrotic cell death, apoptosis is a physiological process, critical to normal development, tissue remodeling and cell turnover, and thought to be non-inflammatory or bland to the immune system, or even tolerigenic. [24-26] The intact membrane and exposure of surface molecules to specific receptors on phagocytes seems to allow the safe disposition of undesirable cellular remains. [27] This idea, however, has been challenged recently. Lopes and colleagues argued that phagocytosis of apoptotic cells plays a previously unrecognized role in regulating the nature of immune responses against pathogens. [28] Restifo proposed that apoptotic death associated with viral infection can trigger innate and adaptive immune responses though activation of caspase-1 (an activator of IL-1β and

IL-18). [29] Recent studies have documented that apoptosis induced by pathogens can elicit potent immune responses. [30-32] These immune responses are associated with the induction of type I cytokines, such as interferon γ (IFN-γ), [30] and the generation of specific cytotoxic T lymphocytes (CTLs). [32] Therefore, when apoptotic cell death is associated with "danger signals", it can be sensed by the immune system and consequently induces active immune responses.

We have reported that vaccination of mice with heat-stressed autologous apoptotic tumor cells induces anti-tumor immunity that significantly retards tumor progression when compared to vaccination with non-stressed apoptotic cells. [62] Moreover, we have found that stressed apoptotic tumor cells are effective immunogens when loaded onto syngeneic DC. [62] In this study, we explore the mechanisms behind this and demonstrate that stressed apoptotic cells have striking effects on DC, such as upregulating co-stimulatory molecules on their surface and stimulating the production of the proinflammatory cytokine IL-12. These DC changes are induced only by stressed apoptotic tumor cells (and not by non-stressed ones), leading to enhanced immunostimulatory functions of DC. Furthermore, we find that vaccination with stressed apoptotic tumor cells induces more prominent IL-2 and IFN-γ secretion by murine splenocytes, generation of tumor-specific CTLs with high lytic activity. In summary, we have documented that apoptotic tumor cells can be immunogenic when stressed, and that DC play a key role in determining whether a T cell response will be generated.

3.3 MATERIALS AND METHODS

Mice: Six to ten week old female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments. The mice were housed in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Generation of apoptosis or necrotic lysate of 12B1-D1: Non-stressed or heat stressed (42 °C, 1 hour) 12B1-D1 cells were treated with 40 nM AP20187 for 6 hours to generate non-stressed or stressed apoptotic tumor cells. The lysate was generated by 5 cycles of freeze and thaw using liquid nitrogen and a 37 °C water bath.

Confocal Microscopy: Non-stressed or heat stressed 12B1-D1 cells were treated with AP20187 for 6 hours as previously described, [62] and then washed in PBS containing 2% heat inactivated fetal bovine serum and 0.1% sodium azide (Sigma Chemical Co, St. Louis, MO). 2×10^5 cells were placed in each well of 96-well U-bottomed microtiter plates. Surface expression of specific antigens was determined by incubating the cells with saturating amounts of monoclonal antibodies for 30 minutes at 4^o C. Antibodies used included anti-HSP72 (clone C92F3A-5, mouse IgG_1, StressGen, Victoria, B.C., Canada), anti-HSP60 (clone LK-1, mouse IgG_1, StressGen), and anti-HSP90 (clone AC88, mouse IgG_1, StressGen). The cells were then washed three times in PBS

containing 2% heat inactivated fetal bovine serum and 0.1% sodium azide. Secondary antibody used was Cy3-conjugated affiniPure F(ab')2 fragment goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). After a 30 min incubation the cells were washed three times and further stained with Annexin V-FITC using the Annexin-V-FLUOS staining kit (Roche). Cells were then washed and transfered onto microscopic slides using Cytospin (Shandon) centrifugation followed by examination under confocal laser microscopy (Bio-Rad, Hercules, CA).

Generation of bone marrow-derived DC: BALB/c mouse bone marrow DC were generated as described previously. [62] Briefly, bone marrow was harvested from femurs and tibiae and filtered through a Falcon 100-□m nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in complete RPMI medium (therapeutic grade; Gibco BRL, Gaithersburg, MD), which contains 10% fetal calf serum, L-glutamine, human serum albumin, 50 μg/mL streptomycin sulfate, and 10 μg/mL gentamicin sulfate. Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/mL) and interleukin (IL)-4 (10 ng/mL) (Peprotech, Rocky Hill, NJ) were added to the culture. After 6 days, the nonadherent and loosely adherent cells were harvested, washed and used for *in vivo* and *in vitro* experiments. Less than 10% of these cells were contaminated by macrophages ($CD14^+$ cells).

Phagocytosis assay: DC and 12B1-D1 cells were stained with PKH 26 and PKH 67 (Sigma, St. Louis, MO) respectively following manufacturer's instruction. PKH 67 stained non-stressed or heat stressed (42° C water bath for 1 hour) 12B1-D1 cells were treated with AP20187 for 6 hours. DC were co-cultured with non-stressed or stressed apoptotic cells for 18 hours, and then examined by flow cytometry or confocal laser microscopy (Bio-Rad).

Examination of DC surface marker expression by flow cytometry: DC were washed in PBS containing 2% heat-inactivated fetal bovine serum and 0.1% sodium azide (Sigma Chemical). A total of 2×10^5 cells were placed in each well of 96-well U-bottom microtiter plates. Surface expression of specific antigens was determined by incubating the cells first with an Fc-receptor-blocking antibody (Pharmingen, San Diego, CA.) for 5 minutes and then with saturating amounts of monoclonal antibodies (all from Pharmingen) for 30 minutes at 4°C. Antibodies used included purified fluorescein isothiocyanate (FITC) conjugated anti-I-A^d (clone AMS-32.1, mouseIgG2b), anti-CD80 (clone 16-10A1, hamster IgG), anti-CD86 (clone GL1, rat IgG2a), anti-CD40 (clone HM40-3, hamster IgM) and purified phycoerythrin (PE) conjugated anti-CD11c (clone HL3, hamster IgG). After a 30 minute incubation the cells were then washed 3 times in PBS containing 2% heat-inactivated fetal bovine serum and 0.1% sodium azide and fixed with PBS containing 1% paraformaldehyde (Polysciences, Warrington, PA). Ten

thousand cells were analyzed using a FACScan (Becton Dickinson Immunocytometry, San Jose, CA).

Mixed leukocyte reaction (MLR): BALB/c (H-2^d) DC were incubated with stressed or non-stressed apoptotic 12B1-D1 cells at 1:1 ratio in the presence of 10 ng/ml murine GM-CSF and IL-4 for 24 hours. DC were collected, treated with 50 μg/ml Mitocycin C (Sigma chemical) for 20 minutes, then washed three times with PBS. Splenocytes (10^5 per well) from naïve C57BL/6 (H-2^b) mice were plated in 96 well plates. DC were serially diluted and incubated with splenocytes at a ratio of splenocytes to DC ranging from 10:1 to 40:1. After a four day co-culture, 20 μl of 50 mCi/ml [^{3}H] thymidine (ICN Pharmaceuticals) was added to each well. The cells were harvested 18 hourslater using a 96-well Packard cell harvester and the radioactivity measured on a Packard beta counter.

T-cell proliferation assay and IL-2 bioassay: BALB/c mice were immunized with 5×10^5 stressed or non-stressed apoptotic 12B1-D1 cells. Control mice were immunized with saline. Splenocytes from immunized mice were harvested 5 days later and co-cultured with Mitomycin-C treated 12B1-D1 cells. The ratio of splenocytes to tumor cells was 10 to 1. After a 72 hour culture, the supernatant from each group was collected and serially diluted in 96-well plates. IL-2 dependent CTLL-2 cells (2×10^3 cells per well) were added to each well. Human recombinant IL-2 was used to generate standard curve. All assays were performed in triplicate wells. After 24 hours culture, [^{3}H]

thymidine (1 μCi per well) was added. For T-cell proliferation assay, splenocytes were co-cultured with apoptotic 12B1-D1 cells for 4 days before the addition of [^{3}H] thymidine. The cells were harvested 18 hourslater using a 96-well Packard cell harvester and the radioactivity was measured on a Packard beta counter.

ELISPOT Assays: ELISPOT assays were performed to measure IFN-γ production by splenocytes and IL-12 production by DC. To measure the IFN-γ secretion, heat-stressed or non-stressed 12B1-D1 cells were treated with 40 nM AP20187 for 6 hours and then injected to BALB/c mice subcutaneously. Splenocytes of the immunized mice were harvested 5 days later and washed. Between 10^5 to 10^6 splenocytes were then cultured in Millipore MultiScreen-HA 96 well plates (MAHA S45, Millipore, Bedford, MA). To measure IL-12 secretion by DC, between 10^5 to 10^6 DC were cultured alone, or with stressed or non-stressed apoptotic 12B1-D1 cells at 1:1 ratio in the presence of 10 ng/ml GM-CSF and IL-4 for 24 hours on Millipore MultiScreen-HA 96 well plates. The plates had been previously coated overnight with anti-IFN-γ capture antibody (10 μg /ml, clone R4-6A2, rat Mab anti-mouse IFN-γ, BD PharMingen, San Diego, CA) or anti-IL-12 capture antibody (10 μg/ml, clone 9A5, rat Mab anti-mouse IL-12 (p70), BD PharMingen). Cells were then washed with copious amounts of PBST (PBS + 0.05% Tween20). Biotinylated anti-IFN-γ antibody (2 μg/ml, clone XMG1.2, rat Mab anti-mouse IFN-γ, BD PharMingen) or anti-IL-12 antibody (2 μg/ml, clone C17.8, rat Mab anti-mouse IL-12 (p40/p70, BD PharMingen) was added for two 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 1 hour, following extensive washing with PBST, and then with PBS. Spots were visualized following the addition of the HRP substrate 3-amino-9-ethylcarbazole (AEC, Sigma Chemical) prepared in acetate buffer (pH 5.0) with 0.015% hydrogen peroxide. Spots were examined using a dissecting microscope. Wells of interest were photographed with a microscope-mounted Cool SNAP20187 CCD camera (RS Photometrics, Tucson AZ), and images captured with RS Image, Version 1.07 (Roper Scientific, Tucson, AZ).

Cytotoxicity assay: BALB/c mice immunized with heat stressed (42°C, 1 hour) or non-stressed 12B1-D1 cells were both treated with 40 nM AP20187 for 6 hours. Splenocytes from immunized mice were harvested 5 days later and then co-cultured with Mitomycin-C treated 12B1-D1 cells for 5 days. Stimulated effector cells were tested for cytolytic activity against 12B1-D1 cells, parental 12B1 cells, or A20 (B-cell leukemia) cells using a 4 to 6 hour cytotoxicity assay (Promega, Madison, WI) following the manufacturer's instruction.

3.4 RESULTS

Heat-stressed apoptotic tumor cells express HSP60 and HSP72 on their surface. Using flow cytometry, we have previously shown that heat-stressed apoptotic 12B1-D1

cells express HSP60 and HSP72 on their surface. [62] We further evaluated the presence of membrane HSPs on apoptotic bodies by confocal microscopy. Figure III-1 demonstrates that both non-stressed and stressed apoptotic cells stained with Annexin V-FITC. However, only the stressed apoptotic cells displayed double staining of green and red dyes on their surface (Figure III-1), confirming that stressed apoptotic 12B1-D1 cells express HSP60 and HSP72 on their surface. We also examined these cells for HSP90 expression by confocal microscopy and found that neither stressed nor non-stressed apoptotic 12B1-D1 cells expressed membrane HSP90 (data not shown).

Immunostimulatory effects of stressed apoptotic 12B1-D1 cells on DC. To explore whether DC can response differently to stressed and non-stressed apoptotic cells, we first examined whether there was differential uptake of non-stressed versus stressed apoptotic bodies by DC. 12B1-D1 cells were stained with PKH67 and then induced to undergo apoptosis by exposure to AP20187. The AP20187-treated cells were then incubated with DC for 18 hours. Using flow cytometry, we found that DC engulfed non-stressed and stressed apoptotic cells at the same rate as illustrated by the double positive staining (Figure III-2A). Confocal microscopy confirmed that internalization was not affected by the stress level of apoptotic tumor cells (Figure III-2B). As expected, phagocytosis was temperature sensitive, since it occurred at 37 °C but not at 4 °C (Figure III-2B).

We next examined whether these groups of apoptotic tumor cells may differ in their abilities to stimulate DC maturation. DC were cultured with non-stressed or stressed apoptotic 12B1-DI cells at 1:1 ratio for 24 hours. The percentage of $CD11c^+$ DC that co-expressed CD40, CD80 (B7-1), CD86 (B7-2), and MHC-II was determined. Both non-stressed and stressed apoptotic 12B1-D1 upregulated the expression of CD40, CD80, CD86, and MHC II on the surface of DC (Figure III-3). However, stressed apoptotic 12B1-D1 cells reproducibly had a more pronounced effect on inducing CD40, CD80 and CD86 expression (Figure III-3). Several groups have reported that necrotic lysate from tumors releases cellular HSPs and matures DC. [96, 97] Although HSP release might be restricted in apoptotic cells even after 24 hours into apoptosis, [96] we incorporated two additional control groups to explore the possibility that the upregulation of co-stimulatory molecules on DC was due to cellular components, such as HSPs released from apoptotic 12B1-D1 cells. 10^6 of heat-stressed or non-stressed 12B1-D1 cells were lysed by freeze-thaw, and then incubated with 10^6 DC for 24 hours. The lysate derived from either heat-stressed or non-stressed 12B1-D1 cells failed to upregulate the co-stimulatory molecules on DC (Figure III-3). This is in agreement with our reported in vivo data that vaccination with tumor lysate generated by freeze-thaw fails to induce significant protective immunity against 12B1-D1 tumor challenge. [62]

IL-12 is one of most important proinflammatory cytokines secreted by DC that determines Th1/Th2 polarization. [98, 99] Using ELISPOT assays, we next investigated the effect of

stressed and non-stressed apoptotic cells on IL-12 production by DC. Apoptotic cells were co-cultured with DC for 24 hours. Stressed apoptotic 12B1-D1 cells had a dramatic effect on stimulating IL-12 production by DC, whereas non-stressed apoptotic tumor cells failed to induce measurable IL-12 (Figure III-4A).

We further examined the effects of apoptotic 12B1-D1 cells on altering the immunostimulatory function of DC in mixed leukocyte reactions (MLR). DC were cultured with non-stressed or stressed apoptotic 12B1-D1 cells for 24 hours and then treated with Mitomycin C before they were added as stimulators to allogeneic ($H\text{-}2^b$) splenocyte responders. Splenocyte proliferation in the MLR was determined by [^{3}H] thymidine incorporation. Compared to non-stressed apoptotic cells, stressed apoptotic 12B1-D1 cells significantly enhanced the immunostimulatory function of DC (Figure III-4B).

Vaccination of mice with stressed apoptotic tumor cells induces secretion of type I cytokines and stimulates the generation of specific CTLs. Cell-mediated immunity plays an essential role in combating tumors [100] and is characterized by the production of type I cytokines, such as IFN-γ, IL-2, and TNF-α, and the induction of CTLs. We next explored whether vaccination with stressed versus non-stressed apoptotic tumor cells may have differential abilities to induce type I cytokine secretion by T cells and to generate tumor-specific CTLs. We found that splenocytes primed in vivo by vaccination with

stressed apoptotic tumor cells responded with increased secretion of IL-2 and IFN-γ upon in vitro restimulation with Mitomycin C treated 12B1-D1 cells (Figure III-5A and 5B). Moreover, proliferation of stressed apoptotic tumor cells primed splenocytes was significantly higher, as assessed by [^{3}H] thymidine incorporation assays (Figure III-5C).

We further examined the CTL activity generated following vaccination with stressed apoptotic tumor cells. Splenocytes from mice immunized with non-stressed or stressed apoptotic 12B1-D1 cells were collected 5 days after immunization, and restimulated in vitro with Mitomycin-C treated 12B1-D1 cells for 5 another days. These effector cells were tested for cytolytic activity against 12B1-D1, parental 12B1, or A20 targets. We found that vaccination with stressed apoptotic cells resulted in generation of potent CTL activity against 12B1-D1 cells (Figure III-6A). In addition, these CTLs were potent and specific enough to lyse the parental 12B1 cells (Figure III-6B). No cytolytic activity above background was observed against the A20 B-cell leukemia targets, confirming the specificity of CTLs (Figure III-6C). In contrast, vaccination with non-stressed apoptotic tumor cells failed to generate CTLs against 12B1-D1 or 12B1.

3.4 DISCUSSION

DC are the most potent antigen presenting cells (APCs) that play a central role in initiating adaptive and innate immune responses. [99] Distributed as sentinels throughout the body, DC are poised to capture antigens, migrate to draining lymphoid organs, and,

after a process of maturation, select antigen-specific lymphocytes to which they present the processed peptides, thereby inducing immune responses. [101] DC are capable of phagocytosing cells dying by apoptosis. [33-35] However, the immunological outcome following the ingestion of apoptotic cells by DC remains an issue of debate. [28, 29] Some reports indicate that the uptake of apoptotic cells may lead to anti-inflammatory signals and possibly tolerance, whereas interaction with necrotic cells will lead to activation of innate and adaptive immune mechanisms. [36, 96] However, some studies have shown that DC can acquire antigens from apoptotic cells and induce specific CTLs [33, 102] in vitro. Others have reported that DC take up apoptotic tumor cells and transport them to lymph node T cell areas where they induce a specific immune response leading to tumor rejection in vivo. [103] We have previously shown that pulsing of stressed apoptotic 12B1-D1 cells, but not non-stressed ones, onto syngeneic DC resulted largely in rejection of coinjected viable 12B1-D1 cells. [62] Mice rejecting the primary 12B1-D1 inoculum were immune to the same but not to a different leukemia challenge. [62] Our current findings indicate that the immune system is capable of distinguishing between stressed and non-stressed cells undergoing programmed cell death. We have demonstrated that both stressed and non-stressed apoptotic cells are efficiently phagocytosed by DC. This indicates that the difference between the immunologic outcome of stressed and non-stressed apoptotic tumor cells is not due to the differential uptake of apoptotic cells by DC, but rather to the maturation or activation that ensues following contact with the stressed apoptotic bodies.

In addition to antigens that can be acquired, processed and presented by APCs, secondary signals are needed to activate local APCs, especially DC, leading to an active immune response against the antigens.[20-23] Are DC differentially activated in response to stressed or non-stressed apoptotic tumor cells? We demonstrated that DC exposed to stressed apoptotic cells upregulate their surface expression of co-stimulatory molecules and secrete of IL-12, both which are key in determining the type of T-cell responses. IL-12 has been shown to have multiple functions, including modulating Th1 versus Th2 switching, which is critical in anti-tumor immune responses. [100] DC activated with stressed 12B1-D1 apoptotic tumor cells had improved immunostimulatory function in MLR. In contrast, non-stressed apoptotic tumor cells displayed limited capacity to induce DC costimulatory molecule expression, IL-12 secretion, and immunostimulatory function. This is consistent with other reports that in the absence of inflammation, infection, and necrosis, apoptotic cells are taken up by immature DC or macrophages without stimulating autoimmune responses against the self antigens from apoptotic cells. [24, 36, 104] This, as proposed by Steinman *et al*, [43] may represent an important mechanism for establishing peripheral tolerance to self. Therefore, it is possible that tumor cells dying of apoptosis may exploit a similar mechanism to induce tolerance and inhibit the development of tumor specific immunity. In agreement with our findings are reports demonstrating that engulfment of non-stressed apoptotic cells does not stimulate

DC maturation. [24, 36, 96] However, when the dying cells are associated with upregulation of HSPs [62, 77, 105] or with infectious pathogens, [30-32] they are capable of stimulating T cells and therefore are highly immunogenic. We demonstrated that stressed apoptotic 12B1-D1 cells up-regulate HSP60 and HSP72 expression on their surface using flow cytometry [62] and confocal microscopy. These membrane expressed HSPs may act as endogenous adjuvants or danger signals, which stimulate DC maturation, antigen presentation, and consequently T-cell priming. Our data confirm that professional APCs can adopt distinct differentiation choices depending upon whether or not cell death is associated with stress, or danger, even if the cells are dying by apoptosis. In fact, we have demonstrated that exogenous HSPs from normal tissue, which are devoid of tumor antigenic peptides, function as adjuvants, enhancing the immunogenicity of non-stressed apoptotic tumor cells and inducing potent long-term tumor specific immunity in vivo (Danger signals provided by multiple chaperone complexes stimulate tumor specific immunity. Feng, et al. Manuscript submitted).

It is essential for DC to mature before they can activate naive T cells. Stressed apoptotic 12B1-D1 cells mature DC and vaccination with stressed apoptotic tumor cells induces a T cell-dependent anti-tumor immunity. [62] This is characterized by the production of type I cytokines and the generation of tumor-specific cytotoxic T cells. We showed that splenocytes from mice immunized with stressed apoptotic 12B1-D1 cells secreted significantly higher amounts of IL-2 and IFN-γ than spleen cells from mice immunized

with non-stressed apoptotic cells. Moreover we clearly demonstrated that stressed apoptotic 12B1-D1 cells, (but not non-stressed ones), induced potent tumor specific CTLs. Our results are in agreement with our previous report that vaccination with stressed, but not non-stressed apoptotic cells significantly retarded the progression of co-injected tumor cells. [62] CTLs are particularly important in tumor immunity. [106, 107] Several reports have shown that APCs can acquire antigens from apoptotic bodies and cross prime CTLs in vitro. [26, 32, 33] But evidence that apoptotic tumor cells can prime CTLs in vivo remains limited. In the current study we have demonstrated that stressed apoptotic tumor cells are capable of inducing potent CTLs in vivo. We do not believe that these CTLs are the result of immunization with cellular components released from secondary necrotic tumor cells. Because apoptotic cells induced by AP20187 keep their membrane integrity before vaccination as determined by trypan blue exclusions and flow cytometry (data not shown). Moreover, we injected mice with only 5×10^5 AP20187 treated cells which is unlikely to overwhelm the phagocytic capacity of host APC. [41] Finally, we have previously shown that necrotic 12B1-D1 lysate failed to generate anti-tumor immunity retarding the progression of co-injected viable tumor cells. [62]

In summary, in the present study, we provided direct evidence that DC differentially respond to stressed and non-stressed apoptotic tumor cells. In contrast to non-stressed apoptotic 12B1-D1 leukemia cells, stressed apoptotic tumor cells induce secretion of IL-12, upregulate the expression of costimulatory molecules on DC, and augment their

immunostimulatory function. This leads to induction of type I cytokines, generation of effective tumor specific CTLs.

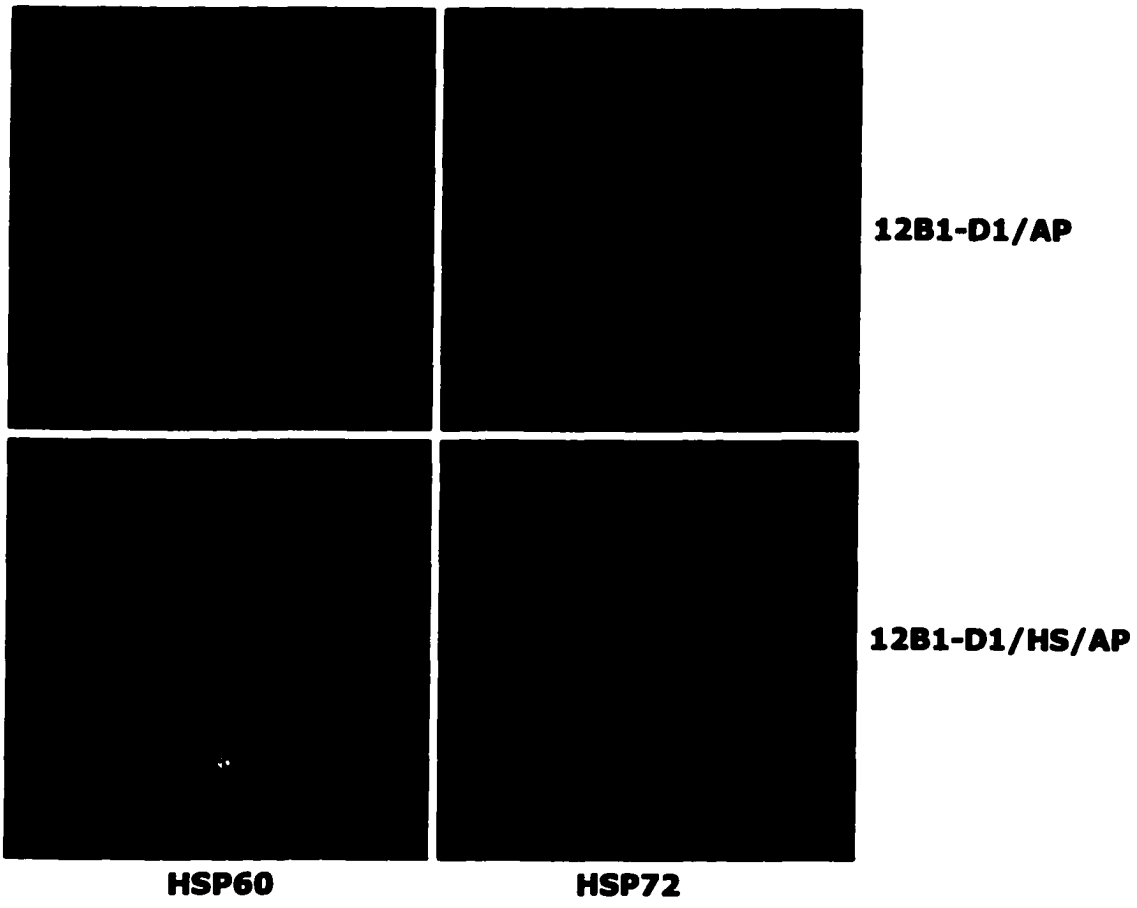


Figure III-1. Stressed apoptotic tumor cells express membrane HSPs. Non-stressed or heat-stressed (42° C, 1 hour) 12B1-D1 cells were treated with AP20187 for 6 hours, and then washed. Surface expression of specific antigens was determined by incubating with anti-HSP72, anti-HSP60, or anti-HSP90 monoclonal antibodies followed by staining with Cy3-conjugated affiniPure F(ab')2 fragment goat anti-mouse IgG. Stained cells were washed and further stained with Annexin V-FITC. Cells were then washed and transfered onto microscopic slides using cytospin centrifugation followed by examination under confocal laser microscopy. (12B1-D1/AP = 12B1-D1 cells were treated with AP20187; 12B1-D1/HS/AP = heat-stressed 12B1-D1 cells were treated with AP20187).

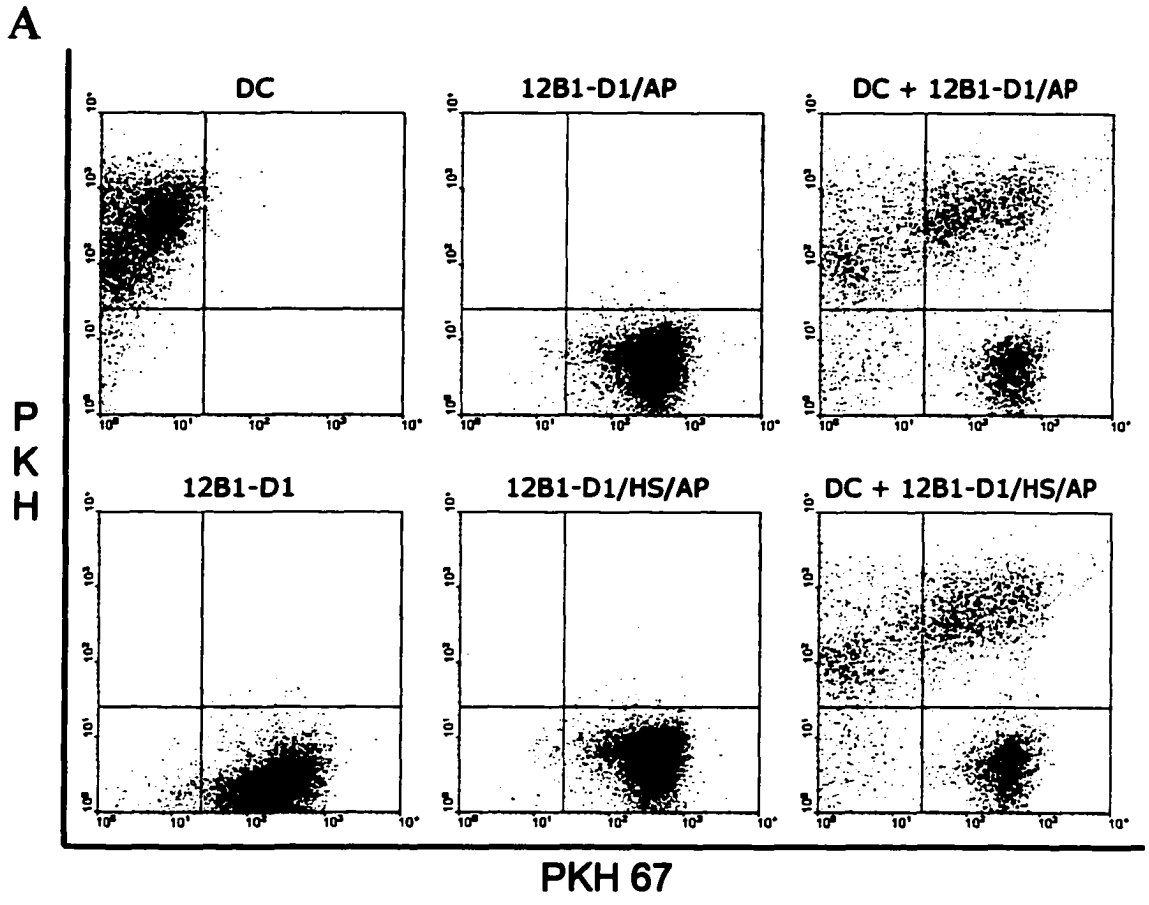


Figure 2. Non-stressed and stressed apoptotic 12B1-D1 cells are taken up by DC. DC and 12B1-D1 cells were stained with PKH 26 and PKH 67 respectively. PKH 67 stained non-stressed or heat-stressed 12B1-D1 cells were then treated with AP20187 for 6 hours to induce apoptosis. DC were co-cultured with non-stressed or stressed apoptotic cells for 18 hours, and then examined by flow cytometry **(A)**

B

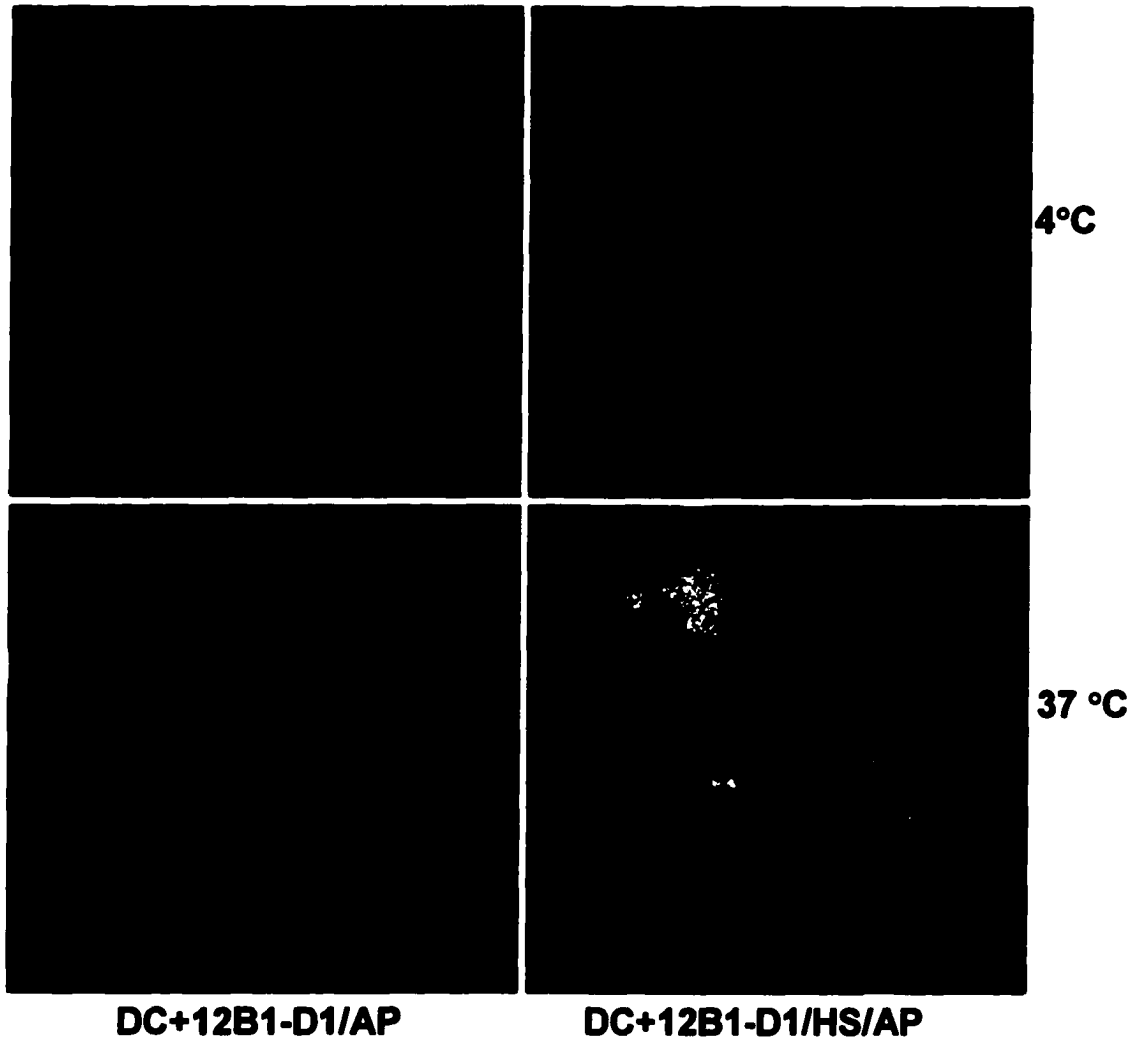


Figure 2. Non-stressed and stressed apoptotic 12B1-D1 cells are taken up by DC. DC and 12B1-D1 cells were stained with PKH 26 and PKH 67 respectively. PKH 67 stained non-stressed or heat-stressed 12B1-D1 cells were then treated with AP20187 for 6 hours to induce apoptosis. DC were co-cultured with non-stressed or stressed apoptotic cells for 18 hours, and then examined by confocal laser microscopy **(B).** Representative data from one of three experiments are shown.

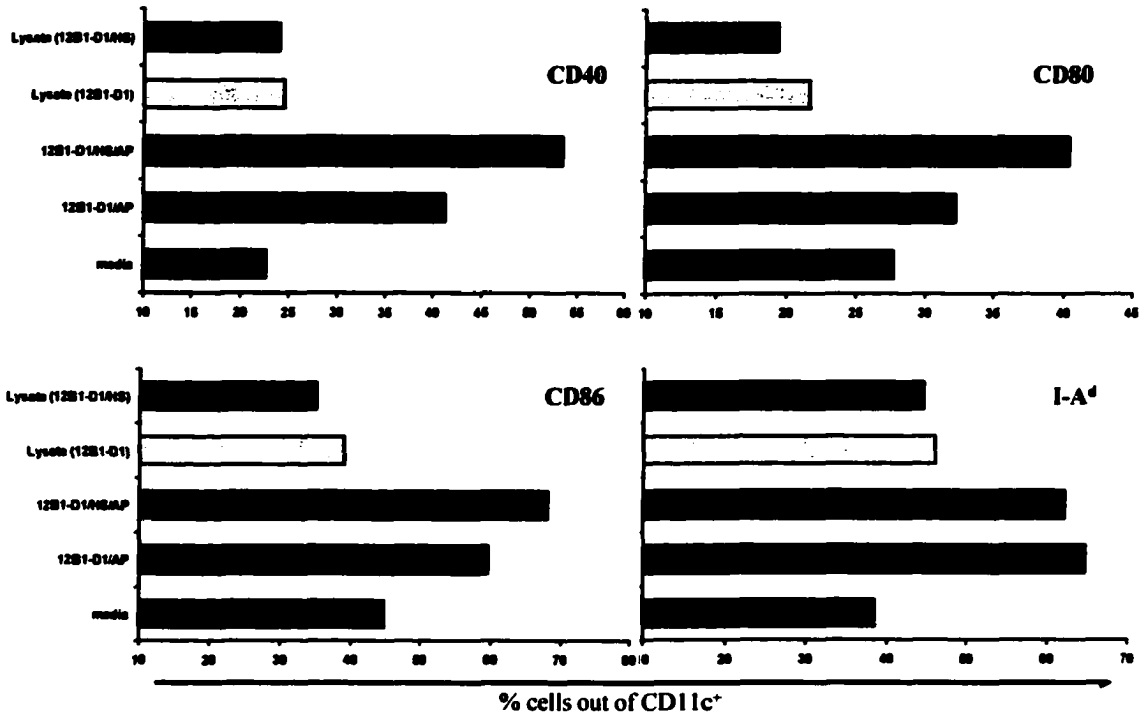


Figure III-3. Stressed apoptotic 12B1-D1 cells upregulate the expression of MHC class II and co-stimulatory molecules on DC. Bone marrow-derived DC were co-cultured heat-stressed or non-stressed apoptotic 12B1-D1 cells at 1:1 ratio for 24 hours. DC were then harvested and analyzed by flow cytometry for expression of the cell surface markers indicated. The numbers in the ordinate represent the percentage of positive cells in the CD11c$^+$ gated population. Representative data from one of four experiments are shown.

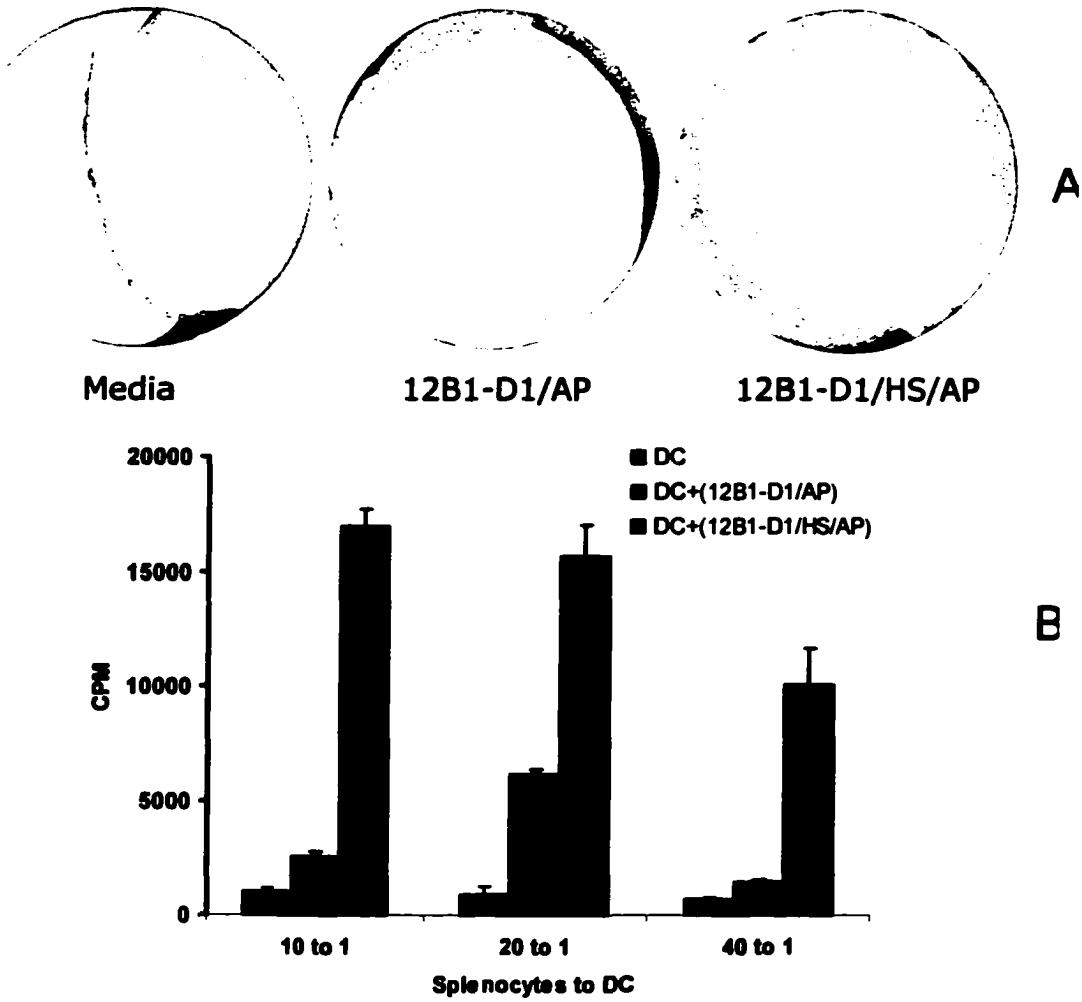


Figure III-4. (A) Stressed apoptotic 12B1-D1 cells induce IL-12 secretion by DC. ELISPOT assays were performed to measure the IL-12 secretion by DC. 3x105 DC were co-cultured with media, or heat stressed or non-stressed apoptotic 12B1-D1 cells at 1:1 ratio for 24 hours in the presence of 10 ng/ml GM-CSF and IL-4 for 24 hours. Representative data from 3 experiments is shown. **(B) Stressed apoptotic 12B1-D1 cells increase DC capacity to stimulate allogeneic splenocyte proliferation.** DC were co-cultured with media, or heat stressed or non-stressed apoptotic 12B1-D1 cells at 1:1 ratio for 24 hours in the presence of 10 ng/ml GM-CSF and IL-4 for 24 hours. DC were then collected and treated with Mitomycin C and washed as described in materials and methods. 105 splenocytes from C57BL6 mice were added per well and cultured with the indicated ratio of pretreated BALB/c DC for 4 days. [^{3}H]- thymidine was added and the cells were cultured for additional 18 hours before the incorporated radioactivity was counted.

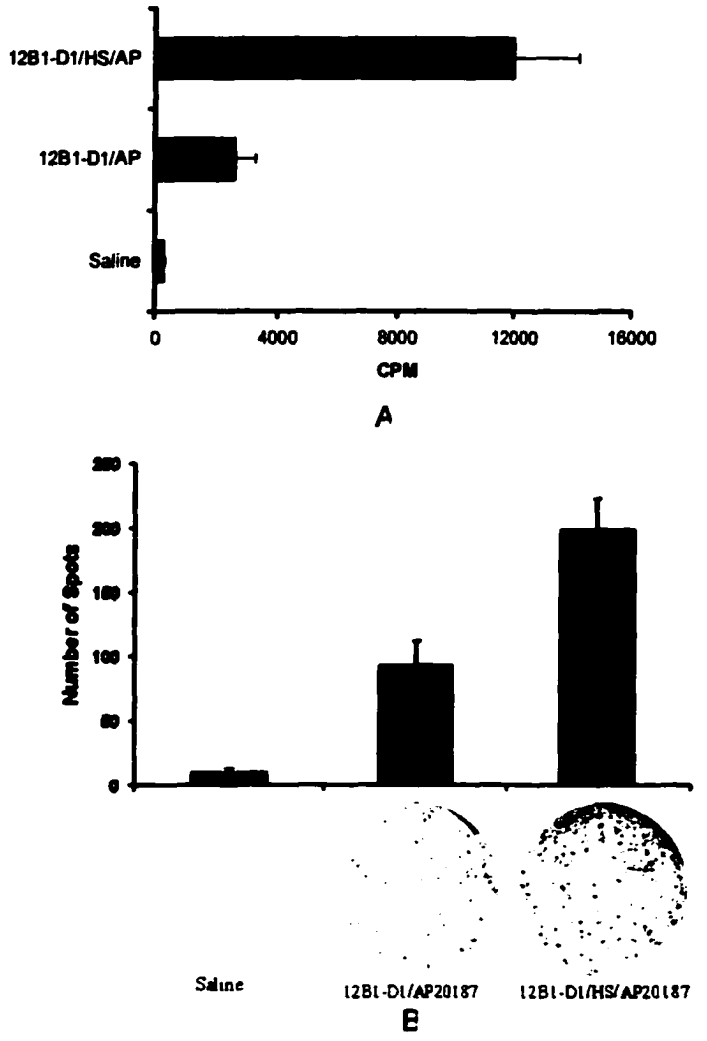
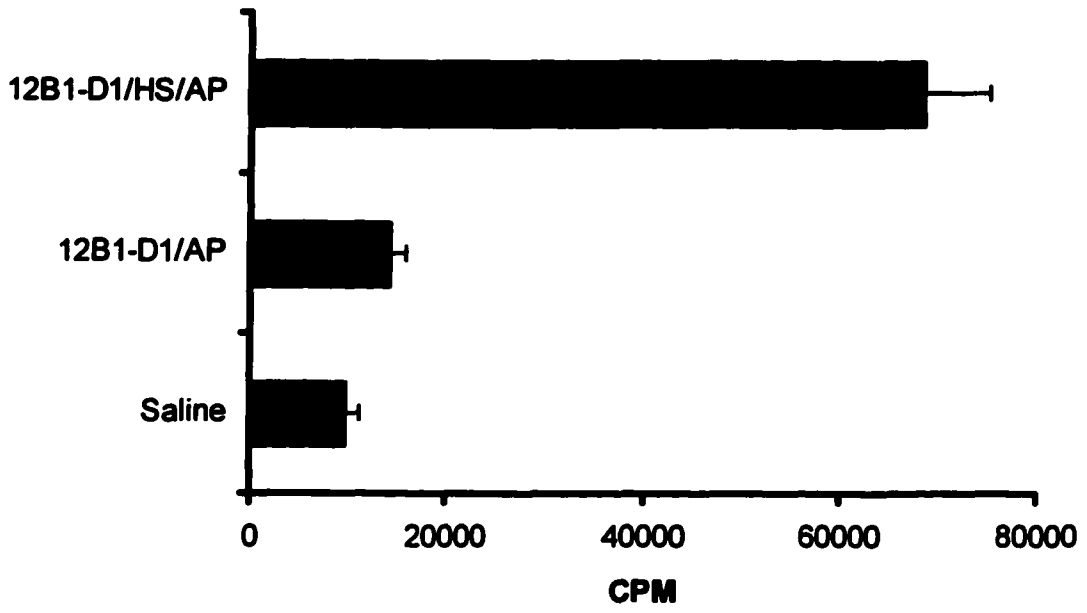


Figure III-5. Immunization of stressed apoptotic 12B1-D1 cells induce IL-2, IFN-γ secretion and T-cell proliferation of splenocytes. Heat stressed (42°C, 1 hour) or non stressed 12B1-D1 cells were treated with 40 nM AP20187 for 6 hours and then injected to BALB/c mice subcutaneously. Splenocytes of the immunized mice were harvested 5 days later and restimulated with Mitomycin-C treated 12B1-D1 cells. **(A)** CTLL-2 bioassay was used to determine the IL-2 production. **(B)** IFN-γ secretion was determined by ELISPOT.



C

Figure III-5. Immunization of stressed apoptotic 12B1-D1 cells induce IL-2, IFN-γ secretion and T-cell proliferation of splenocytes. Heat stressed (42°C, 1 hour) or non stressed 12B1-D1 cells were treated with 40 nM AP20187 for 6 hours and then injected to BALB/c mice subcutaneously. Splenocytes of the immunized mice were harvested 5 days later and restimulated with Mitomycin-C treated 12B1-D1 cells. **(C)** T cell proliferation was determined by [^{3}H] thymidine incorporation. Experiments have been repeated three times.

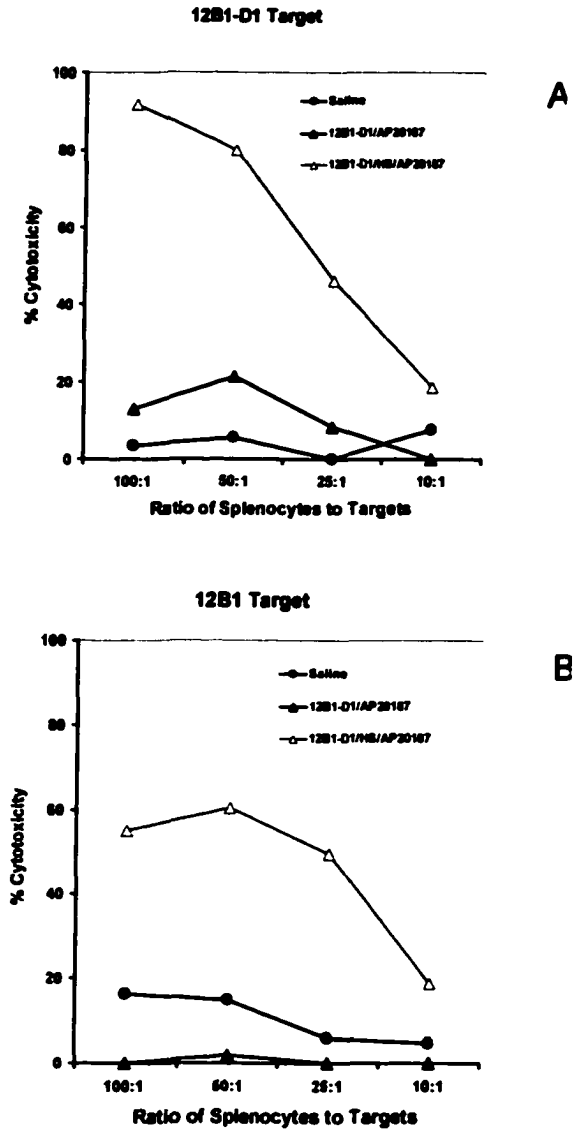


Figure III-6. Immunization of stressed apoptotic 12B1-D1 cells induce specific CTL. BALB/c were immunized with saline, heat stressed (42°C, 1 hour) or non stressed 12B1-D1 cells that had been pre-treated with 40 nM AP20187 for 6 hours. Splenocytes of the immunized mice were harvested 5 days later and restimulated with Mitomycin C treated 12B1-D1 cells for 5 days. Stimulated effector cells were tested for cytolytic activity against 12B1-D1 cells **(A)**, parental 12B1 cells **(B)**

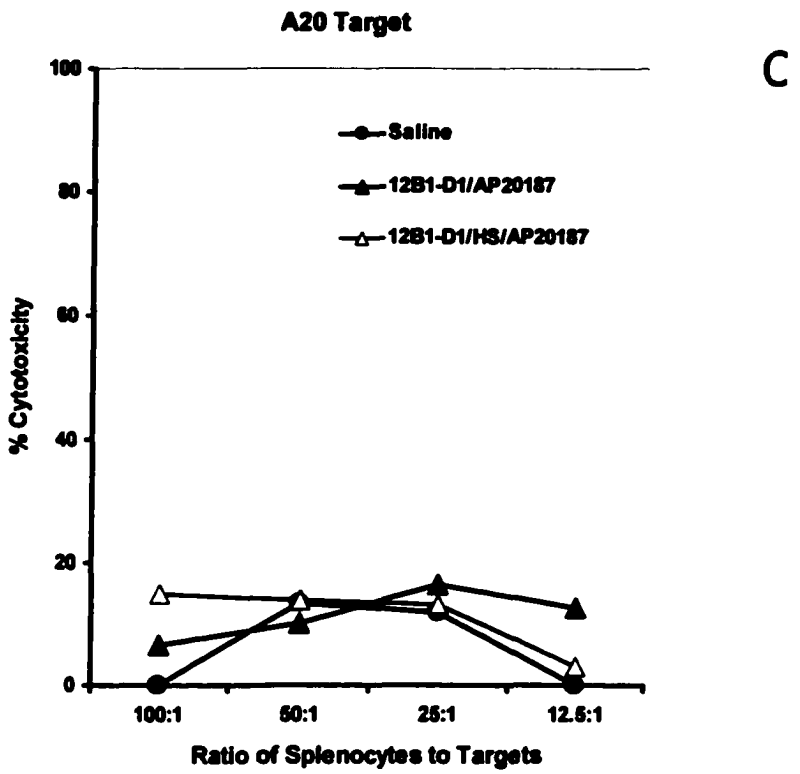


Figure III-6. Immunization of stressed apoptotic 12B1-D1 cells induce specific CTL. BALB/c were immunized with saline, heat stressed (42°C, 1 hour) or non stressed 12B1-D1 cells that had been pre-treated with 40 nM AP20187 for 6 hours. Splenocytes of the immunized mice were harvested 5 days later and restimulated with Mitomycin C treated 12B1-D1 cells for 5 days. Stimulated effector cells were tested for cytolytic activity against A20 cells **(C)** by using 4 to 6 hrs cytotoxicity assay. The figure depicts representative data from one of three similar experiments.

CHAPTER IV: DANGER SIGNALS PROVIDED BY MULTIPLE CHAPERONE COMPLEXES STIMULATE TUMOR IMMUNITY

Hanping Feng, Yi Zeng, Michael W. Graner, Anna Likhacheva and Emmanuel Katsanis

4.1 ABSTRACT

We have previously reported that apoptotic tumor cells can be either immunogenic or non-immunogenic in vivo, depending upon whether or not these cells are heat stressed before apoptosis induction. Stressed apoptotic cells express heat shock proteins on their plasma membranes, and dendritic cells, are capable of distinguishing them from non-stressed apoptotic cells. Here we provide evidence that when purified heat shock protein 70 or multiple chaperone complexes from syngeneic normal tissue are used as adjuvants with non-immunogenic apoptotic tumor cells in vaccination, potent anti-tumor immunity can be generated. This anti-tumor immunity is T-cell mediated, since anti-tumor effects were not observed in either severe combined immunodeficiency or T-cell-depleted mice. We further demonstrated that vaccination of mice with apoptotic tumor cells mixed with liver-derived multiple chaperone complexes as adjuvant were capable of enhancing the production of Th1 cytokines, inducing specific cytotoxic T lymphocytes and eliciting long-lasting anti-tumor immunity. Stress proteins from autologous normal tissue components therefore can serve as danger signals to the immune system. Our studies provide new insights into combining immunotherapy with agents that induce apoptosis of tumor cells for more effective cancer treatments.

4.2 INTRODUCTION

It is well accepted that antigen presenting cells (APC) efficiently acquire antigens from apoptotic tumor cells and present them to T cells [26, 33-35]. However, the immunological consequences of this remain controversial [28, 29]. Macrophages and dendritic cells (DC) phagocytose apoptotic tumor cells through a receptor mediated pathway [33-35] that results in tumor antigen access to the cytoplasm and cross presentation on APC MHC class I molecules [26, 33]. Therefore, apoptotic tumor cells induced by chemotherapy or radiotherapy can theoretically be a suitable antigen source for stimulation of anti-tumor responses. We have previously reported that apoptotic 12B1-D1 (*BCR-ABL*$^+$) leukemia cells can be either immunogenic or non-immunogenic in vivo depending upon whether or not these cells are heat stressed prior to apoptosis induction [62]. Furthermore, we have found that DC are capable of distinguishing stressed apoptotic tumor cells from non-stressed ones (unpublished data). We have also demonstrated that stressed apoptotic tumor cells express heat shock proteins (HSPs) on their surface, which appear to play a critical role in enhancing their immunogenicity [62] (and unpublished data).

Tumor derived HSPs also called chaperone proteins, when used as vaccines, can induce protective immunity against their tumors of origin [44]. In our studies we found that vaccination with multiple HSPs/chaperone proteins enriched from tumor lysate by free solution isoelectric focusing (FS-IEF) induced specific anti-tumor immunity in various

tumor models [45] (and unpublished data[2]). Some important chaperone proteins such as gp96, HSP90, HSP70, and calreticulum enriched by this method tend to complex together [46]. We therefore refer to the FS-IEF derived preparation as multiple chaperone complexes (MCC). We have previously reported that tumor derived MCC have superior abilities to stimulate DC compared to purified individual HSPs such as HSP70 and gp96 (unpublished data[3]), which have been used as tumor vaccines in mice and humans [47, 48].

Several mechanisms have been proposed to explain how these HSP complexes, which carry tumor derived peptides as part of their chaperoning functions, can elicit immune responses [49-51]. One possible hypothesis is that chaperone complexes supply both antigens and danger signals to the immune system [49-51]. These adjuvant effects/danger signals activate APC, such as DC, leading to more efficient processing and presentation of HSP chaperoned peptides [52, 53]. We have previously reported that heat stress induces HSP expression on the surface of apoptotic 12B1-D1 cells and increases their immunogenicity [62] (and unpublished data[1]). We therefore reasoned 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. To test our hypothesis, normal syngeneic liver derived chaperone proteins, (devoid of tumor specific antigenic peptides), were co-injected with non-stressed apoptotic tumors. This resulted in the reproducible generation of durable and

specific T-cell-mediated anti-tumor immunity. Non-stressed apoptotic cells alone or when combined with liver lysate generated by freeze-thaw were ineffective vaccines. Therefore, we have demonstrated that normal tissue (liver) MCC may function as an effective danger signal to the immune system. MCC enriched by FS-IEF is a simple and rapid method for the generation of natural vaccine adjuvants active in enhancing immunogenicity of apoptotic tumor cells, which allow the generation of a potent anti-tumor immunity when combined with apoptotic tumor cells.

4.3 MATERIALS AND METHODS

Mice. Six to ten week old female BALB/c (H-2^d) mice (Harlan Sprague Dawley, Indianapolis, IN) and C.B-17 severe combined immunodeficiency (SCID) *scid/scid* (University of Arizona animal breeding facility) were used for the experiments. The animals were housed in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

FS-IEF for chaperone enrichment and conventional purification of HSP70. Methods to enrich MCC from tumors or naïve BALB/c mouse liver have been described previously [46]. Briefly, tumor or liver tissue was homogenized in lysis buffer and a 100,000g supernatant was obtained. The high-speed supernatant was subjected to FS-IEF in a Bio-Rad Rotofor cell for 5 h at 15 W constant power. Twenty fractions were harvested, and each fraction was analyzed by SDS-PAGE and Western blot probing with

specific antibodies for the chaperones HSP60, 70, 72, 90, gp96, and calreticulin (CRT). Fractions from FS-IEF that contained substantial amounts of above chaperon proteins, as determined by SDS-PAGE and Western blotting, were pooled and dialyzed stepwise out of urea and detergents. Pooled fractions were then concentrated using Centricon devices, reconstituted in PBS and stored in -70 °C until use. Purification of liver HSP70 was done via conventional and nucleotide-affinity chromatography as previously described [45].

Induction of apoptotic cell death in 12B1-D1 cells. To induce 12B1-D1 cells to undergo apoptosis, cells were treated with 40nM of AP20187 for 6 hours as discribed previouly [62], or with 100 μg/ml of mitomycin C (Mit-C) for 1 hour. To confirm that 12B1-D1 cells were dying by apoptosis after Mit-C treatment, we performed Annexin V-FITC and Propidum Iodide (PI) staining using the Annexin-V-FLUOS staining kit (Roche, Indianapolis, IN) followed by flow cytometric analysis of cells. DNA fragmentation analysis was performed using the apoptotic DNA ladder kit from Roche.

In vivo tumor growth experiments. In the prophylactic model, mice were immunized with 2×10^6 Mit-C treated cells, with or without 20 μg/mouse per vaccination of MCC or HSP70, by subcutaneous injection into the left groin on days -14 and -7. On day 0, mice were challenged with 2×10^4 (LD_{100}) viable 12B1-D1 cells. Tumor size was measured every other day with calipers once the tumors became palpable. Tumor volume was calculated using the formula: length $\times$ width^2 $\times$ $\pi/6$. Differences in mean tumor volume

between groups were compared using an unpaired *t* test. Mice with tumor were euthanized at the end points listed. Tumor free mice were kept for rechallenge experiments. In rechallenge experiments, 2×10^4 12B1-D1, 10^3 12B1, or 10^6 A20 leukemia cells, (all LD_{100} doses as determined by dose titration experiments, data not shown), were injected into the right groin 56 to 80 days after the first challenge.

In the pre-established tumor model, mice were injected with 2×10^4 (LD_{100}) viable 12B1-D1 cells in right groin on day 0. On day +1 or +3, mice were vaccinated as indicated by subcutaneous injection into the left groin. Tumor volume was measured at indicated time points.

In depletion experiments, on days –3, -1, +1, and +7, mice were intraperitoneally injected with 200 μg/mouse anti-CD4 (clone GK1.5) [108], and/or anti-CD8 (clone 2.43) [109] monoclonal antibodies. Flow cytometry of splenocytes confirmed depletion of T cell subsets.

ELISPOT Assay. ELISPOT assays were performed to assess the interferon-gamma (IFN-γ) production of splenocytes from vaccinated mice following in vitro stimulation with Mit-C treated apoptotic 12B1-D1 cells. Splenocytes/apoptotic cells (10:1 ratio) were co-cultured for 24 hours on Millipore MultiScreen-HA 96 well plates (MAHA S45, Millipore, Bedford, MA). The plates had been previously coated overnight with anti-

IFN-γ capture antibody (10 μg/ml, clone R4-6A2, rat mAb anti-mouse IFN-γ, BD PharMingen, San Diego, CA). Cells were then washed out with copious amounts of PBST (PBS + 0.05% Tween20). Biotinylated anti-IFN-γ antibody (2 μg/ml, clone XMG1.2, rat mAb anti-mouse IFN-γ, BD PharMingen) was added for two 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 1 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 captured with RS Image, Version 1.07 (Roper Scientific, Tucson, AZ).

T-cell proliferation assays and bioassay to determine IL-2 production. BALB/c mice were immunized with Mit-C treated 12B1-D1 cells that were mixed with liver lysate, liver-derived HSP70 or liver MCC (20μg/mouse) on days –14 and –7. For controls, mice were immunized with an equal number of Mit-C treated 12B1-D1 cells, or 20μg/mouse of liver-derived MCC or saline. On day -2, splenocytes from the immunized mice were harvested and co-cultured with Mit-C treated apoptotic 12B1-D1 cells. The ratio of splenocytes to apoptotic cells was 10 to 1. After 72 hours of culture, the

supernatant from each group was collected and serially diluted in a 96-well plate. IL-2 dependent CTLL-2 cells were added to each well. Human recombinant IL-2 was used to generate a standard curve. All assays were performed in triplicate wells. After 24 hours culture, [^{3}H] thymidine (1 μCi per well) was added. For T-cell proliferation assays, splenocytes were co-cultured with apoptotic 12B1-D1 cells for 4 days before the addition of [^{3}H] thymidine. The cells were harvested 18 hours later using a 96-well Packard cell harvester and the radioactivity measured on a Packard beta counter.

Cytotoxicity assay. BALB/c mice were immunized as indicated above. Five days after the second immunization, splenocytes from the immunized mice were harvested. The in vivo primed splenocytes were cultured for 5 days with Mit-C treated apoptotic 12B1-D1 cells. The ratio of splenocytes to apoptotic cells was 10 to 1. Stimulated effector cells were tested for cytolytic activity against 12B1-D1, parental 12B1, or A20 cells by non-radioactive cytotoxicity assay (Promega, Madison, WI) following the instructions provided. The percentage of cytotoxicity was determined according to the formula provided in the kit instructions.

4.4 RESULTS

MCC from normal tissue enhance the immunogenicity of apoptotic 12B1-D1 tumor cells. We have previously reported that 12B1-D1 leukemia cells undergo apoptosis in response to AP20187 treatment in vitro [62]. A 6 hour exposure to 40 nM AP20187

induces apoptosis in greater than 90% of 12B1-D1 cells, while 10% of cells remain clonogenic. When we inoculated mice with 5×10^5 AP20187-treated 12B1-D1 cells, rapid tumor growth occurred. This indicated that the apoptotic cells failed to induce an active immune response against the surviving clonogenic cells. To test whether syngeneic naïve mouse liver-derived HSP70 or MCC would provide adjuvant effects that may enhance the immunogenicity of AP20187-induced apoptotic 12B1-D1 tumor cells, we subcutaneously injected mice with AP20187-treated 12B1-D1 cells that were mixed with either liver-derived HSP70 or MCC (20 μg/mouse, based on previous dose titration experiments, data not shown). We found that co-injection of HSP70 significantly delayed tumor growth compared with mice that were injected with AP20187 treated cells alone (Figure IV-1A). Moreover, MCC provided superior adjuvant effects compared to HSP70, resulting in significant delay of tumor growth with rejection of tumors in 75% of mice (Figure IV-1A). In additional experiments, we compared the adjuvant effects of LPS with those of liver-derived MCC and found that they were comparable (Figure IV-1B), with both significantly delaying tumor progression when compared to no MCC adjuvant.

We next investigated whether liver-derived MCC would have similar adjuvant effects in enhancing the immunogenicity of apoptotic 12B1-D1 cells induced by other agents, such as chemotherapy drugs. We used mitomycin-C (Mit-C) (100 μg/ml for 1 hour) to induce apoptosis in 12B1-D1 cells. This resulted in more than 80% cells to undergo apoptosis

within 6 hours as determined by annexin V-FITC/ Propidum Iodide (PI) staining (Figure IV-2A). DNA from Mit-C treated 12B1-D1 cells displayed electrophoretic ladder patterns typical of apoptotic cells (Figure IV-2B). We then tested whether liver-derived MCC can enhance the immunogenicity of Mit-C induced apoptotic 12B1-D1 cells. To avoid secondary necrosis, we injected 12B1-D1 cells into mice immediately follow Mit-C treatment of the cells while 12B1-D1 cells maintained their membrane integrity as determined by trypan blue exclusion and flow cytometry (data not shown). Compared to 6 hours of AP20187 treatment, 1 hour of 100 μg/ml Mit-C treatment generated no clonogenic cells. Mice were immunized on day -14 and -7, and challenged with a lethal dose of live 12B1-D1 cells on day 0 by subcutaneous injection, as described in the materials and methods. Vaccination with Mit-C treated 12B1-D1 cells that were mixed with liver-derived MCC induced potent anti-tumor immunity. All mice immunized with MCC-adjuvant apoptotic cells rejected a lethal dose of 12B1-D1 tumor challenge (Figure IV-3A). However, immunization with Mit-C-induced apoptotic tumor cells alone did not delay tumor progression, compared to mock vaccinated mice (Figure IV-3A). Immunization with liver-derived MCC without a source of antigen, such as apoptotic tumor cells, provided no protection (data not shown).

We have previously demonstrated that vaccination with MCC derived from 12B1 (the parental cell line of 12B1-D1) elicited specific anti-tumor immunity (unpublished data[3]). 12B1-derived MCC (presumably contain tumor antigenic peptides) have similar adjuvant

effects to liver MCC. Vaccination with either 12B1 tumor MCC or liver MCC as adjuvant plus apoptotic 12B1-D1 tumor cells induced potent anti-tumor immunity that protected 100% of mice from a lethal dose of 12B1-D1 challenge (Figure IV-3B).

We further investigated whether vaccination with apoptotic 12B1-D1 cells plus MCC as adjuvant could elicit therapeutic effects on pre-established tumors. Mice received a lethal dose of 12B1-D1 on day 0, and were then immunized with Mit-C treated apoptotic 12B1-D1 cells plus MCC as adjuvant in the opposite groin on day +1 or day +3. Vaccination on day +1 with MCC adjuvant apoptotic tumor cells significantly delayed tumor growth as compared with mock vaccination (Figure IV-4). As expected, when vaccination was delayed until day +3, tumor progression was suppressed but to a lesser degree (Figure IV-4).

Immunity generated by MCC adjuvant-apoptotic tumor cells is T-cell dependent. We next investigated whether the anti-tumor response induced by apoptotic tumor cells with MCC adjuvant was T-cell dependent. SCID mice were subcutaneously injected with 5×10^5 AP20187 treated 12B1-D1 cells, with or without liver-derived MCC adjuvant. Mice developed 12B1-D1 tumors at comparable rates in both groups (Figure IV-5A), suggesting that MCC have no adjuvant effects in T-cell deficient mice. Moreover, tumor growth in mice co-injected with apoptotic cells plus LPS as adjuvant was not significantly delayed (Figure IV-5A).

To further evaluate the role of T cell subsets in the immunity induced by MCC adjuvant plus apoptotic tumor cells, we performed in vivo T-cell depletion experiments. $CD4^+$, $CD8^+$, or both subsets of T cells were depleted by intraperitoneal injection with 200 μg of anti-CD4, and/or anti-CD8 monoclonal antibodies into mice on days -3, -1, +1, and +7, as described in materials and methods. Mice immunized with apoptotic tumor cells plus MCC completely rejected a lethal dose of 12B1-D1 tumor challenge (Figure IV-5B). However, when either $CD4^+$ or $CD8^+$ T cells were depleted (as confirmed by flow cytometry of splenocytes; data not shown), anti-tumor immunity was partially abrogated, since 12B1-D1 tumor developed, but at significantly slower rates than that of mock vaccinated mice (Figure IV-5B). The anti-tumor immunity was completely abolished when both subsets of T cells were depleted by antibodies (Figure IV-5B). These results indicate that the potent anti-tumor immunity induced by MCC-adjuvant apoptotic tumor cell immunization is T-cell dependent, and that both $CD4^+$ and $CD8^+$ T cells contribute to this immunity.

Vaccination with MCC adjuvant-apoptotic tumor cells induces tumor-specific CTL. Cell-mediated immunity, which is particularly important in suppressing tumors, is characterized by production of type I cytokines, activation of macrophages, and induction of cytotoxic T lymphocytes (CTLs). To explore whether vaccination with apoptotic tumor cells plus MCC as adjuvant can induce type I cytokine secretion by T cells and

generate tumor-specific CTLs, we examined interferon γ (IFN-γ) and interleukin 2 (IL-2) production as well as the cytolytic activities of splenocytes derived from vaccinated mice. We found that vaccination with apoptotic tumor cells plus MCC adjuvant substantially increased the secretion of IL-2 (Figure IV-6A) and IFN-γ (Figure IV-6B) by splenocytes upon re-stimulation in vitro as measured by IL-2 bioassay and ELISPOT assay respectively. Moreover, spleen T-cell proliferation in MCC-adjuvant apoptotic tumor cell vaccinated mice also increased dramatically upon re-stimulation with apoptotic tumor cells (Figure IV-6C), compared to mock immunized mice. The induction of cytokine secretion or T-cell proliferation was not due to non-specific stimulation since splenocytes from mice immunized with Mit-C induced apoptotic cells alone or liver MCC alone produced limited amounts of cytokines and those immunizations resulted in almost no T cell proliferation (Figure IV-6). We also found that vaccination with apoptotic cells plus liver-derived HSP70 as adjuvant induced IFN-γ and IL-2 secretion by splenocytes, and increased T-cell proliferation, but at lower levels when compared to MCC as adjuvant (Figure IV-6). This may explain the weaker in vivo protective effects following immunization with HSP70 adjuvant plus apoptotic 12B1-D1 cells induced by AP20187 (Figure IV-1A) or Mit-C (data not shown). Since both HSP70 and MCC were derived from whole liver lysate, we explored whether the liver lysate can also confer adjuvant effects. We found that apoptotic cells co-injected with liver lysate induced minimal cytokine production and T cell proliferation (Figure IV-6A, B, and C).

CTLs are important in controlling tumor growth [107]. Several reports have shown that APC can acquire antigens from apoptotic bodies and can cross prime CTLs in vitro [26, 32]. However, evidence that those apoptotic cells prime CTLs in vivo remains limited. We therefore investigated whether or not vaccination with apoptotic tumor cells plus MCC as adjuvant induces measurable CTL activity. Splenocytes were collected from immunized mice and restimulated in vitro with Mit-C treated 12B1-D1 cells for 5 days, and then tested for cytolytic function against different tumor targets. Cytotoxic activity was determined by LDH release from target cells. Vaccination with apoptotic cells plus MCC elicited CTL activity (Figure IV-7A). In contrast, vaccination with Mit-C induced apoptotic tumor cells alone or with liver lysate failed to generate CTL activity, whereas liver-derived HSP70 had less impressive adjuvant effects in eliciting CTL activity (Figure IV-7A).

The 12B1-D1 cell line is derived from 12B1 by transfection of a plasmid encoding a death construct [62]. 12B1 cells therefore may contain all other tumor antigens of 12B1-D1 except the plasmid products. One of the advantages of using apoptotic cells as a vaccine is that they should contain the whole repertoire of tumor antigens. Theoretically, the CTLs induced by MCC adjuvant plus apoptotic 12B1-D1 cells should also lyse 12B1 cells. We demonstrated that the CTL activity induced by MCC adjuvant plus apoptotic 12B1-D1 cells was specific and potent enough to lyse the parental 12B1 targets (Figure IV-7B). Furthermore, we used another leukemia cell line, A20, as a target for CTLs to

confirm the specificity of the CTL activity. Figure IV-7C shows that no lysis above background was detected when A20 cells were used as targets, confirming that the CTL activity induced by MCC adjuvant apoptotic cells was tumor specific. In summary, our data provide direct evidence that apoptotic leukemia cells, when combined with syngeneic tissue-derived MCC as adjuvant, induced potent and specific CTLs in vivo.

MCC adjuvant-apoptotic tumor cells induce long term, specific immunity. Finally, we investigated whether the anti-tumor immunity induced by vaccination with MCC adjuvant plus apoptotic tumor cells was long lasting. Mice surviving vaccination with either liver-derived or 12B1 tumor-derived MCC adjuvant plus apoptotic 12B1-D1 cells were rechallenged with a lethal dose of 12B1-D1, 12B1, or A20 tumor cells 56 or 80 days after the initial challenge. All these mice rejected the rechallenge of 12B1-D1 tumor cells (Figure IV-8A). In contrast, age-matched naïve mice developed 12B1-D1 tumor rapidly (Figure IV-8A). The long term immunity also held true when mice were rechallenged with the parental 12B1 tumor (Figure IV-8B), but not when challenged with A20 B-cell leukemia (Figure IV-8C), confirming that the anti-tumor immunity induced by MCC adjuvant plus 12B1-D1 apoptotic cells was long lasting, potent, and tumor-specific.

4.5 DISCUSSION

The deficiency of self/non-self paradigms led to new hypotheses proposed by Janeway [20, 21] and Matzinger [22, 23] to explain the immune response: The immune system is thought to react to "danger" associated with certain molecules of infectious organisms, or cell products released during tissue damage or stress [51]. In addition to antigens that can be acquired, processed and presented by APC, these danger signals are needed to activate local APC, potentiating the immune responses against the antigens. In our studies, we found that 12B1-D1 leukemia cells, induced to undergo apoptosis by either AP20187 or Mit-C, were poorly immunogenic. In vivo immunization using these apoptotic cells induced no detectable anti-tumor immunity. In vitro, apoptotic 12B1-D1 cells had poor immunostimulatory activities on DC in terms of inducing IL-12 production and enhancing imunostimulatory functions of DC (unpublished data). Our results are consistent with other studies [24, 36]. Therefore, our data contribute the notion that apoptotic cells are, by default, non-immunogenic to the immune system, since normal cell turnover must not induce active immune responses. Although apoptotic tumor cells can be efficiently taken up and their antigens presented by APC [26, 110], an active immune response is seldom generated in vivo because of lack of danger signals. However, when apoptotic cells are under stress, such as heat-stress apoptosis [62] or pathogen-induced apoptosis [30-32], they are able to induce potent immune responses. How can the immune system recognize "stressful" apoptotic cells? We found that heat-stressed apoptotic 12B1-D1 cells expressed HSPs on their surface [62] and these stressed apoptotic cells activated DC

(unpublished data). Since heat shock proteins are emerging to be key danger signals to the immune system [51], we hypothesized that, by providing exogenous heat shock proteins, the default (immune silent) pathway of apoptotic cells could be bypassed.

In order to test our hypothesis, we purified HSP70 and enriched MCC from a syngeneic naïve mouse liver. In vivo immunization with liver-derived MCC provided no protection against subsequent autologous 12B1-D1 tumor challenge (data not shown). In addition, repeated injection of these syngeneic tissue components into BALB/c mice resulted in no apparent autoimmune phenomena (data not shown). However, when we co-injected mice with HSP70 and apoptotic tumor cells, anti-tumor immunity and specific CTLs were generated. We therefore demonstrated that HSP70 derived from naïve mouse liver had adjuvant effects enhancing the immunogenicity of apoptotic tumor cells. Danger signals provided by HSP70, together with antigens from apoptotic tumor cells, induced specific anti-tumor immunity.

Tumor-derived HSP70 has been used as a cancer vaccine against a variety of tumors [47, 48]. The antigen-chaperoning properties of HSP were highlighted in the tumor vaccine setting by the work of Srivastava [44]. How does this soluble tumor-derived HSP70, with its chaperoned peptides, elicit strong cell-mediated anti-tumor immunity in the absence of adjuvants? Blachere *et al.* showed for different peptide antigens (viral and nonviral CTL epitopes) that vaccination with gp96 or HSP70 induced a peptide-specific

CTL response, whereas vaccination with peptides alone did not [111]. Ciupitu *et al.* reported that vaccination with HSP70/LCMV peptide complexes elicited LCMV-specific CTL and protective immunity against LCMV [112]. Our results demonstrate that autologous naïve mouse liver-derived HSP70, devoid of tumor antigens, can serve as an adjuvant to enhance the immunogenicity of apoptotic tumor cells.

We have demonstrated that vaccination with tumor-derived MCC induced specific anti-tumor immunity against autologous tumor challenge in a variety of mouse models [46] (unpublished data[2]). In all models, we found that MCC were generally more effective than purified individual HSPs in generating anti-tumor immunity. We also demonstrated that tumor-derived MCC had a stronger ability to stimulate DC than purified HSP70 or gp96 (unpublished data[3]). Here, we have documented that naïve mouse liver-derived MCC have superior adjuvant effects when compare to HSP70 derived from the same tissue. This may partially explain why tumor-derived MCC are generally superior to individual HSPs in terms of inducing anti-tumor immunity [46] (unpublished data[2]). A secondary "danger signal" is apparently important to activate APC, which consequently activate specific T cells. Why liver-derived MCC provide better adjuvant effects than does purified liver HSP70 is an area of active research in our laboratory. One possible explanation is that MCC contain multiple HSPs or chaperone proteins that synergistically activate APC. Several studies have documented that APC bind and internalize gp96 through receptor-mediated endocytosis, which leads to MHC-I-restricted re-presentation

of gp96-chaperoned peptides and CTL activation [113, 114]. The CD91 molecule (α2-macroglobulin receptor), which was initially identified as a protein related to the low-density lipoprotein receptor, has been recently shown to be a cell surface receptor for gp96 [115]. Hsp60 was reported [88] to be a putative ligand of the toll-like receptor-4 (TLR-4) complex and triggers TLR-2 and TLR-4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells [116]. Exogenous HSP60 has also been shown to stimulate macrophages to express IL-12 and IL-15 and rapidly release TNF-α [87]. Hsp70 can assume dual roles as a chaperone and a cytokine [86] to activate monocytes and upregulate the expression of pro-inflammatory cytokines. MCC are enriched for multiple stress proteins, including HSP60, 70, and gp96 [45, 46], which may bind to a distinct set of receptors on APC and trigger different signaling pathways, resulting in synergistic effects on APC. In fact, we have demonstrated that tumor-derived MCC have superior immunostimulatory activities on DC (compared to HSP70), increasing DC co-stimulatory molecule expression and production of IL-12 (unpublished data[3]).

Cell-mediated immunity, which is particularly important in suppressing tumors, is characterized by production of type I cytokines, activation of macrophages, and induction of CTLs. Previous reports have shown that apoptotic cells are associated with induction of type II immune suppressive cytokines, such as TGF-β and IL-10 [25, 104]. In our studies, we found that vaccination with MCC adjuvant plus apoptotic tumor cells induced

IL-2 and IFN-γ production. This indicates that MCC steer the immune system toward a Th1 type response, which is critical in suppressing tumors [99].

Cytotoxic T lymphocytes (CTLs) are particularly important in tumor immunity [107]. Several reports have shown that professional APC can acquire antigens from apoptotic bodies and cross prime CTLs in vitro [26, 32]. However, evidence that those apoptotic cells prime CTLs in vivo remains limited. We found that vaccination with MCC adjuvant plus apoptotic tumor cells induced potent and specific CTLs, which appear to play an important role against 12B1-D1 tumor. The CTLs induced by vaccination with MCC adjuvant plus apoptotic 12B1-D1 cells also lysed parental 12B1 cells. In fact, when we depleted $CD8^+$ cells by using specific antibodies, we found that the anti-tumor immunity was partially abolished, suggesting that $CD8^+$ T cells played an important role in tumor killing in vivo. This anti-tumor immunity was not abolished completely by depletion of $CD8^+$ T cells alone, indicating that $CD4^+$ T cells through a direct or indirect action contributed to the immune response. Accordingly, we found that depletion of $CD4^+$ T cells significantly impaired the anti-tumor immunity, indicating that CTLs require $CD4^+$ help.

It is surprising that coinjection of liver lysate with apoptotic tumor cells did not induce anti-tumor immunity in vivo (data not shown). The pathologic necrotic cell death has been proposed to be dangerous to immune system, since it releases cellular components,

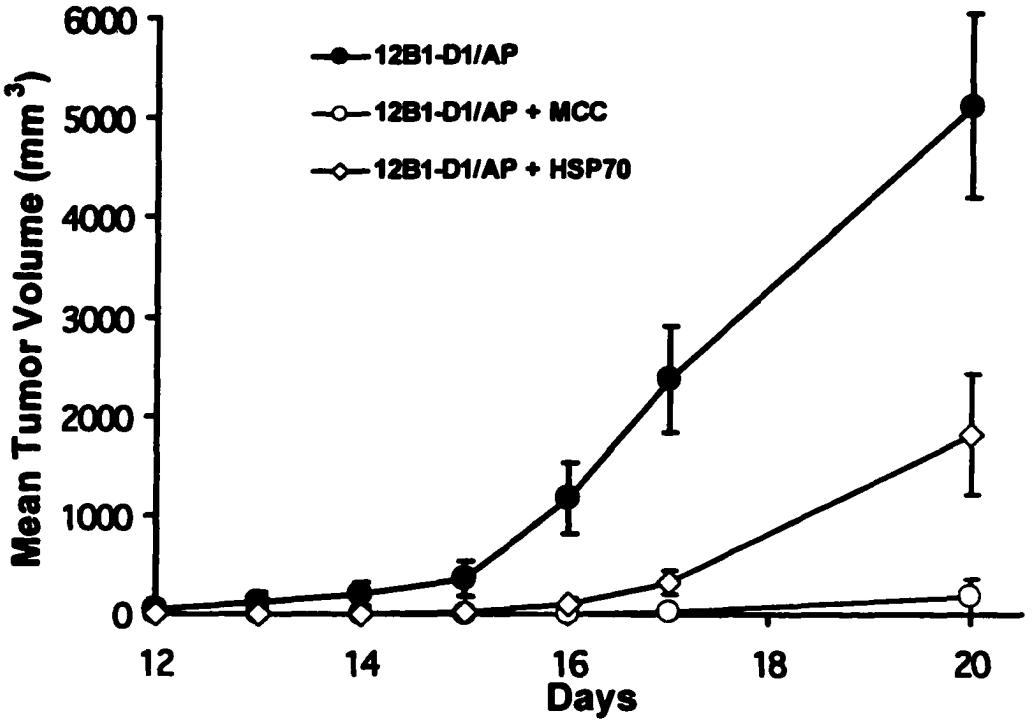
such as HSPs, mitochondria, double-strained DNA, etc., which may act as danger signals [24, 117]. It is possible that the lysate, generated simply by several cycles of freeze-thaw, can not represent the true necrotic cell death occurring in pathological conditions. In addition, the local concentrations of these "danger signals" released from dying cells may be important [51, 105]. It has been shown that tumor immunogenicity is associated with increased expression of HSP70 when tumor cells are undergoing necrotic death [77]. Tumors that were genetically modified to express HSP or when exogeneous HSP70 was provided during tumor cell killing decreased the immunosuppressive cytokine IL-10 expression [118]. Furthermore, lysate from primary cells contains less HSPs than their transformed counterparts and fail to mature DC [105]. MCC, which contain at least a 20 fold of enrichment of major HSPs [45, 46] appear to provide a higher concentration of local danger signals.

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 [119]. These apoptotic tumor cells are attractive tumor antigen sources. However, without proper danger signals, they are largely ignored by the immune system, or may even induce tolerance [25, 26, 43]. We have demonstrated that MCC, enriched from normal or tumor tissues, provide potent adjuvant effects for enhancing the immunogenicity of apoptotic tumor cells that can induce potent, long lasting anti-tumor immunity. Using FS-IEF, a relatively simple and rapid method, one can enrich large

quantities of chaperone proteins from tissues in a less laborious and time consuming manner compared to conventional purification of individual HSP [45, 46]. These findings, together with the superior adjuvant effects, confer significant advantages of MCC in terms of clinical applications. Our studies may provide new insights for the combination of immunotherapy with conventional therapies for the fight against cancer.

4.6 ACKNOWLEDGEMENTS

The authors wish to thank Dr. Douglas Lake for his helpful comments.



A

Figure IV-1. The adjuvant effects of liver-derived MCC, hsp70, or LPS for enhancing the immunogenicity of apoptotic 12B1-D1 cells induced by AP20187. 12B1-D1 cells were treated with 40nM AP20187 for 6 hours. **(A)** 20 μg/mouse liver-derived MCC or HSP70 was added to the cells and the mixture was injected to the groin of BALB/c mice subcutaneously. Control mice were injected with AP20187 treated cells only. (Control *versus* HSP70 $p<0.05$ from day 16 onward; Control *versus* MCC $p<0.05$ from day 15 onward in onward; HSP70 *versus* MCC $p<0.05$ from day 17 onward; n=8 mice per group; data represent two individual experiments.).

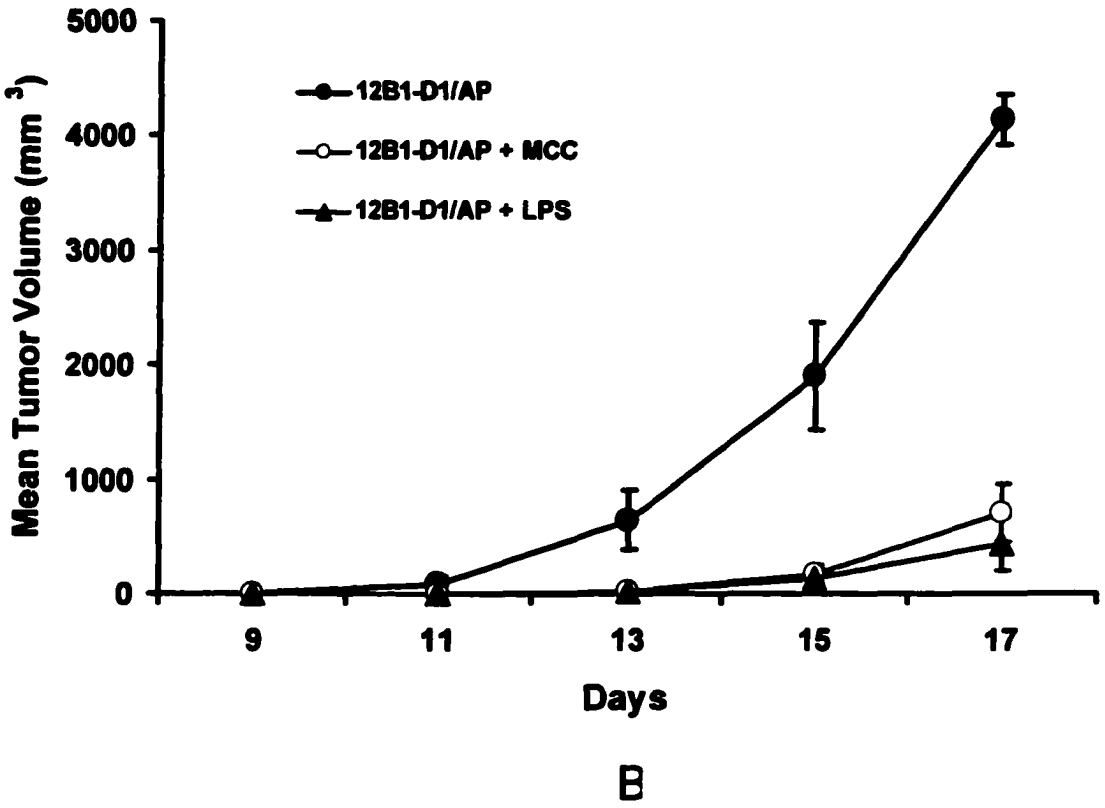


Figure IV-1. The adjuvant effects of liver-derived MCC, hsp70, or LPS for enhancing the immunogenicity of apoptotic 12B1-D1 cells induced by AP20187. 12B1-D1 cells were treated with 40nM AP20187 for 6 hours **(B)** 20 μg/mouse liver-derived MCC or 10 μg/mouse LPS was added to the cells and the mixture was injected to the groin of BALB/c mice subcutaneously. Control mice were injected with AP20187 treated cells only. (Control *versus* MCC $p<0.05$ from day 13 onward; Control *versus* LPS $p<0.05$ from day 13 onward; n=8 mice per group; data represent three individual experiments.).

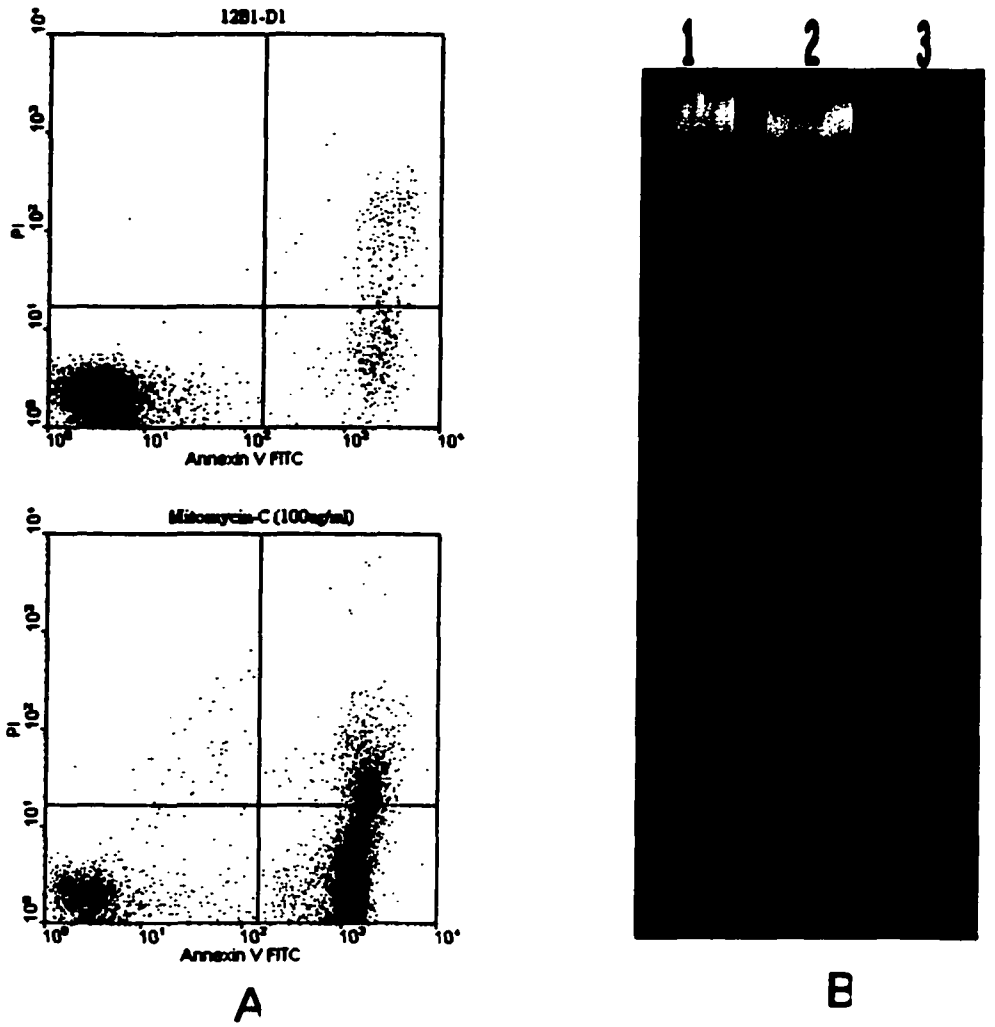


Figure IV-2. Apoptosis induction of 12B1-D1 cells by Mitomycin C treatment. **(A)** 12B1-D1 cells were treated with 100µg/ml mit-C for 1 hour, washed and then cultured in complete media for additional 6 hours. Induction of apoptosis was assessed by Annexin V and PI staining. **(B)** DNA fragmentation analysis. DNA extracted from either 12B1-D1 cells (lane 1), or from 12B1-D1 cells that had been treated with mit-C for 1 hour and re-cultured in complete media for additional 6 hours (lane 2) or 24 hours (lane 3).

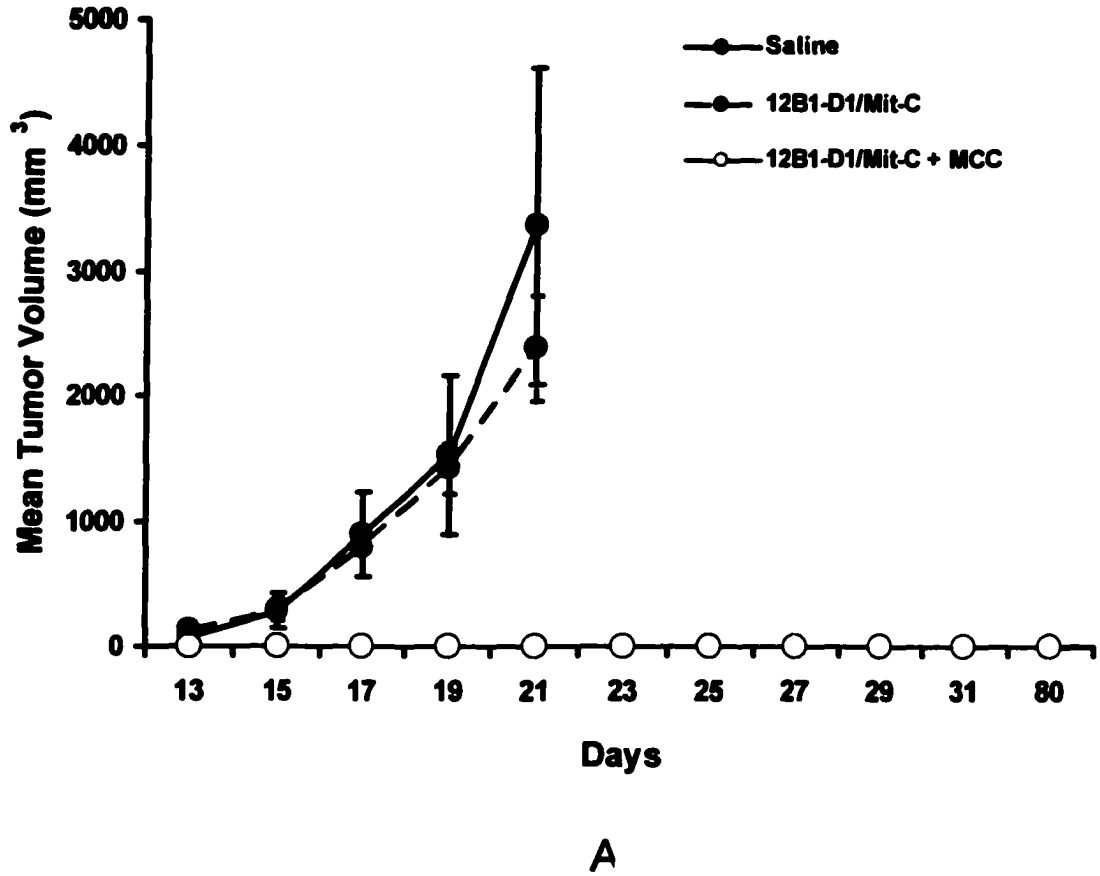


Figure IV-3. Both liver and 12B1 tumor-derived MCC enhance the immunogenicity of mit-C-induced apoptotic tumor cells. 12B1-D1 cells (2×10^6 per mouse) were treated with 100μg/ml mit-C for 1 hour and then extensively washed. (A) 20μg/mouse liver-derived MCC was added to the cells and the mixture was subcutaneously injected to the groin of BALB/c mice on days –14 and –7. Control mice were immunized with equal number of mit-C treated 12B1-D1 cells alone or saline. On day 0, mice were challenged with 2×10^4 (LD100) 12B1-D1 cells subcutaneously. Mice that vaccinated with saline or mit-C treated 12B1-D1 alone were sacrificed on day 21, whereas mice that were vaccinated with apoptotic cells plus MCC as adjuvant survived with tumor free up to day 80. (Saline *versus* 12B1-D1/mit-C p = ns; Saline, or 12B1-D1/mit-C *versus* MCC $p<0.05$ from day 15 onward; n=8 mice per group; data represent three individual experiments.).

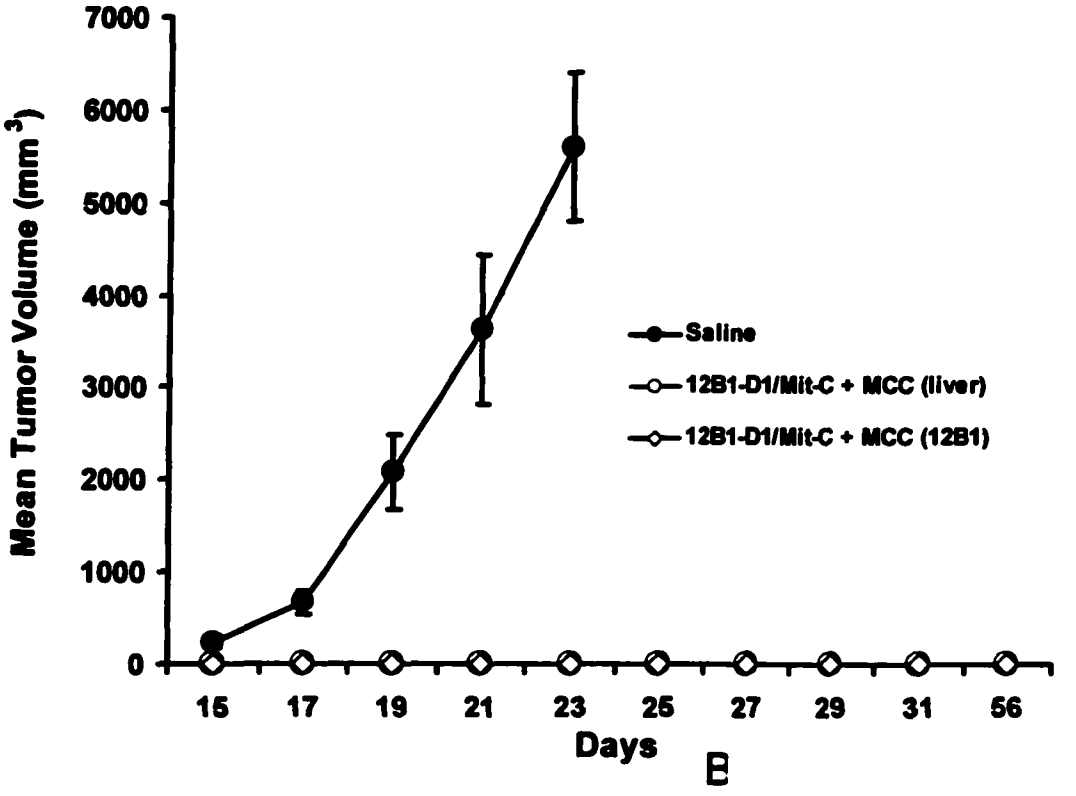


Figure IV-3. Both liver and 12B1 tumor-derived MCC enhance the immunogenicity of mit-C-induced apoptotic tumor cells. 12B1-D1 cells (2×10^6 per mouse) were treated with 100μg/ml mit-C for 1 hour and then extensively washed **(B)** 20μg/mouse liver or 12B1 tumor-derived MCC was added to the cells and the mixture was subcutaneously injected to the groin of BALB/c mice on days –14 and –7. Control mice were immunized with saline. On day 0, mice were challenged with 2×10^4 (LD100) 12B1-D1 cells subcutaneously. Mice that vaccinated with saline or mit-C treated 12B1-D1 alone were sacrificed on day 23, whereas mice that were vaccinated with apoptotic cells plus MCC as adjuvant survived with tumor free up to day 56. (Saline *versus* MCC $p<0.05$ from day 15 onward; n=8 mice per group; data represent two individual experiments.).

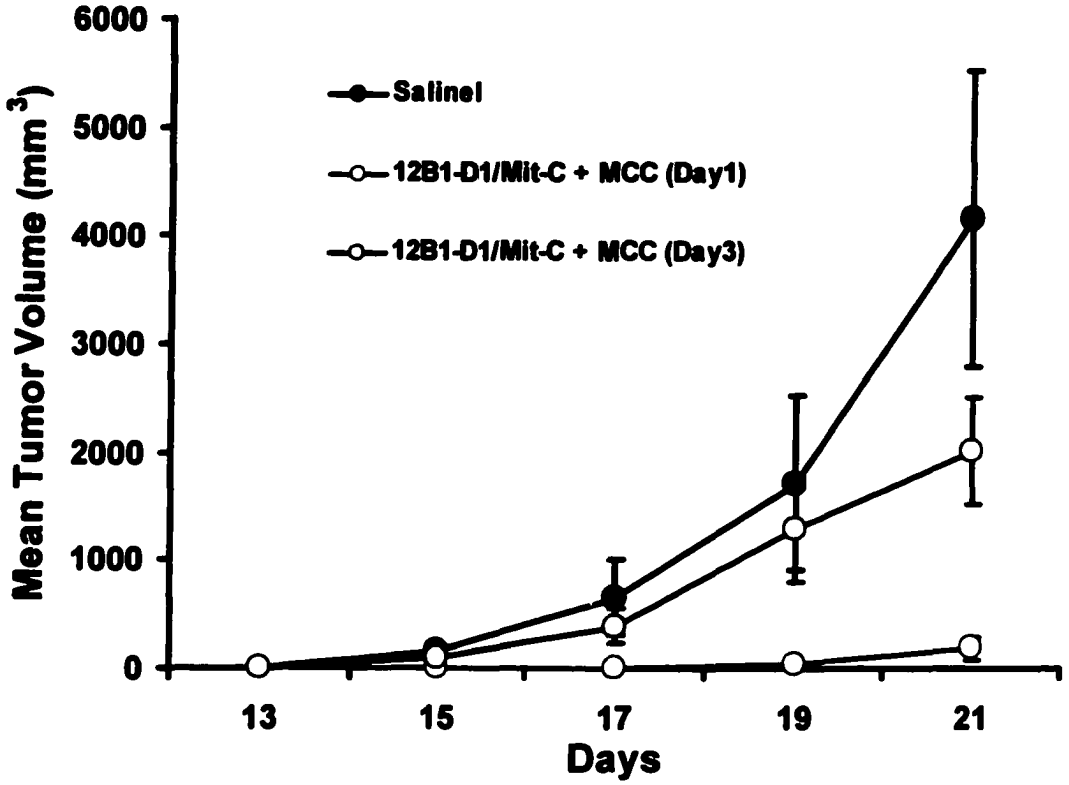


Figure IV-4. MCC-adjuvanted apoptotic tumor cells provide therapeutic effects in pre-established tumor model. BALB/c mice were subcutaneously injected with 2×10^4 (LD100) 12B1-D1 cells at right groin on day 0. On day 1 or 3, mice were vaccinated with mit-C treated 12B1-D1 cells (2×10^6 per mouse) plus 20μg/mouse liver-derived MCC adjuvant by s.c. injection at the opposite groin. (Saline *versus* day3 p = ns; Saline *versus* day1 $p<0.05$ from day 17 onward; n=4 mice per group; data represent three individual experiments.).

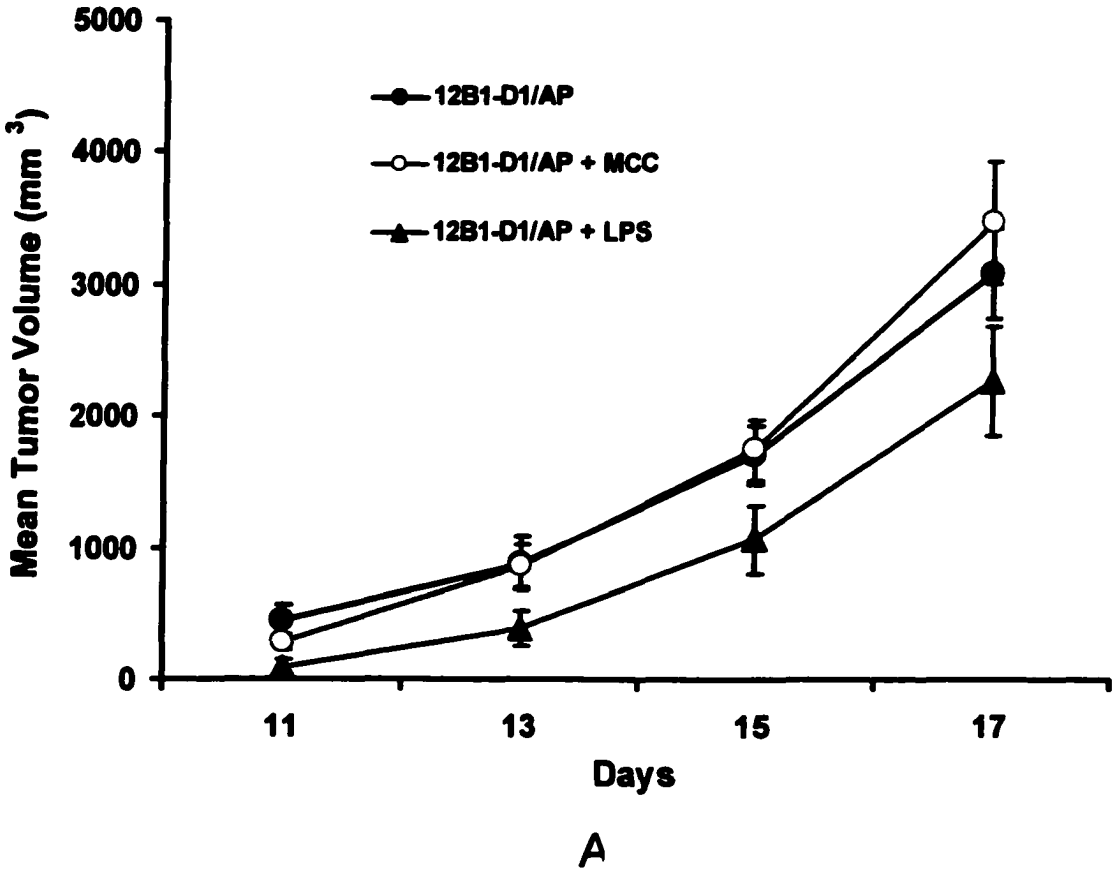


Figure IV-5. MCC adjuvant effects are T cell dependent. (A) MCC adjuvant effects are abrogated in SCID mice. 12B1-D1 cells were treated with 40nM AP20187 for 6 hours and then washed. 20μg/mouse liver-derived MCC or 10μg/mouse LPS was added to the cells and the mixture was subcutaneously injected to the right groin of SCID mice. Control mice were immunized with equal number of AP20187 treated 12B1-D1 cells alone. (p = ns; n=8 mice per group; data represent two individual experiments.).

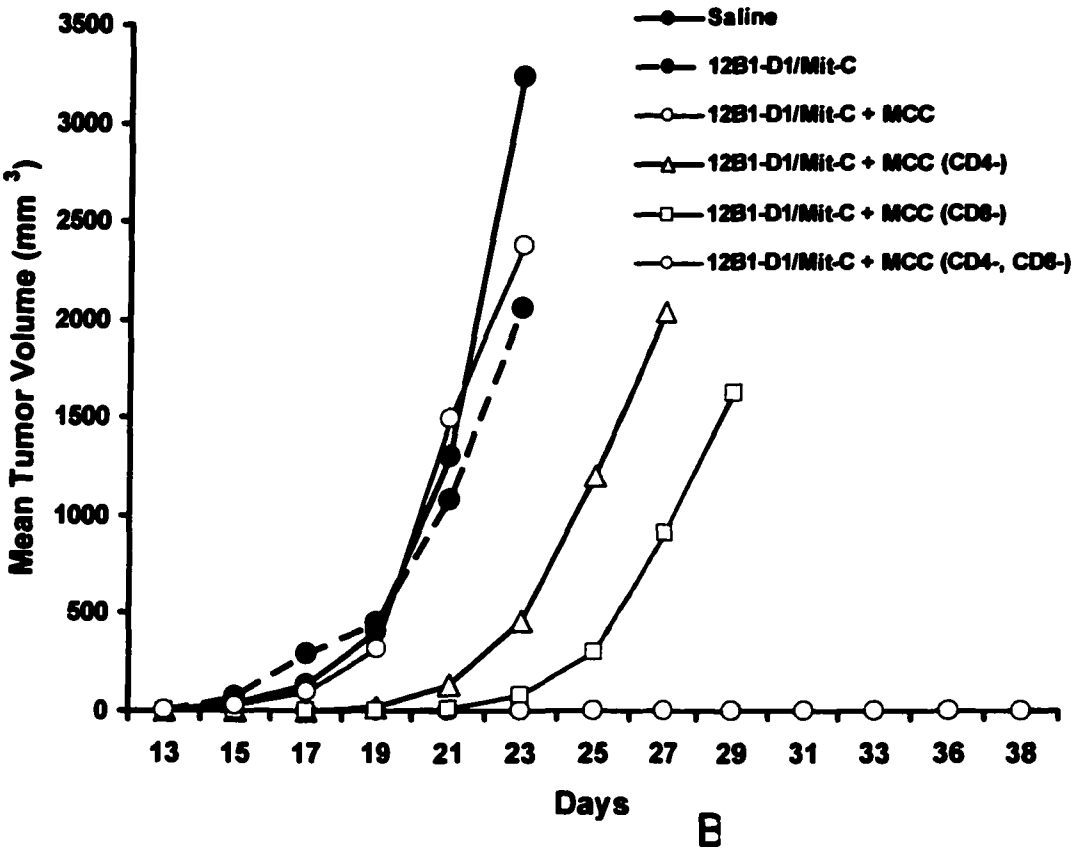


Figure IV-5. MCC adjuvant effects are T cell dependent. (B) The anti-tumor immunity of MCC-adjuvanted apoptotic tumor cells is both CD4+ and CD8+ T cell dependent. On days –14 and –7, BALB/c mice were subcutaneously injected with 2×10^6 mit-C treated 12B1-D1 cells that were mixed with 20μg/mouse liver-derived MCC at the right groin. On days –3, -1, +1, and +7, mice were intrapretoneally injected with 200 μg/mouse anti-CD4, and/or anti-CD8 mAb, or same volume of saline. Control mice were immunized with equal number of mit-C treated 12B1-D1 cells or saline. On day 0, mice were injected with 2×10^4 12B1-D1 cells subcutaneously. (Saline *versus* 12B1-D1/mit-C, or double depletion p = not significant; Saline *versus* MCC $p<0.05$ from day 15 onward; Saline *versus* CD4 or CD8 depleteion $p<0.05$ from day 17 onward; CD4 depletion *versus* CD8 depletion p = ns except day23 $p=0.04$; n=8 to 24 mice per group; data represent two individual experiments.).

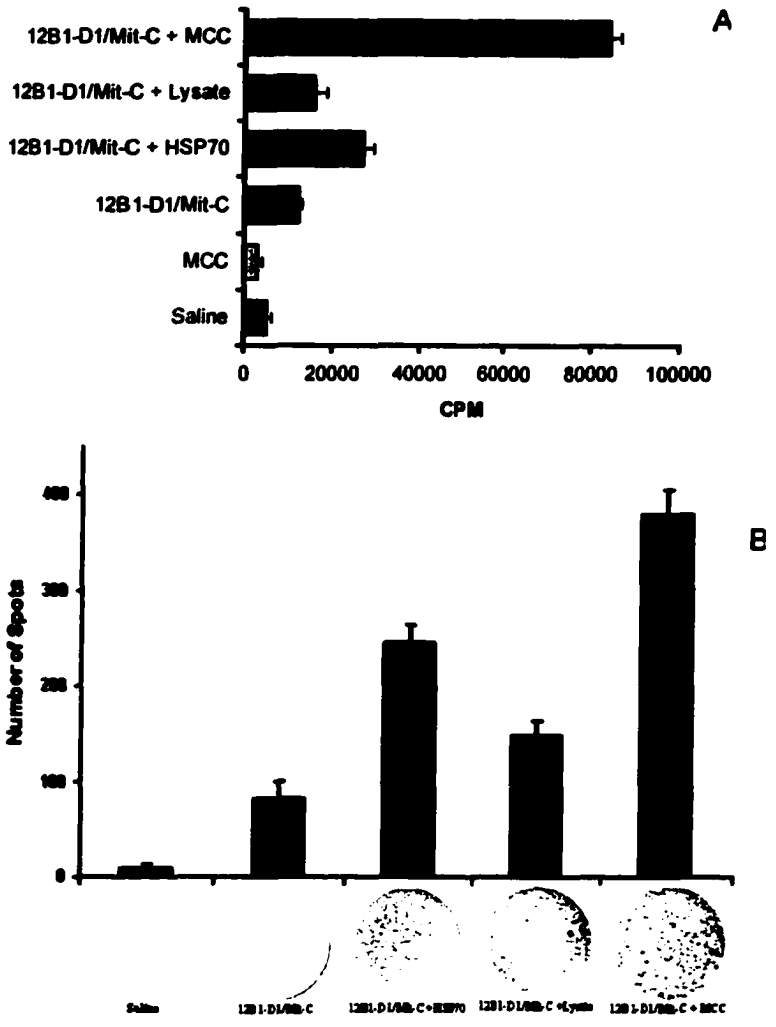
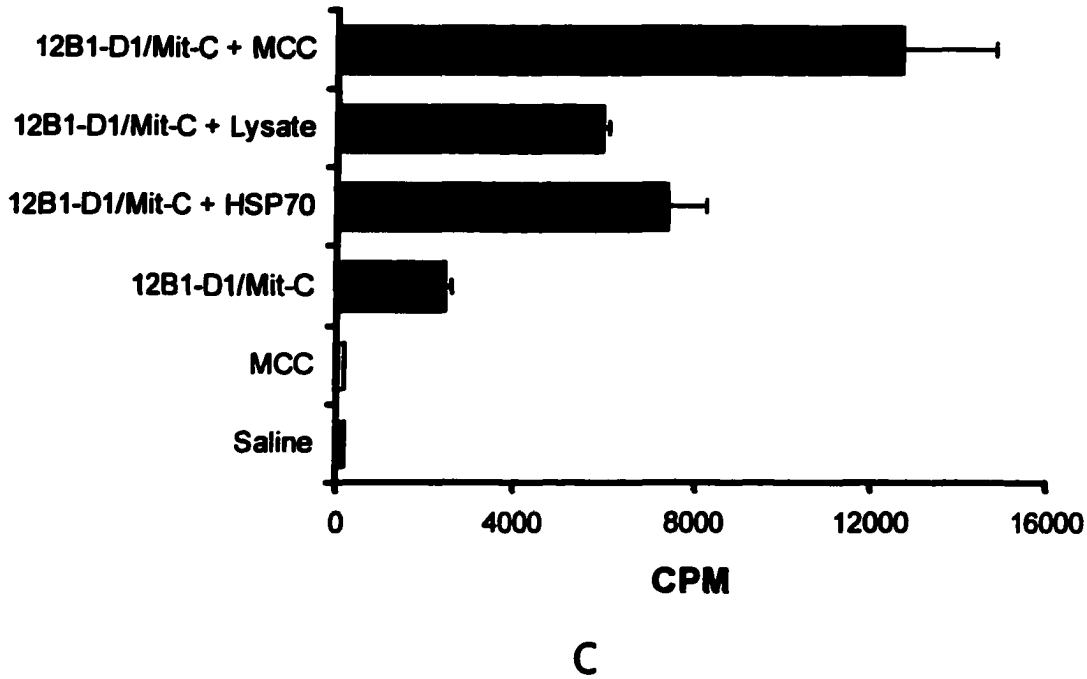


Figure IV-6. Immunization of apoptotic 12B1-D1 cells plus MCC adjuvant induce IL-2, IFN-γ secretion and T-cell proliferation of splenocytes. 12B1-D1 cells were treated with 100μg/ml mit-C for 1 hour and then extensively washed. 20μg/mouse liver lysate, liver-derived HSP70 or MCC was added to the cells and the mixture was injected to BALB/c mice subcutaneously on days –14 and –7. Control mice were immunized with equal number of mit-C treated 12B1-D1 cells or saline. On day -2, splenocytes of the immunized mice were harvested restimulated with mit-C treated 12B1-D1 cells. **(A)** CTLL-2 bioassay was used to determine the IL-2 production. **(B)** IFN-γ secretion was determined by ELISPOT.



C

Figure IV-6. Immunization of apoptotic 12B1-D1 cells plus MCC adjuvant induce IL-2, IFN-γ secretion and T-cell proliferation of splenocytes. 12B1-D1 cells were treated with 100μg/ml mit-C for 1 hour and then extensively washed. 20μg/mouse liver lysate, liver-derived HSP70 or MCC was added to the cells and the mixture was injected to BALB/c mice subcutaneously on days –14 and –7. Control mice were immunized with equal number of mit-C treated 12B1-D1 cells or saline. On day -2, splenocytes of the immunized mice were harvested restimulated with mit-C treated 12B1-D1 cells. **(C)** T cell proliferation was determined by [^{3}H] thymidine incorporation. (Experiments have been repeated three times).

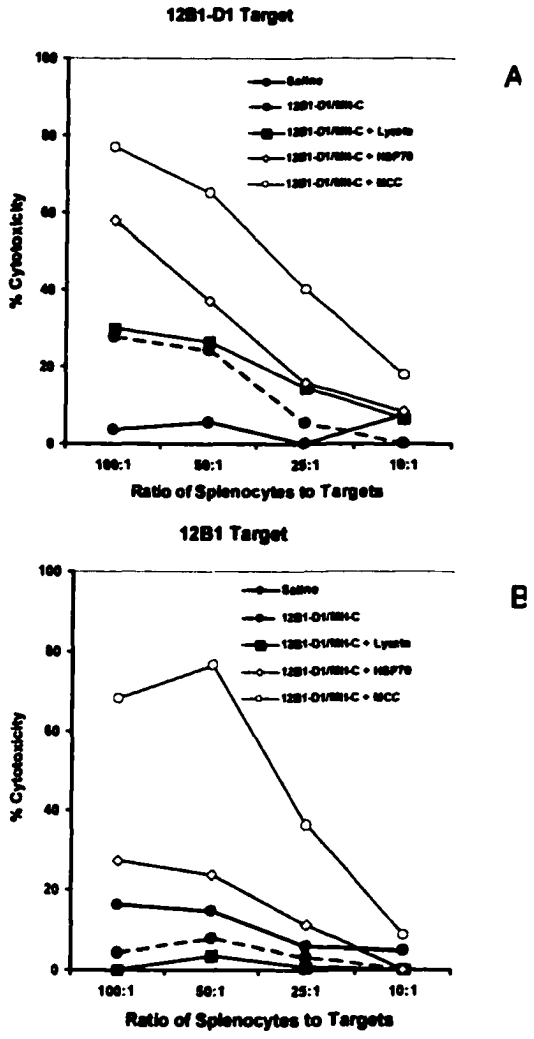


Figure IV-7. Immunization of stressed apoptotic 12B1-D1 cells induce specific CTL. BALB/c were immunized with mit-C treated 12B1-D1 cells that were mixed with 20μg/mouse liver lysate, liver-derived HSP70 or MCC on days –14 and –7. For controls, mice were immunized with equal number of mit-C treated 12B1-D1 cells or saline. On day -2, splenocytes of the immunized mice were harvested and co-cultured with mit-C treated 12B1-D1 cells for 5 days. Stimulated effector cells were tested for cytolytic activity against 12B1-D1 cells **(A)**, parental 12B1 cells **(B)**

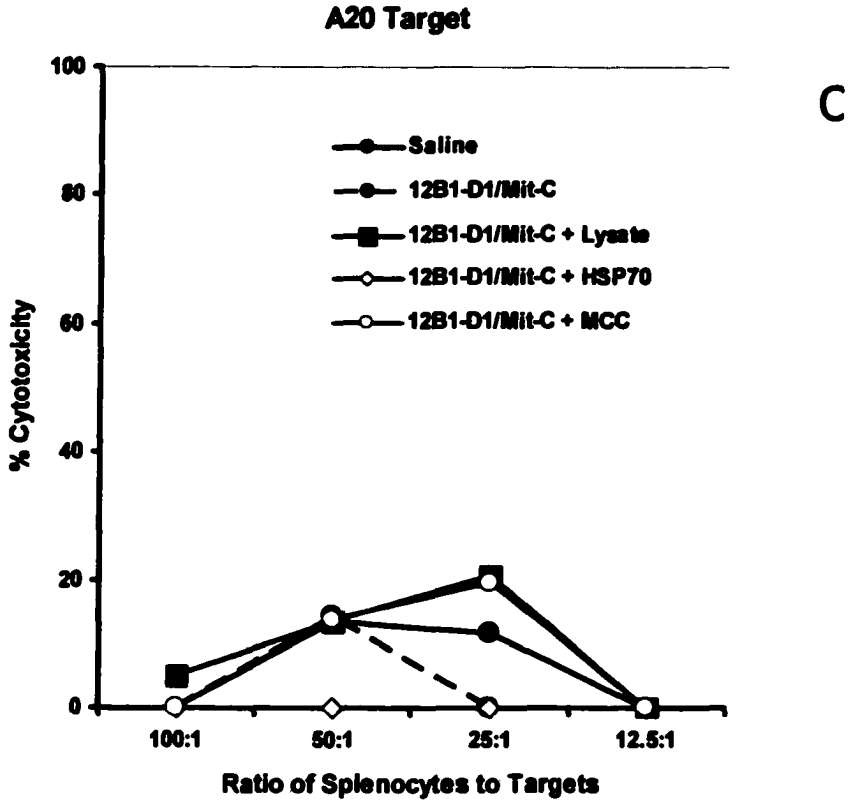


Figure IV-7. Immunization of stressed apoptotic 12B1-D1 cells induce specific CTL. BALB/c were immunized with mit-C treated 12B1-D1 cells that were mixed with 20μg/mouse liver lysate, liver-derived HSP70 or MCC on days –14 and –7. For controls, mice were immunized with equal number of mit-C treated 12B1-D1 cells or saline. On day -2, splenocytes of the immunized mice were harvested and co-cultured with mit-C treated 12B1-D1 cells for 5 days. Stimulated effector cells were tested for cytolytic activity against A20 cells (C) by non-radioactive cytotoxicity assay. The figure depicts representative data from one of three similar experiments.

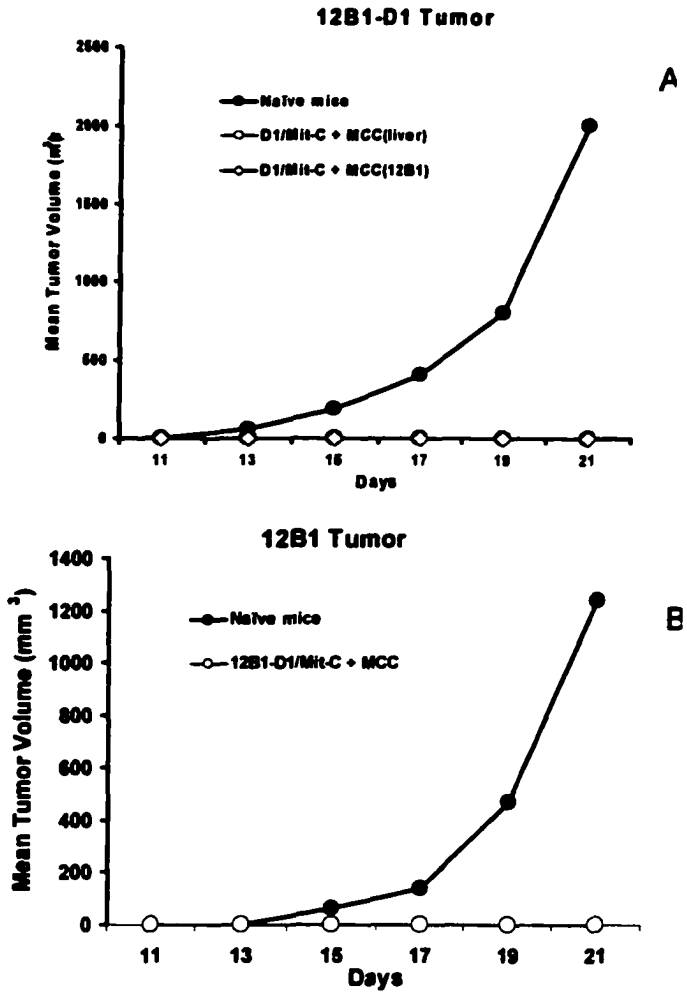


Figure IV-8. Vaccination with stressed apoptotic 12B1-D1 cells induces long term, specific anti-tumor immunity. Naïve and surviving mice were rechallenged 56 or 80 days later with 2×10^4 (LD100) 12B1-D1 cells **(A)**, or 10^3 (LD100) parental 12B1 cells **(B)**

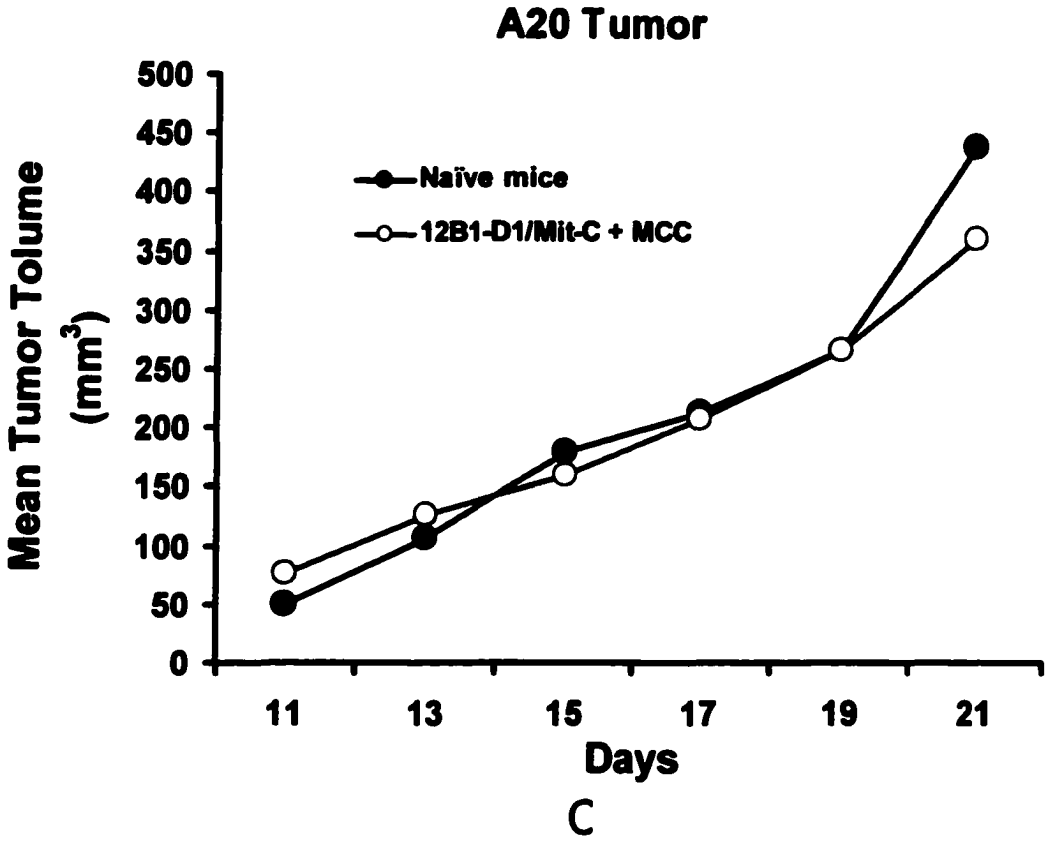


Figure IV-8. Vaccination with stressed apoptotic 12B1-D1 cells induces long term, specific anti-tumor immunity. Naïve and surviving mice were rechallenged 56 or 80 days later with 10^6 (LD100) A 20 cells **(C)** in the groin. (In 12B1 or 12B1-D1 rechallenged mice, $p<0.05$ from day 15 onward; in A20 rechallenged mice, p = ns; n = 8 to 16 surviving and 8 naïve mouse, pooled data from two experiments.).

DISCUSSION

The first part of this study indicates that FasDD-oligomerization can trigger a novel caspase-8-independent apoptotic pathway. This pathway is activated by FasDD, but is independent of Bid and the proteolytic activity of caspase-8. It appears to target mitochondria directly by a Z-VAD-FMK-inhibitable mechanism, suggesting the existence of a novel protease(s) that we are now attempting to identify.

In the second part of this study, we have demonstrated that apoptotic tumor cells can be either immunogenic or non-immunogenic. Vaccination with heat-stressed apoptotic 12B1-D1 leukemia cells induced a potent and tumor specific T-cell-mediated immune response that retarded co-injected viable tumor cell growth, while non-stressed apoptotic cells were non-immunogenic. The immunogenicity of non-stressed apoptotic tumor cells can be significantly enhanced by supplying exogenous HSPs in the vaccination site, resulting in the generation of potent anti-tumor immunity and specific CTLs. Futhermore, we examined the mechanisms behind the increased immunogenicity of stressed apoptotic tumor cells. We demonstrated that stressed apoptotic cells were more potent in stimulating DC which expressed higher levels of co-stimulatory molecules on their surface and produced more IL-12. Our data indicate that stressed apoptotic tumor cells are capable of providing the necessary danger signals to DC, likely through their increased surface expression of HSPs.

Apoptosis, or programmed cells death, is important in normal development and in maintaining physiological homeostasis. *In vivo*, apoptotic cells can be rapidly phagocytosed by antigen presenting cells (APCs) [27, 104, 110]. Exogenous antigens acquired from apoptotic cells can gain access to the cytoplasm and be cross-presented on MHC class I molecules [26]. The fact that professional APCs are involved in phagocytosis, processing, and presentation of apoptotic cell derived antigens suggests that there may be immunological consequences of apoptosis. *In vitro* studies have demonstrated measurable cellular responses induced by apoptotic cells. However, a potent immune response was seldom generated *in vivo* [39-42]. In our studies, we found that leukemia 12B1-D1 cells, induced to undergo apoptosis by either AP20187 or mit-C, were poorly immunogenic. *In vivo* immunization using these apoptotic cells induced no detectable anti-tumor immunity. *In vitro*, non-stressed apoptotic 12B1-D1 cells had poor immunostimulatory activities on DC in terms of induction of IL-12 production and enhancing imunostimulatory functions of DC in MLRs. Our results are consistent with other studies [24, 36]. Therefore, it seems that apoptotic cells are, by default, non-immunogenic to the immune system, which makes sense since normal cell turnover must not induce active immune responses. When immature DCs phagocytose apoptotic bodies, they are not stimulated to mature, and consequently may present their processed antigens in the absence of adequate co-stimulation thereby inducing tolerance [39, 42, 43]. This appears to be an important protective physiologic process since normal cell

turnover must not induce autoimmune responses. Similarly, cancer cells undergoing apoptosis typically do not elicit immune responses against their own antigens.

Although apoptotic tumor cells can be efficiently taken up by APCs [110], and their antigens can be presented on MHC molecules [26], an active immune response is seldom generated *in vivo* because of lack of danger signals. Heat shock protein induction following stress can provide the danger signals required for generation of a more effective immune responses [51]. We reasoned that by inducing the expression of endogenous HSPs, or supplying exogenous HSPs to apoptotic tumor cells, hence supplying danger signals, which may be critical to enhance the immunogenicity of apoptotic tumor cells, and consequently, for the generation of tumor specific immunity.

In order to inducing HSPs upregulation by apoptotic tumor cells, we heat stress (42°C, 1 hour) 12B1-D1 cells before the induction of apoptosis by AP20187 treatment. We found that heat stress resulted in upregulation of membrane HSP72 and HSP60 as determined by flow cytometry and confocal microscopy. The exogenous HSPs are supplied by the enriched MCC from a syngeneic naïve mouse liver, which are devoid of tumor-specific antigenic peptides. We found that vaccination of mice with either stressed or MCC adjuvant-apoptotic tumor cells induced a potent cell-mediated anti-tumor immunity that suppresses tumor growth, whereas vaccination with non-stressed apoptotic cell induced no immune protection.

When apoptotic cells are under stress, such as heat-stress apoptosis or pathogen-induced apoptosis [30-32], they are able to induce potent immune responses. According to the new hypothesis proposed by Janeway [20] and Matzinger [22] in immunology, besides antigens that can be acquired, processed and presented by APCs, secondary danger signals are needed to activate local APCs, especially DC, leading to an active immune response against the antigens. Are DC differentially activated in response to stressed or non-stressed apoptotic tumor cells? We demonstrated that DC exposed to stressed apoptotic cells upregulate their surface expression of co-stimulatory molecules and have increased secretion of proinflammatory cytokines (IL-12), both which are key in determining subsequent immune responses. IL-12 has been shown to have a myriad of functions, including modulating Th1 vs Th2 switching, which is critical in antitumoral immune response [100]. DC activated with stressed apoptotic tumor cells had improved immunostimulatory function in MLR. We further examined whether DCs incubated *ex-vivo* with stressed and non-stressed apoptotic cells could induce rejection of co-injected viable leukemia cells and generate long lasting specific immunity. Injection of syngeneic DCs that had been pulsed with stressed apoptotic leukemia cells resulted largely in rejection of co-injected viable leukemia cells. Mice rejecting the primary 12B1-D1 inoculum were immune to the same but not to a different leukemia challenge, confirming the long term and specific anti-tumor immunity.

It is essential for DC to mature before they can activate naive T cells. Stressed apoptotic tumor cells mature DC and their vaccinations consequently induce a T cell-dependent anti-tumor immunity. Cell-mediated immunity plays an essential role in combating tumors [100] and is characterized by the production of type I cytokines and generation of cytotoxic T cells. We found that the splenocytes from mice immunized with stressed, or MCC adjuvant-apoptotic 12B1-D1 cells secreted significantly higher amount of IL-2 and IFN-γ than that from mice immunized with non-stressed apoptotic cells. Moreover we demonstrated that stressed or MCC adjuvant-apoptotic 12B1-D1 cells, but not non-stressed ones, induced potent tumor specific CTLs. The results may explain our finding that vaccination with stressed, but not non-stressed apoptotic cells significantly retarded the progression of co-injected tumor cells. CTLs are particularly important in tumor immunity [106, 107]. Several reports have shown that APCs can acquire antigens from apoptotic bodies and cross prime CTLs *in vitro* [26, 32, 33]. But evidence that apoptotic tumor cells can prime CTLs *in vivo* remains limited. In the current study we have demonstrated that both stressed or MCC adjuvant-apoptotic tumor cells are capable of inducing potent CTLs *in vivo*.

Lysis or necrosis of cells leads to release of intracellular danger stimuli [24]. To exclude the possibility that the delayed tumor growth we observed was the result of necrotic 12B1-D1 cells from which cytoplasmic components were released, heat stressed or non-stressed viable or apoptotic cells were lysed. Co-injection of lysates with viable tumor

cells did not retard tumor growth when compared with mice injected with an equal number of viable cells only. This was consistent with our previous studies using a B cell leukemia/lymphoma model [45, 46]. In addition, we found that the 12B1-D1 tumor lysates failed to upregulate co-stimulatory molecules on the surface of DC. Furthermore, coinjection of liver lysate with apoptotic tumor cells did not induce anti-tumor immunity *in vivo*. It is possible that the lysate, generated simply by several cycles of freeze and thaw, can not represent the true necrotic cell death occurring in pathological conditions. In addition, the local concentrations of these "danger signals" released from dying cells may be important [51, 105]. MCC, which contain at least a 20 fold of enrichment of major HSPs (Graner et al. data not shown) should provide a much higher concentration of local danger signals.

In summary, our studies conclusively demonstrate that apoptotic tumor cells can be either immunogenic or non-immunogenic *in vivo*, dependent upon whether they are associated with endogenous or exogenous HSPs. DC can efficiently phagocytose both stressed and non-stressed apoptotic cells. However, in contract to non-stressed apoptotic ones, stressed apoptotic tumor cells induce secretion of IL-12 and upregulate expression of costimulatory molecules by DC, which further augmenting their immunostimulatory function. This leads to induction of type I cytokines, generation of tumor specific CTLs. Currently, chemotherapy and radiotherapy remain the main treatments to many cancers. Most of

these therapies are thought to induce tumor cells to undergo apoptosis [119]. These apoptotic tumor cells are attractive tumor antigen sources. However, without proper danger signals, they are largely ignored by the immune system, or may even induce tolerance [25, 26, 43]. We have demonstrated that MCC, enriched from normal or tumor tissues, provide potent adjuvant effects for enhancing the immunogenicity of apoptotic tumor cells and induce potent, long lasting anti-tumor immunity. Using FS-IEF, a relatively simple and rapid method, one can enrich large quantity of chaperone proteins from tissues in a less laborious and time consuming manner in comparison with conventional purification of individual HSP [45, 46]. These, together with the superior adjuvant effects, confer significant advantages of MCC in terms of clinical applications. Our studies may provide new insights for the combination of immunotherapy with conventional therapies for treatments of cancers.

APENDIX I

Dendritic Cell-Peptide Immunization Provides Immunoprotection Against Bcr-Abl Positive Leukemia In Mice

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A1.1 ABSTRACT

Chronic myelogenous leukemia (CML) is a clonal disorder characterized by proliferation of cells that possess the *bcr-abl* fusion gene resulting in the production of one of two possible chimeric 210kD tyrosine kinase proteins. Since these chimeric proteins are expressed only in leukemic cells they have the potential to serve as tumor specific antigens for cytotoxic T lymphocytes (CTLs). Using the 12B1 murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the *bcr-abl* (b_3a_2) fusion gene we have demonstrated that intravenous inoculation of 12B1 cells into BALB/c mice results in a disseminated acute leukemia analogous to human CML in blast crisis. Histologic sections of liver and spleen and PCR analysis of peripheral blood, bone marrow, liver, spleen and lymph nodes confirmed the presence of *bcr-abl*$^+$ leukemia cells in these murine tissues, while Western blot data demonstrated the expression of the fusion protein in 12B1 cells. Immunization of mice with dendritic cells (DC) loaded with the synthetic *bcr-abl* chimeric nonapeptide, GFKQSSKAL, increased the frequency of *bcr-abl* specific CTL precursors in the spleen 150-fold as compared to mice immunized with peptide alone. *In vitro* re-stimulation of DC-peptide primed splenocytes resulted in substantial secretion of IFN-γ and augmented cytolytic activity against 12B1 targets. Finally, vaccination with peptide loaded-DC significantly prolonged survival of BALB/c mice that were challenged with 12B1 leukemia. The capacity to generate *bcr-abl* specific CTLs *in vivo* using DC based immunization may have clinical implications in the treatment of CML.

Key words: bcr-abl, dendritic cell, peptide, vaccine

A1.2 INTRODUCTION

Chronic myelogenous leukemia is a clonal disorder characterized by proliferation of cells that possess the Philadelphia chromosome, a reciprocal translocation between chromosomes 9 and 22 leading to the fusion of the breakpoint cluster region (*bcr*) on chromosome 22 with the Abelson (*abl*) oncogene on chromosome 9 [120]. This *bcr-abl* fusion results in the production of one of two possible chimeric 210kD tyrosine kinase proteins (b2a2 or b3a2 breakpoints). Since these chimeric proteins are expressed only on leukemic cells they could serve as tumor specific antigens for cytotoxic T cells. In fact, *in vitro* studies using human cells have demonstrated that synthetic *bcr-abl* chimeric peptides can bind to certain HLA alleles on antigen presenting cells (APCs) and that the CTLs thereupon generated can lyse targets that have been pre-incubated with these peptides. However, reports are conflicting on whether CTLs can lyse leukemic cells displaying endogenous *bcr-abl* peptides on their MHC class I molecules[106, 121, 122].

Murine studies have documented that peptides spanning the *bcr-abl* joining region have the appropriate motifs to bind the MHC class I molecule, H-2K^d [121]. CTLs specific for *bcr-abl* joining region peptides were generated, but they failed to lyse murine leukemia

cells expressing the p210 *bcr-abl* protein. This finding suggested that chimeric peptides were not naturally processed and presented in a form recognized by CTLs. As a result, whether peptide-based tumor vaccines can induce *in vivo* immunoprotection against *bcr-abl* expressing leukemia remains a critical unanswered question. We therefore established a murine model of *bcr-abl*$^+$ leukemia to determine whether immunization of mice with a *bcr-abl* peptide can impact survival. This clinically relevant model has many of the hallmarks of CML such as the presence of *bcr-abl*$^+$ cells in the peripheral blood, bone marrow and leukemic infiltration of other tissues resulting in hepatosplenomegaly and lymphadenopathy. Using DC loaded with the synthetic *bcr-abl* chimeric nonapeptide, GFKQSSKAL (lysine results from the bcr-abl fusion), we provide evidence that an immune response can be elicited, that prolongs survival of mice bearing this aggressive leukemia. Moreover, using this approach we demonstrate that DC-peptide primed splenocytes can be stimulated *in vitro* to become cytolytic against leukemia targets endogenously expressing *bcr-abl* gene products.

A1.3 MATERIALS AND METHODS

Bcr-abl positive leukemia cell line: 12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the *bcr-abl* (b_3a_2) fusion gene and expresses the p210 *bcr-abl* protein [78]. This cell line was kindly provided by Dr. Wei Chen (Cleveland Clinic, Cleveland, OH). 12B1 cells were cultured at 37° C under 5% CO_2 in RPMI media containing 10% heat inactivated fetal calf serum and

supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 □g/ml streptomycin sulfate, 0.025 □g/ml amphotericin B, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (2-ME) all from Gibco/BRL (Gaithersburg, MD).

Mice: Six to ten week old female BALB/c ($H\text{-}2^d$) mice (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments. The animals were housed in a specific pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Immunoblotting: The presence of p210 *bcr-abl* protein in the 12B1 cell line was confirmed by Western blotting and compared to the *bcr-abl*$^+$ human leukemia line, K562, and the *bcr-abl*$^-$ murine B cell line A20. An equivalent amount of protein from each cell line was loaded onto the gel. Following SDS-PAGE, the gels were electroblotted to nitrocellulose using an Idea Scientific electroblotter (Minneapolis, MN). Gels were transferred in 25 mM Tris, 200 mM glycine, 20% methanol overnight at 60 V, stained with Ponceau Red and destained in TBST (50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.4). Blots were blocked in 3% non-fat dried milk in TBST for 20-60 min, followed by 3 x 5 min rinses in TBST. The protein of interest was identified using the monoclonal antibody to bcr (AB-2) clone #7C6 (Oncogene Research Products, Cambridge, MA). Primary antibody solutions were prepared in blocking solution, and

blots were incubated in primary antibody for 1 hr at room temperature or 12 hr at 4° C, followed by 3 x 5 min rinses in TBST. Peroxidase conjugated goat anti mouse secondary antibody was applied for 1 hr at room temperature or 12 hr at 4° C followed by chemiluminescent detection (Super Signal, Pierce, Rockford, IL).

Polymerase chain reaction: Presence of the *bcr-abl* fusion gene (b3a2) was confirmed through the PCR amplification and sequencing of the *bcr-abl* fusion region. DNA extracts from organs of BALB/c mice that had received injection of 12B1 cells were obtained using QIAamp DNA Mini Kit (QIAgen, Valencia, CA). PCR cocktail of Gibco reagents, forward primer 5'-CCTCTGACTATGAGCGTG-3' and reverse primer 5'-TCACACCATTCCCCATTG-3' (Sigma Genosys, The Woodlands, TX) were thermocycled in an Eppendorf mastercycler gradient (Eppendorf, Westbury, NY) under the following conditions: 1 minute at 94 degrees; 1 minute at 54 degrees; 2 minutes at 72 degrees for 35 cycles. The resultant 456 bp fragment was purified using a QIAquick PCR Purification Kit (QIAgen, Valencia, CA) and sequenced on an ABI 377 (Perkin Elmer Corp., Foster City, CA) instrument (Laboratory of Molecular Systematics and Evolution, in the Division of Biotechnology at the University of Arizona).

Immunohistochemistry: Mice with advance leukemia were euthanized. Immediately upon sacrifice, the livers and spleens were harvested. Frozen blocks were prepared by arranging tissues in an aluminum foil cup, using O.C.T. compound (Miles, Inc., Elkhart,

IN) as an embedding medium and snap-freezing in liquid nitrogen. Blocks were stored at -70°C.

Serial frozen sections were cut 4 □m thick on an IEC microtome cryostat, thaw-mounted onto glass slides and fixed for 5 minutes in acetone. One section from each block was stained with hematoxylin and eosin for morphological and pathological assessment. Other sections were blocked with 10% normal goat serum (Sigma) followed by incubation with biotinylated moAbs from PharMingen: CD4 (clone RM4-5), CD8 (clone 5H10-1), and Mac-1 (clone M1/70). Staining by an immunoperoxidase procedure was performed as described previously [123] using avidin-biotin blocking reagents, avidin-biotin-complex and DAB as chromogen (Kirkegaard and Perry, Gaithersburg, MD).

Flow cytometry: Cells were washed in phosphate buffered saline (PBS) containing 2% heat inactivated fetal bovine serum and 0.1% sodium azide (Sigma). 2 x 10^5 cells were placed in each well of 96-well U-bottomed microtiter plates. Surface expression of distinct molecules was determined by incubating the cells first with an Fc receptor blocking antibody for 5 min (PharMingen, San Diego CA), and then with saturating amounts of monoclonal antibodies for 30 minutes at 4° C. Except where otherwise indicated, antibodies used were from PharMingen, San Diego CA, and included; anti-H-2K^d (clone SF1-1.1, mouse IgG_{2a}), anti-H-2D^d (clone 34-2-12, mouse IgG_{2a}), anti-I-A^d (clone AMS-32.1, mouse IgG_{2b}), anti-Mac-1 (clone M1/70, rat IgG_{2b}), anti-CD11c (clone HL3, hamster IgG), F4/80 (clone C1:A3-1, rat IgG_{2b}; Serotec, Indianapolis, IN),

anti-Gr-1 (clone RB6-8C5, rat IgG_{2b}), anti-CD45R/B220 (clone RA3-6B2, rat IgG_{2a}), anti-CD54 (clone 3E2, hamster IgG), anti-CD80 (clone 16-10A1, hamster IgG), anti-CD86 (clone GL1, rat IgG_{2a}), anti-CD4 (clone GK1.5, rat IgG_{2b}), and anti-CD8 (clone 53-6.7, hamster IgG). The cells were then washed three times and fixed with PBS containing 1% formaldehyde (Polysciences, Warrington, PA). Ten thousand cells were analyzed using a Becton Dickinson FACScan.

Generation of bone marrow derived dendritic cells: Murine bone marrow DC were generated using a slightly modified protocol from that previously described [79]. Bone marrow was harvested from femurs and tibiae and filtered through a nytex screen. Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in AIM V medium (therapeutic grade, GIBCO BRL) which contains L-glutamine, human serum albumin, 50 μg/ml streptomycin sulfate, 10 □g/ml gentamicin sulfate. Murine GM-CSF (10 ng/ml, kindly provided by Immunex, Seattle. WA) and IL-4 (10 ng/ml, Peprotech, Rocky Hill, NJ) were added to the culture. After four days, the non- and loosely-adherent cells were harvested and layered onto a metrizamide gradient (14.5% metrizamide solution, Sigma Chemical Co, St. Louis, MO), and centrifuged. Enriched DC were washed and cultured with GM-CSF, IL-4 and murine CD40L trimer (1 μg/ml, Immunex) for an additional 24 hr. The DC were then washed and used for *in vitro* or *in vivo* experiments.

Murine immunizations: In all experiments BALB/c mice were immunized subcutaneously in the left and right groin, the base of the tail and both hind foot pads with peptide, empty DC, or peptide pulsed DC. The synthetic peptides used for loading DC were; a) GFKQSSKAL, a *bcr-abl* peptide and b) HYLSTQSALSK, a green fluorescent protein (GFP) peptide (both from Sigma- Genosys). These peptides were added to 10^6 DC at a concentration of 100 μg/ml with α2 microglobulin 5 μg/ml, incubated at 37° for 3 hr and washed in PBS prior to injection into mice. Mice were given a total of 0.5 – 1 x 10^6 DC twice at weekly intervals (i.e., day -14 and day -7).

IFN-γ and IL-2 production by splenocytes of immunized mice: Splenocytes from naïve or immunized mice were placed in microtiter plates and serially diluted and in some cases stimulated with 5 μg/ml of *bcr-abl* peptide. Culture supernatants were collected and assayed for IFN-γ using an ELISA kit (R & D Systems, Minneapolis, MN).

The IL-2 dependent murine T cell line, CTLL-2 (American Type Culture Collection) was used to determine the IL-2 bioactivity present in the supernatants [124]. Samples were added in triplicate to 96 well flat-bottom plates. CTLL-2 cells (5,000 cells per well), washed free of IL-2, were then added. The microtiter plates were incubated for 24 hr at 37° C and 5% CO_2, then pulsed for another 24 hr with 1 Ci ^{3}H-thymidine (ICN Pharmaceuticals). The cells were then harvested using a 96 well Packard cell harvester

and the radioactivity measured on a Packard beta counter. The IL-2 bioactivity was calculated by comparing to an IL-2 standard curve.

Cytotoxicity assay: ^{51}Cr release assays were performed as described previously [125]. In brief, 12B1 target cells (1-2 x 10^6 in 0.5 ml tissue culture media) were incubated with 500 μCi $Na^{51}CrO_4$ (5 mCi/ml, ICN Pharmaceuticals, Irvine CA) for 1 hour at 37° C. Targets were washed 3 times and resuspended at a concentration of 10^5 cells/ml. Fifty microliters (5,000 targets/well) were then added in triplicate to 96-well V-bottomed microtiter plates into which effectors had been previously added and serially diluted to yield effector to target ratios from 20:1 to 2:1. Spontaneous release wells contained only tissue culture media and ^{51}Cr labeled targets; maximal release wells contained 5% Triton X-100 (Sigma) and ^{51}Cr labeled targets. The microtiter plates were centrifuged at 200 x g for 2 min and incubated at 37° C and 5% CO_2 for 4 hours. The plates were then centrifuged at 200 x g for 10 minutes after which 75 μl aliquots of supernatant were harvested, added to 150 μl of scintillation fluid and the radioactivity measured on a Packard beta counter. Cytotoxicity was determined by the formula:

$$\% \text{ cytotoxicity} = 100 \times \frac{\text{experimental mean cpm - spontaneous release mean cpm}}{\text{maximal release mean cpm - spontaneous release mean cpm}}$$

One lytic unit (LU) was defined as the number of effectors required to lyse 20% of targets; cytotoxicity is presented as LU per 10^6 effector cells.

Estimation of CTL precursor (CTLp) frequencies: Splenocytes from naïve or immunized mice were placed in microtiter U-bottomed plates in a limiting dilution fashion (titrations from 200,000 to 823 cells per well) with 30 replicate wells for each dilution and stimulated with 5 μg/ml of *bcr-abl* peptide. On days 3 and 6 of culture, 20 U/ml of IL-2 (Chiron, Emeryville CA) were added to the wells. After 8 days in culture, wells were tested for cytotoxicity against ^{51}Cr-labeled 12B1 pulsed or not for 1 hr with *bcr-abl* peptide (5 μg/ml). Wells were considered positive when cytotoxicity (i.e. ^{51}Cr release) exceeded three standard deviations over the mean value of control wells without responder cells. The CTLp frequency estimations were calculated using the Poisson equation [126].

***In vivo* immunoprotection experiments:** Seven days following the last immunization (day 0), the mice were challenged via tail vein injection with 66 viable 12B1 leukemia cells using a 27 gauge needle. 12B1 cells used for challenge were harvested 2-3 hr prior to i.v. injection from mice bearing subcutaneous tumors. These solid tumors were removed under sterile conditions, placed in PBS, finely minced with a scalpel, gently crushed with the piston of a syringe, gathered in a pipette, filtered through a 100 μm

Nytex mesh, washed twice, resuspended in PBS and injected intravenously in a volume of 0.2 ml.

Statistical analysis: The Kaplan-Meier product-limit method was used to plot the survival of mice inoculated with the *bcr-abl*$^+$ leukemia and the log-rank statistic to test differences between groups [127, 128].

A1.4 RESULTS

Murine model of bcr-abl positive leukemia: 12B1 was derived by transducing BALB/c bone marrow cells with the *bcr-abl* (b_3a_2) fusion gene [78]. Western blot analysis was used to confirm the presence of *bcr-abl* protein in the 12B1 cell line (Figure AI-1). These cells express substantial quantities of the *bcr-abl* fusion protein when compared to another *bcr-abl*$^+$ human leukemia line, K562. We established the 12B1 leukemia model in BALB/c mice to study whether peptide loaded DC can induce immunoprotection, This is an aggressive leukemia with a cell dose as low as 66 cells i.v. being uniformly lethal resulting in a median time to death of 22 days. Mice receiving 12B1 cells by i.v. injection display leukemic infiltration of spleen, liver, lymph nodes, bone marrow and blood as detected by PCR analysis of tissues for the *bcr-abl* joining region (Figure AI-2). Histologic evaluation of tissues revealed marked leukemic cell infiltration of the spleens resulting in destruction of its normal architecture (Figure AI-3). There was evidence of increased extramedullary hematopoiesis with large numbers of

megakaryocytes present throughout the spleens. 12B1 leukemia cells were also evident albeit less prominent in the livers (data not shown). Immunohistochemistry demonstrated a diffuse dense macrophage infiltration (Mac-1$^+$) in spleens of leukemia bearing mice. CD4$^+$ (Figure AI-3) and CD8$^+$ (data not shown) were decreased in numbers and dispersed throughout the leukemic mouse spleens instead of concentrated in the white pulp.

Use of peptide loaded DC for immunization of BALB/c mice: The GFKQSSKAL chimeric peptide spanning the *bcr-abl* joining region has previously been shown to bind to the MHC class I allele, H-2K^d [121]. 12B1 cells have high expression of H-2K^d (data not shown) and consequently can serve as targets of CTLs if sufficient endogenous peptides are presented on their cell surfaces. The 12B1 system therefore provides an *in vivo* model to study whether DC pulsed with GFKQSSKAL can generate leukemia specific CTLs with high affinity T cell receptors able to recognize potentially low concentrations of presented *bcr-abl* peptides. As a control peptide we used a GFP peptide (HYLSTQSALSK). This 11-mer is processed by DC and the resulting 9-mer HYLSTQSAL is predicted to bind with high affinity to H-2K^d [129].

The DC used for our studies were generated from BALB/c mouse bone marrow in serum free medium (as described in materials and methods). These antigen presenting cells reproducibly expressed high levels of MHC class I (H-2K^d; 96%, H-2D^d; 90%; data not shown), MHC class II (I-A^d; 96%), CD54 (ICAM-1; 95%), CD80 (B7-1; 94%), CD86

(B7-2; 87%), CD11c (96%), CD11b (Mac-1; 96%) and had low expression of the macrophage marker, F4/80 (9%), and the granulocyte marker, Gr-1 (16%).

DC-peptide primed splenocytes secrete IFN-γ and have augmented cytolytic activity against 12B1 leukemia targets: BALB/c mice were immunized twice (days -14, -7), their spleens were harvested (day 0) and the splenocytes stimulated *in vitro* with GFKQSSKAL peptide as described in materials and methods. The production of IFN-γ was highest in DC-GFKQSSKAL primed splenocytes (Figure AI-4A). Suprisingly, *in vivo* exposure to unpulsed DC resulted in substantial IFN-γ production by cultured spleen cells. *In vivo* priming with GFKQSSKAL or saline failed to elicit significant IFN-γ secretion.

In additional experiments we examined fresh spleen cells of mice previously given saline or unpulsed DC in order to analyze further how immunization with "empty" DC may stimulate IFN-γ production. Mice received saline or 10^6 DC subcutaneously and their spleens were harvested 7 days later. The spleens of DC treated animals were clearly larger in size (data not shown) and contained more $CD25^+$, $CD4^+$, $CD8^+$ T cells, $CD11^+$, and Mac-1^+ cells (Table 1), while the numbers of $B220^+$ and Gr-1^+ cells were comparable to spleens of saline treated controls. Splenocytes from DC treated mice cultured without additional stimulation for 24 hours produced detectable amounts of IFN-γ and IL-2, while spleen cells from saline treated mice did not (Table 1). These observations were

reproducible in two additional mouse strains, namely C3H/HeJ and C57BL/6, and were uniformly present irrespective of the route of inoculation of unpulsed DC (s.c., i.v. or i.p.).

Further experiments were performed to evaluate the cytolytic function of *in vivo* primed splenocytes restimulated *in vitro* with GFKQSSKAL. Splenic effectors from GFKQSSKAL pulsed DC were more potent killers of 12B1 leukemia cells (Figure AI-4B). Similar to the cytokine production assays, effector cells generated from DC primed mice also had some cytolytic activity against the *bcr-abl*$^+$ target, albeit less than that seen in DC-GFKQSSKAL immunized mice. GFKQSSKAL immunization without DC was ineffective in priming cytotoxic effectors. Taken together these data indicate that immunization of mice with DC, cultured under the conditions described in the materials and methods, appears to direct T cells toward a Th1 phenotype which in turn produce cytokines such as interferon-γ and IL-2 inducing nonspecific cytolytic activity.

Immunization of mice with chimeric bcr-abl peptide loaded DC increases the frequency of specific CTLp: Limiting dilution assays were done next to determine whether DC based immunizations would increase the *bcr-abl* specific CTL precursor frequencies. Mice were immunized as described in materials and methods. Groups of mice received saline, irrelevant GFP peptide HYLSTQSALSK, bcr-abl peptide GFKQSSKAL, unpulsed DC or DC loaded with HYLSTQSALSK or GFKQSSKAL.

Immunization with unpulsed DC or DC loaded with irrelevant peptide nonspecifically increased cytolytic activity of splenocytes resulting in an increase in "CTLp frequencies" 31-fold and 29-fold respectively over saline controls (Figure AI-5). However, immunization with GFKQSSKAL pulsed DC resulted in a 367-fold increase in CTLp. GFKQSSKAL peptide immunization without DC did not augment CTLp over saline immunized controls. As with previous experiments immunization with unpulsed DC stimulated cytotoxicity by nonspecific lymphoid effectors presumably through stimulation of IFN-□ and IL-2 production. These studies clearly demonstrate that DC-GFKQSSKAL significantly increased CTLp over empty DC or DC loaded with irrelevant peptide. Interestingly, susceptibility to lysis was not substantially increased in 12B1 cells incubated with exogenous *bcr-abl* peptide compared to 12B1 only, suggesting that the generated effector cells may recognize endogenously processed *bcr-abl* fusion peptides (data not shown).

Efficacy of bcr-abl peptide pulsed DC in generating protective immunity: To determine whether or not DC pulsed with the *bcr-abl* fusion peptide, GFKQSSKAL, are effective in stimulating protective immunity, DC-peptide immunized mice were challenged i.v. with 12B1 cells one week after their second immunization. Since DC-HYLSTQSALSK was not superior to unpulsed DC in generating cytotoxicity *in vitro*, this irrelevant peptide was not included in the *in vivo* survival experiments. Figure AI-6 depicts survival data comparing the effect of GFKQSSKAL-loaded DC vaccination to

that of DC or GFKQSSKAL alone. *Bcr-abl* GFKQSSKAL-loaded DC immunization significantly prolonged survival compared to mice immunized with GFKQSSKAL or unpulsed DC.

To determine whether the addition of exogenous peptide can increase the susceptibility of 12B1 to lysis by the *in vivo* generated CTL, we challenged groups of naïve and immunized mice with 12B1 cells that were previously incubated with the *bcr-abl* peptide, GFKQSSKAL. Animals challenged with "peptide coated" 12B1 cells did not survive longer than those receiving 12B1 cells alone (data not shown). This would indicate that exogenous peptide did not increase the susceptibility of 12B1 cells to killing *in vivo* by effector cells generated following DC-GFKQSSKAL immunization.

Additional experiments were performed to assess whether immunization of mice bearing 12B1 leukemia would result in elimination of preexisting disease. Mice were given 12B1 cells on day 0 and immunized with saline, DC-HYLSTQSALSK, DC-GFKQSSKAL on days +2 and +4. This regimen failed to significantly prolong survival of leukemic mice (data not shown).

A1.5 DISCUSSION

12B1 is a murine leukemia cell line derived by retroviral transduction of BALB/c bone marrow cells with the *bcr-abl* (b_3a_2) fusion gene and consequently expresses the p210 *bcr-abl* protein [78]. The susceptibility of this cell line to lysis by CTLs was first described by Chen et al. [121]; however, an *in vivo* model of 12B1 has not previously been described. The experiments presented herein demonstrate that intravenous inoculation of 12B1 *bcr-abl*$^+$ cells into BALB/c mice results in a disseminated acute leukemia analogous to human chronic myelogenous leukemia in blast crisis. Histologic sections of liver and spleen and PCR analysis of peripheral blood, bone marrow, liver, spleen and lymph nodes confirmed the presence of *bcr-abl*$^+$ cells in these murine tissues, while Western blot data demonstrated the expression of the fusion protein in 12B1 cells.

Previous reports have documented that *bcr-abl* specific CTL clones can be generated in BALB/c mice, but these effectors could only lyse 12B1 cells that had been incubated with synthetic peptides (i.e. "coating" their surfaces) [121]. In those experiments, priming mice with a mixture of twenty-one *bcr-abl* peptides emulsified with complete Freund's adjuvant failed to generate effective killers. It was speculated that such priming yielded low affinity CTLs with an inability to lyse leukemic cells displaying low levels of endogenously processed peptide on their surface. In our studies, we used DC loaded with a single *bcr-abl* chimeric nonapeptide GFKQSSKAL, which has the highest binding affinity for H-2K^d (Dr. Kenneth Parker, http://www-

bimas.dcrt.nih.gov/molbio/hla_bind/index.html). We found that GFKQSSKAL loaded DC can generate cytolytic effectors capable of lysing 12B1 cells suggesting that when the most effective of APCs are pulsed with a suitable peptide they can elicit high affinity effector cells which may recognize endogenous *bcr-abl* peptides on leukemic cells. Moreover, we have provided new *in vivo* evidence that the cellular response generated by DC-peptide immunization was potent enough to prolong survival of mice challenged with 12B1 leukemia. We were not able however, to demonstrate efficacy of therapeutic vaccination using DC-GFKQSSKAL. This is likely due to the fact that in an established leukemia setting the time required to generate an immune response is overwhelmed by the rapidly progressing leukemia.

Immunization with unpulsed DC has been previously described to provide some protection against tumor challenge [130]. This is secondary to tissue culture calf serum components, which may be processed and presented to T cells *in vivo* resulting in generation of CTLs against serum proteins. If immunized mice are challenged with *in vitro* propagated tumor cells, anti-calf serum CTLs may cross react with tumor cells carrying bovine serum epitopes on their MHC molecules. Porgador et al. reported that induction of CTL responses to OVA peptides were obscured by the high levels of nonspecific lysis [130]. They attributed this to concurrent generation of CTLs against fetal calf serum antigens. In order to avoid these nonspecific T cell responses we grew our DC in serum-free media. Furthermore, in all the experiments described herein mice

were challenged with 12B1 cells grown *in vivo* as subcutaneous tumors, processed and washed in serum-free medium prior to i.v. leukemia induction. Despite the precautions taken to minimize nonspecific responses, exposure to unpulsed DC increased the number of splenic T cells in mice. These cells produced considerable amounts of Th1 type cytokines, IFN-γ and IL-2, and had cytolytic activity against 12B1. It is feasible that the production of IFN-γ and IL-2 may augment antileukemia responses by inducing lymphokine activated killer (LAK) activity *in vivo*. Earlier studies have documented that APCs such as DC or macrophages can promote LAK activity mediated by T cells [131-133]. *In vitro* studies demonstrated somewhat increased lytic activity of splenocytes from mice receiving unpulsed DC. This effect was also evident *in vivo* with empty DC moderately increasing survival of mice when compared those receiving saline.

The importance of T cells in preventing relapse following allogeneic stem cell transplantation has been well documented in CML [134-137]. Relapse rates are higher in syngeneic transplants when compared to allogeneic and in related matched transplants when compared to unrelated. T cell depletion has decreased the incidence and severity of graft versus host disease at the expense of increasing relapse rates [136]. The role of T cells in eliciting a graft versus leukemia effect in CML is further supported by induction of sustained remissions following donor leukocyte infusions in patients relapsing after allogeneic BMT [138-142]. The capacity therefore to generate leukemia specific CTLs may have clinical implications in CML. Several laboratories have documented that

human $CD4^+$ and $CD8^+$ T cells can be elicited against *bcr-abl* peptides [106, 121, 122, 143-146]. Moreover, DC from patients with CML, which constitutively express *bcr-abl* protein and thus present *bcr-abl* peptides, have been shown to be capable of stimulating anti-leukemic CTL responses *in vitro* [147].

Our data indicate that vaccination with DC pulsed with a chimeric peptide spanning the *bcr-abl* joining region can induce significant immunoprotection against leukemia *in vivo*. Our model more closely resembles blast crisis that is typically difficult to treat. As a result we are optimistic that this form of immune therapy may be even more effective in chronic phase CML. We are however cognizant that the relevance of the murine immune response is unclear. Whether this strategy will be effective in human CML remains to be determined and may be dependent additional factors. For example, the ability of a *bcr-abl* peptide to stimulate an immune response is dependent on its binding affinity to certain MHC class I molecules thus restricting this treatment to a subset of patients. Moreover, as opposed to mice, patients with CML may be tolerant to *bcr-abl* fusion protein. Despite these potential limitations, a DC based peptide vaccine appears to be a promising new immunobiologic approach that warrants further investigation.

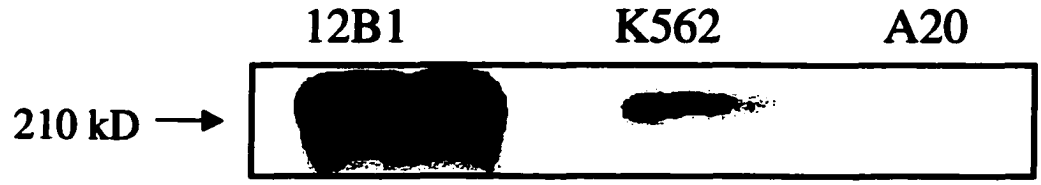

Figure AI-1. The presence of p210 *bcr-abl* protein in the 12B1 cell line confirmed by Western blotting using the monoclonal antibody to *bcr* (AB-2) as described in materials and methods. The K562 human leukemia cell was used as a positive control and the murine B cell leukemia line A20 as a negative control.

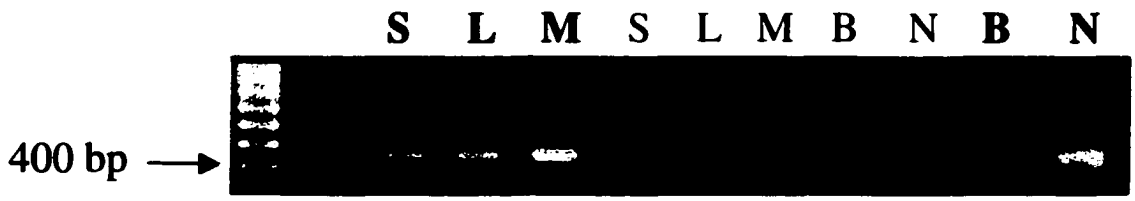

Figure AI-2. Presence of the *bcr-abl* fusion gene (b3a2) in tissues of mice inoculated with 12B1 confirmed by PCR amplification of the *bcr-abl* fusion region. S = spleen, L = liver, M= bone marrow, B= blood, N= lymph nodes, *bold type* depicts tissues from mice inoculated i.v. with 12B1 cells, *outline type* depicts tissues from non tumor bearing mice.

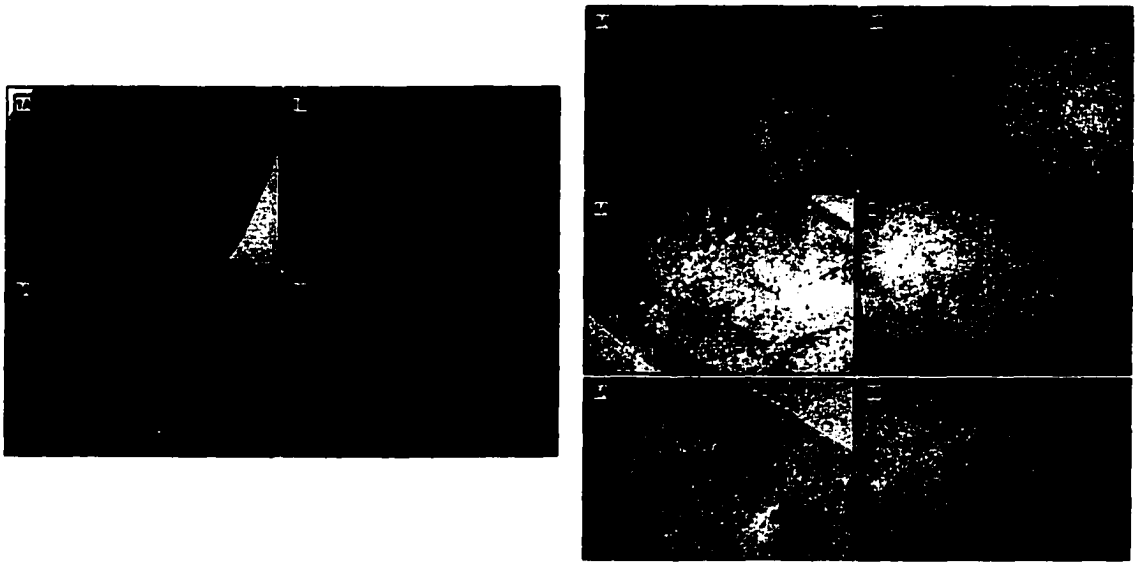

Figure A1-3. Histologic sections of spleen from **(A)** healthy and **(B)** 12B1 leukemia bearing BALB/c mice. Hematoxylin and eosin stained sections **(1.** 50X, **2.** 100X). **1A. & 2A.** Normal spleen histology with clearly defined white and red pulp. **1B. & 2B.** Diffuse leukemic infiltration of spleen resulting in loss of normal splenic architecture. Evidence of increased extramedullary hematopoiesis with large numbers of megakaryocytes. **3A. & 3B.** Immunohistochemical control staining; secondary antibody only (blue is from the counter stain used). **4A.** Immunoreactive brown staining Mac-1+ cells predominately in red pulp of spleen. **4B.** Increased numbers of Mac-1+ cells diffusely infiltrating spleen. **5A.** CD4+ T cells concentrated in white pulp of normal spleen. **5B.** Scattered infiltration of splenic parenchyma with CD4+ T cells in leukemia bearing mice.

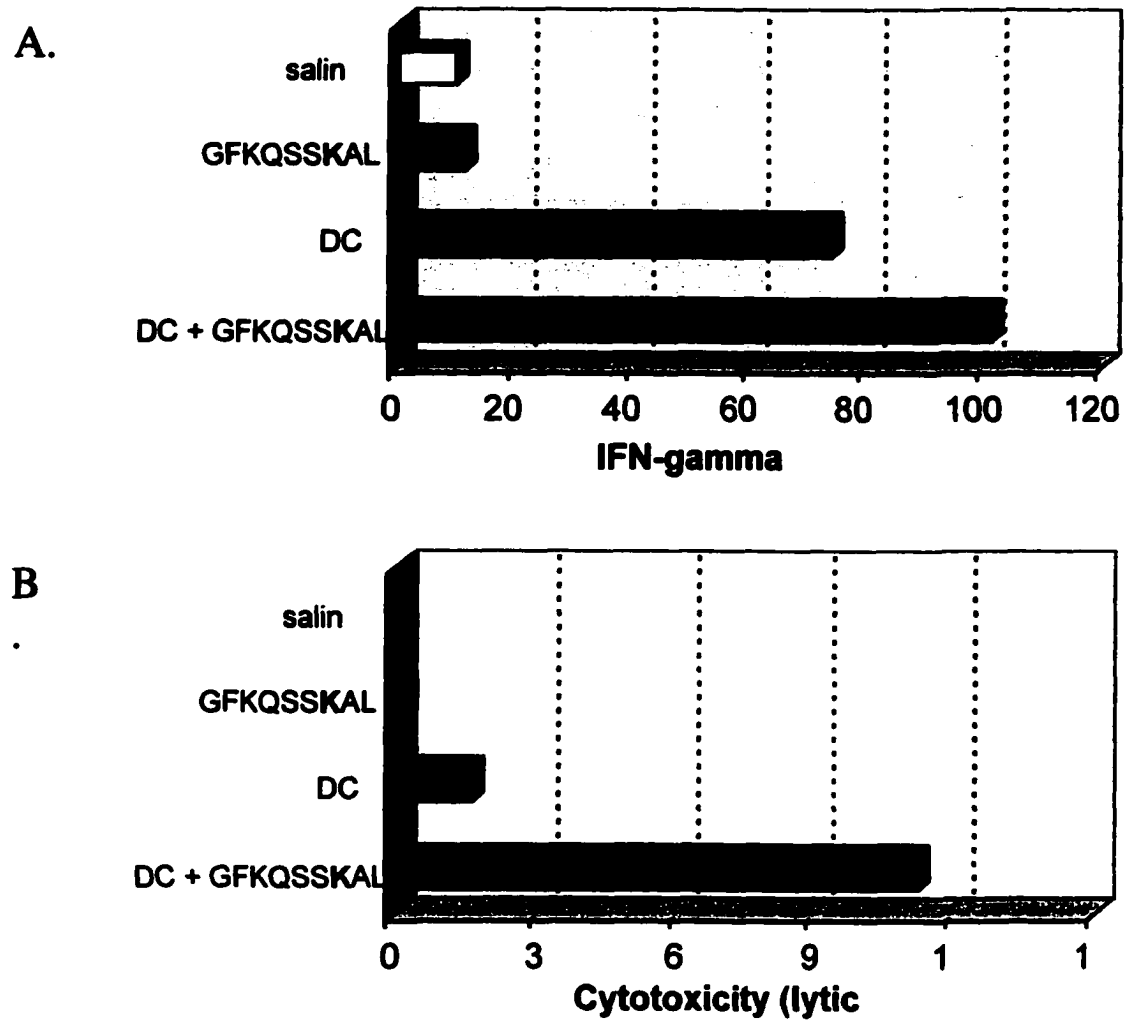

Figure AI-4. A. Effect of *bcr-abl* loaded DC vaccination on IFN-g production by splenocytes. Mice were immunized twice (days -14, -7), their spleens were harvested (day 0) and the splenocytes stimulated *in vitro* with *bcr-abl* peptide. Culture supernatants were collected after 24 hours and assayed for IFN-g using ELISA. **B.** Effect of *bcr-abl* loaded DC vaccination on cytotoxicity. Mice were immunized twice (days -14, -7) and their spleens were harvested on day 0. The *in vivo* primed splenocytes were cultured for 8 days with *bcr-abl* peptide and low concentrations of IL-2 as described in materials and methods. Stimulated effector cells were tested for cytolytic activity against 12B1 cells using 51Cr release assays. One lytic unit (LU) was defined as the number of effectors required to lyse 20% of targets; cytotoxicity is presented as LU per 106 effector cells. Figures depict representative data from one of three similar experiments.

Table 1 Characteristics of splenocytes from saline and unpulsed DC treated mice

	saline	DC
Cells per spleen (x 10^6)[1]	33.2 ± 7.2	55.6 ± 4.7
CD4+	3.7	13.4
CD8+	1.6	4.1
CD25+	0.3	3.9
B220+	21.7	26.0
Mac-1+	4.2	7.1
F4/80	6.2	8.7
CD11c	0.6	2.4
Gr-1+	4.4	4.2
IFN-γ (pg/ml)[2]	<1	132
IL-2 (U/ml)	<1	25

[1]Three mice per group were analyzed
Phenotype and cytokine data derived from pooled spleens from 3 mice in each group
[2]Cytokines produced by 10^6 cells/ml in 24 hours

Table 1: Characteristics of splenocytes from mice treated with saline and unpulsed dendritic cells (DC). Phenotype and cytokine data are derived from pooled spleens from three mice in each group. IFN γ: interferon γ, IL-2: interleukin-2 (cytokines produced by 10^6 cells/ml in 24 hrs).

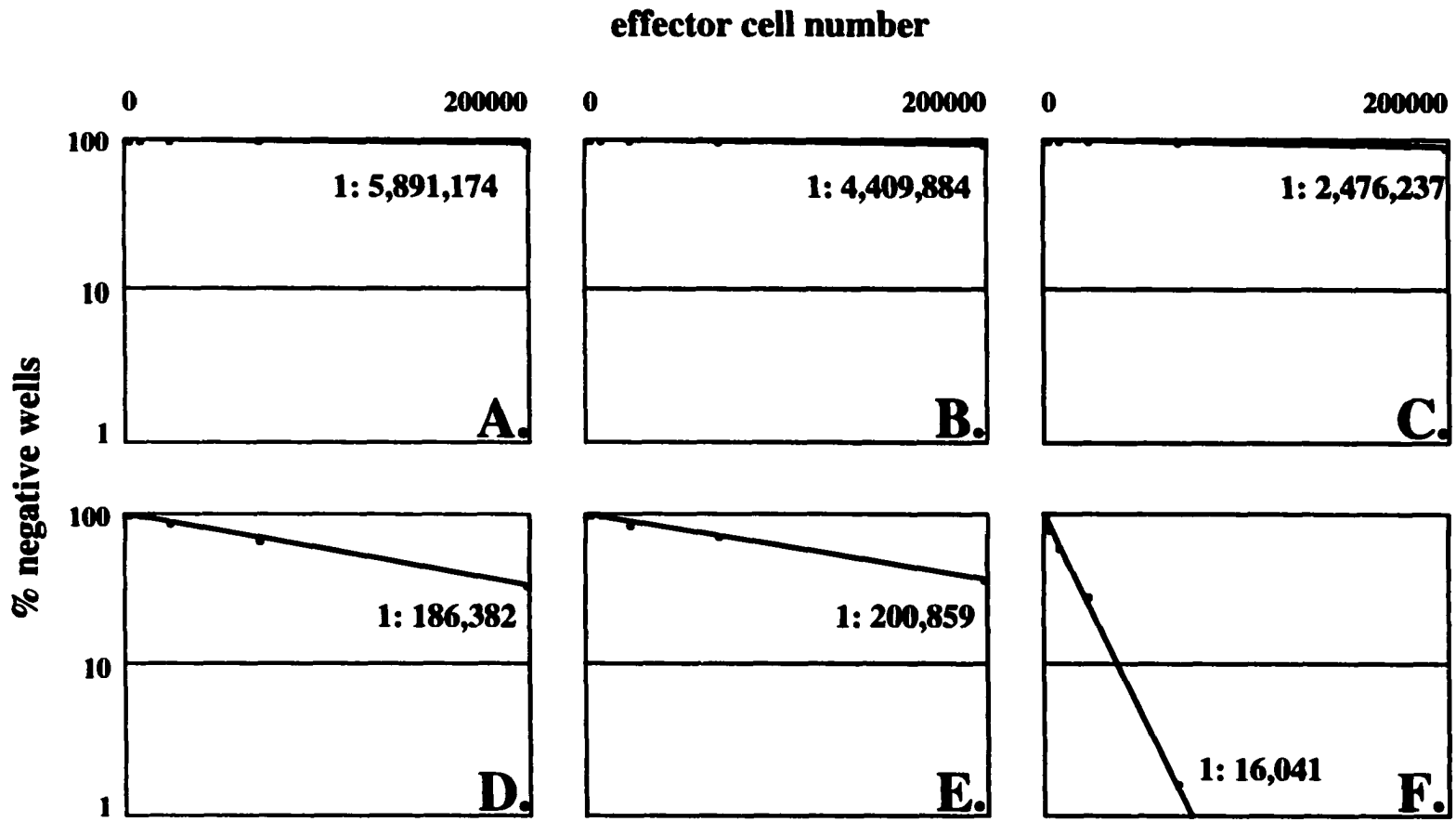
effector cell number
0
200000
0
200000
0
200000
100
10
1
% negative wells
1: 5,891,174
A.
1: 4,409,884
B.
1: 2,476,237
C.
1: 186,382
D.
1: 200,859
E.
1: 16,041
F.

Figure AI-5. Effect of immunization of mice with *bcr-abl* peptide, GFKQSSKAL, and peptide pulsed dendritic cells on CTLp frequency estimation. BALB/c mice were immunized with **A.** saline, **B.** irrelevant GFP peptide HYLSTQSALSK, **C.** *bcr-abl* peptide GFKQSSKAL, **D.** unpulsed DC or **E.** DC loaded with HYLSTQSALSK or **F.** DC pulsed with GFKQSSKAL peptide on days -14 and -7. On day 0 responder splenocytes from immunized mice were placed in microtiter plates in a limiting dilution fashion and stimulated with 5 mg/ml of *bcr-abl* peptide and low concentration of IL-2 as described in materials and methods. After 8 days in culture, wells were tested for cytotoxicity against ^{51}Cr labeled 12B1 pulsed or not for 1 hr with *bcr-abl* peptide (5 mg/ml). Wells were considered positive when cytotoxicity exceeded three standard deviations over the mean value of control wells without responder cells. The CTLp frequency estimations were calculated using the Poisson equation. Figures depict representative data from one of two similar experiments.

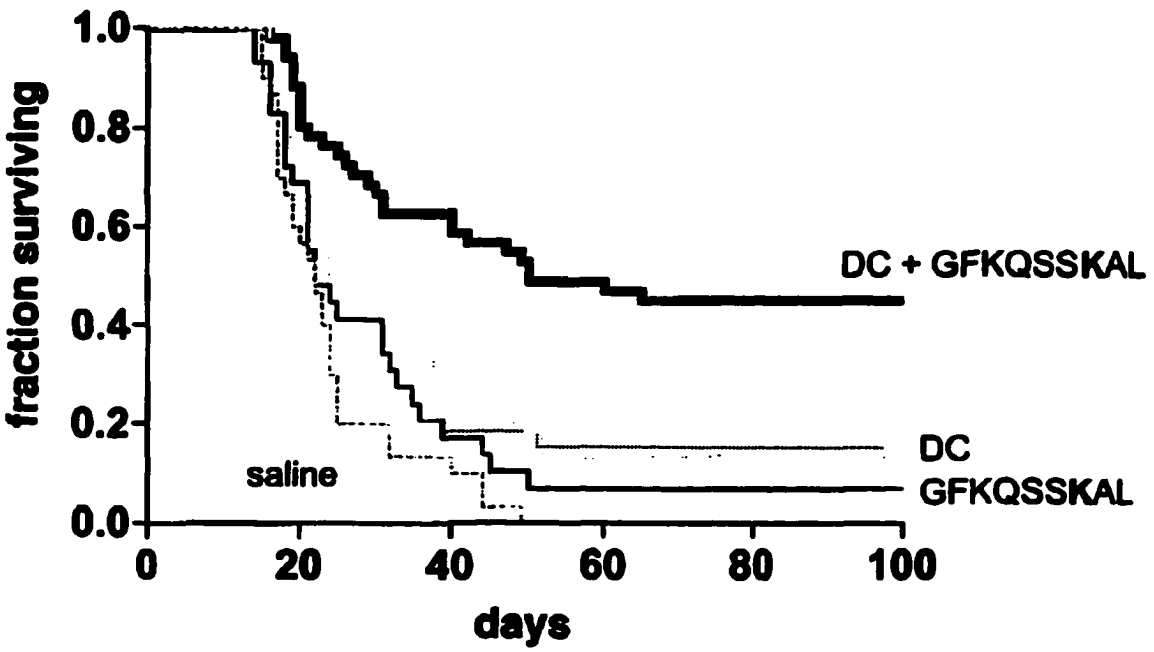

Figure AI-6. Effect of *bcr-abl* loaded DC vaccination on survival following 12B1 leukemia challenge. Mice were immunized s.c. (as depicted using the protocol described in materials and methods) on days -14 and -7 and challenged on day 0 with 66 12B1 cells given i.v. Data represent pooled data from 3 experiments (n=26-30 mice per group). *P* values *versus* saline control; GFKQSSKAL = ns; DC = 0.01; DC + GFKQSSKAL <0.0001, *versus* GFKQSSKAL; DC = 0.29; DC + GFKQSSKAL =0.0001, *versus* DC; DC + GFKQSSKAL = 0.001

APENDIX II

DENDRITIC CELLS LOADED WITH TUMOR-DERIVED MULTIPLE CHAPERONE COMPLEXES ELICIT THERAPEUTIC ANTI-TUMOR IMMUNITY

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Running Title: multiple chaperone complex loaded DC vaccines

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A2.1 ABSTRACT

We have utilized a free-solution-isoelectric focusing technique (FS-IEF) to obtain fractions rich in multiple chaperone complexes (MCC) from clarified tumor lysate and have previously reported on their vaccinating potential. To improve on the immunizing efficacy of tumor derived chaperone complexes, in the present study we examined the effects of MCC loaded dendritic cells (DCs) against 12B1, an aggressive *bcr-abl* positive murine leukemia tumor. We found that DCs incubated with 12B1 derived MCC had higher expression of CD40 on their cell surface, produced more interleukin-12 (IL-12) and had superior immunostimulatory capacity in a mixed leukocyte reactions (MLR) when compared to DCs exposed to unfractionated tumor lysate or purified heat shock protein 70 (HSP70). Vaccination of mice with 12B1 MCC pulsed DCs significantly prolonged their survival following lethal challenge with live 12B1 as compared to those immunized with tumor lysate or HSP70 loaded DCs. The protective immunity generated was tumor specific, long lasting, and both $CD4^+$ and $CD8^+$ T cell dependent. Moreover, immunization with MCC loaded DCs resulted in eradication of 12B1 growth in three-fourths of mice with pre-established 12B1 tumors. Our findings indicate that MCC have prominent adjuvant effects, are a very effective source of tumor antigen for pulsing DCs and therefore warrant further research and development.

A2.2 INTRODUCTION

Developing more effective anti-cancer vaccines has been one of the major goals of cancer immunotherapy. Purified tumor derived chaperone proteins such as heat-shock protein 70 and 90 (HSP70 and 90), GRP94/gp96, and calreticulin (CRT) have shown promise as vaccines, capable of generating tumor specific T cell responses and protective anti-tumor immunity in numerous animal models[45, 148-152]. These studies have indicated that it is not the chaperone proteins per se but rather the tumor antigen repertoire (i.e peptides) carried by the chaperones, which is immunogenic. In the normal cellular environment, chaperone proteins perform their intracellular functions as multi-protein complexes consisting of chaperones, co-chaperones, substrate molecules, etc. Vaccination studies have demonstrated that when purified away from their normal cellular environment, individual chaperone proteins retain effective anti-tumor activity. However, a remaining question has been whether or not multi-chaperone/co-chaperone vaccines would be more effective than single-component HSP vaccines.

To address this question, we have utilized a free-solution isoelectric focusing (FS-IEF) technique to obtain fractions rich in multiple chaperone complexes (MCC) from clarified tumor lysate. Using this method we are able to obtain much higher quantities of immunogenic material in a timely fashion. MCC contain chaperone proteins carrying a broad repertoire of antigenic peptides, which may offer significant advantages over

individual antigenic peptide vaccines. In our previous studies, we have shown that vaccines prepared from chaperone-rich fractions were capable of providing protective immunity in mice against tumor challenge in an A20 B-cell leukemia model[46]. To explore the effectiveness of MCC as a vaccine in other tumor models, to improve on its efficacy, to study its potency in a pre-established tumor setting, as well as to examine its effects on DCs, we enriched for chaperone proteins by FS-IEF from 12B1, a *bcr-abl* positive murine leukemia. We found that vaccination of mice with 12B1-derived MCC provides significant protection against lethal subcutaneous challenge with autologous tumor. The protective effect is significantly enhanced when MCC are loaded onto DCs and is superior to that achieved with tumor lysate or HSP70 pulsed DC immunization. The T cell immunity elicited by MCC loaded DCs is tumor specific and long lasting. Moreover, MCC loaded DCs can be used effectively to treat mice with pre-established tumors. Our findings indicate that 12B1-derived FS-IEF enriched MCC are capable of both efficient antigen delivery and of DC activation resulting in generation of potent anti-tumor immunity.

A2.3 MATERIALS AND METHODS

***Bcr-abl*-positive leukemia cell line:** 12B1 is a murine leukemia cell line derived by retroviral transformation of BALB/c bone marrow cells with the human *bcr-abl* (b3a2) fusion gene, and these cells express the p210 bcr-abl protein. This is an aggressive leukemia, with the 100% lethal dose (LD100) being 10^2 cells after tail vein injection and

10^3 cells after subcutaneous injection. The 12B1 cell line was kindly provided by Dr Wei Chen (Cleveland Clinic, Cleveland, OH). The cell line was tested monthly and found to be free of Mycoplasma contamination.

Mice: Six- to 10-week-old female BALB/c ($H2^d$) mice (Harlan Sprague Dawley, Indianapolis, IN) were used for the experiments. The animals were housed in a dedicated pathogen-free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Tumor generation: All tissue/cell culture reagents were purchased from Gibco/BRL (Gaithersburg, MD). 12B1 cells were cultured at 37 °C and in 5% CO_2 in RPMI medium containing 10% heat-inactivated fetal calf serum and supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.025 μg/ml amphotericin B, 0.5 × minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Cells were prepared for injection by washing and resuspending in Hanks' balanced salt solution. The cells were counted and adjusted to a concentration of 25×10^6 cells/ml. Female BALB/c mice were injected with 0.2 ml (5×10^6 cells) subcutaneously in both flanks and were monitored for tumor development. Tumors greater than 1 cm in diameter were harvested from euthanized mice. *In vivo* passaging of tumors involved harvesting and mincing the tumor to produce a cell suspension. The cell suspension was filtered through a Nitex screen to remove

debris and centrifuged. The cell pellet was resuspended, washed, counted, and injected into mice.

FS-IEF for chaperone enrichment and conventional purification of HSP70: Tumor tissue grown *in vivo* was harvested from mice and homogenized at 4 °C in a motor-driven glass/Teflon homogenizer; the buffer was 10 mM Tris/Cl (pH 7.4)/ 10 mM NaCl, 0.1% Triton X-100/0.1% Triton X-114/ 0.1% Igepal CA-630 (equivalent to Nonidet P-40; all detergents were from Sigma Chemical, St Louis, MO), with the following protease inhibitors (Roche Molecular Biochemicals, Indianopolis, IN): leupeptin (2 μg/ml), pepstatin A (1 μg/ml), phenylmenthylsulfonyl fluoride (0.5 mM) and a Complete protease inhibitor cocktail tablet. This buffer was chosen for its low ionic strength and ability to solubilize membranes. The homogenate was centrifuged at 10,000 x g for 30 min at 4°C to obtain a "low-speed" supernatant. That supernatant was centrifuged at 100,000 x g for 60 min at 4°C to obtain a "high speed" supernatant. This was then dialyzed against 5 mM Tris/Cl (pH 7.4)/ 5 mM NaCl, 0.05% Triton X-100/0.05% Triton X-114/0.05% Igepal CA-630. Protein concentration of this dialysate was determined by BCA (bicinchoninic acid) method (Pierce Endogen, Rockford, IL) using bovine serum albumin as a standard. This dialysate was frozen in aliquots containing 25 mg of total protein. To generate vaccine one aliquot was filtered through a 0.8 μm filter and prepared for isoelectric focusing by adding urea to 6 M, the detergents Triton X-100, Triton X-114, and Igepal each to 0.05%, and a mixture of Rotolytes (Bio Rad

Laboratories, Hercules, CA; 5 ml each solution A and B for each pH range; pH 3.9-5.6; 4.5-6.1; and 5.1-6.8) to a total volume of around 40-50 ml. FS-IEF was carried out in a Rotofor device (Bio Rad Laboratories). Isoelectric focusing was conducted for 5 h at 15 W constant power while the apparatus was cooled with recirculating water at 4 °C; the anode compartment contained 0.1 M H_3PO_4, while the cathode compartment contained 0.1 M NaOH. Twenty fractions were harvested; the pH of each fractions was determined with a standard pH meter, and the protein content was analyzed by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described[45]. Purification of 12B1 HSP70 was done via conventional and nucleotide-affinity chromatography as previously described[45].

Preparation of chaperone-enriched vaccines: Fractions from FS-IEF that contained substantial amounts of four chaperone proteins (HSP70, HSP90, gp96, and calreticulin), as determined by SDS-PAGE and Western blotting, were pooled and dialyzed stepwise out of urea and detergents (starting in 0.1 × PBS), 4 M urea, and 0.025% detergents, ending with 0.1 × PBS). Pooled fractions were then concentrated using Centricon devices and reconstituted in PBS. Vaccines were then passed onto an Extracti-gel D column (Pierce Endogen) to remove detergent. Protein concentrations were determined by the BCA method and the concentrated proteins were diluted to appropriate concentration for *in vivo* and *in vitro* experiments.

Generation of bone marrow-derived DCs: BALB/c mouse bone marrow DCs were generated using a slightly modified protocol from that described previously[79]. Bone marrow was harvested from femurs and tibiae and filtered through a Falcon 100-□m nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). Red blood cells were lysed in a hypotonic buffer and the marrow was cultured in complete RPMI medium (therapeutic grade; Gibco BRL, Gaithersburg, MD), which contains 10% fetal calf serum, L-glutamine, human serum albumin, 50 μg/mL streptomycin sulfate, and 10 μg/mL gentamicin sulfate. Murine granulocyte-macrophage colony-stimulating factor (10 ng/mL; Peprotech, Rocky Hill, NJ) and interleukin (IL)-4 (10 ng/mL) were added to the culture. After 6 days, the nonadherent and loosely adherent cells were harvested, washed and used for *in vivo* and *in vitro* experiments. Less than 10% of these cells were contaminated by macrophages ($CD14^+$ cells).

Flow cytometry: DCs were washed in PBS containing 2% heat-inactivated fetal bovine serum and 0.1% sodium azide (Sigma Chemical). A total of 2×10^5 cells were placed in each well of 96-well U-bottom microtiter plates. Surface expression of specific antigens was determined by incubating the cells first with an Fc-receptor-blocking antibody (Pharmingen, San Diego, CA.) for 5 minutes and then with saturating amounts of monoclonal antibodies (Pharmingen) for 30 minutes at 4°C. Antibodies used included purified fluorescein isothiocyanate (FITC) conjugated anti-$I\text{-}A^d$ (clone AMS-32.1, mouseIgG2b), anti-CD80 (clone 16-10A1, hamster IgG), anti-CD86 (clone GL1, rat

IgG2a), anti-CD40 (clone HM40-3, hamster IgM) and purified phycoerythrin (PE) conjugated anti-CD11c (clone HL3, hamster IgG). After a 30 minute incubation the cells were then washed 3 times in PBS containing 2% heat-inactivated fetal bovine serum and 0.1% sodium azide and fixed with PBS containing 1% paraformaldehyde (Polysciences, Warrington, PA). Ten thousand cells were analyzed using a FACScan (Becton Dickinson Immunocytometry, San Jose, CA)

Mixed leukocyte reaction (MLR): Day 6 BALB/c ($H2^d$) DCs were incubated with 50 µg/ml derived 12B1 lysate, HSP70, or FS-IEF enriched MCC in the presence of 10 ng/ml murine GM-CSF and IL-4 for 24 hours. DCs were collected, treated with 50 µg/ml Mitocycin C (Sigma chemical) for 20 minutes, then washed three times with PBS. Splenocytes (10^5 per well) from naïve C57BL/6 ($H2^b$) mice were plated in U-bottom 96 well plates. DCs were serially diluted and incubated with splenocytes with the ratio of splenocytes to DCs ranging from 1:1 to 27:1. After a four day co-culture, 20 µl of 50 mCi/ml [^{3}H] thymidine (ICN Pharmaceuticals) was added to each well. The cells were harvested 18 hr later using a 96-well Packard cell harvester and the radioactivity measured on a Packard beta counter.

ELISPOT Assays: ELISPOT assays were performed to measure the IL-12 secretion by DCs. Between 10^5 to 10^6 day 6 DCs were cultured with 50 µg/ml 12B1-derived lysate, HSP70 or MCC in the presence of 10 ng/ml GM-CSF and IL-4 for 24 hours on Millipore

MultiScreen-HA 96 well plates (MAHA S45, Millipore, Bedford, MA). The plates had been previously coated overnight with anti-IL-12 capture antibody (10 μg / ml, clone 9A5, rat Mab anti-mouse IL-12 (p70), BD PharMingen). DC were then washed out with copious amounts of PBST (PBS + 0.05% Tween20). Biotinylated anti-IL-12 antibody (2 μg / ml, clone C17.8, rat Mab anti-mouse IL-12 (p40/p70, BD PharMingen) was added for two 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 1 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) prepared in acetate buffer (pH 5.0) with 0.015% hydrogen peroxide. Spots were examined using a dissecting microscope. Wells of interest were photographed with a microscope-mounted Cool SNAP CCD camera (RS Photometrics, Tucson AZ), and images captured with RS Image, Version 1.07 (Roper Scientific, Tucson, AZ).

***In vivo* tumor growth experiments:** In the prophylactic model, mice were immunized with indicated vaccines (20 μg/mouse per vaccination) by subcutaneous injection into the left groin on days -14 and -7. On day 0, mice were challenged with 10^3 (LD_{100}) viable 12B1 cells obtained from one single *in vivo* passage. For dosage escalation studies, 5, 10, 20, and 50 μg/mouse were used for each vaccination. In experiments with DCs, DCs were incubated with 50 μg/ml indicated vaccines in the presence of 10 ng/ml murine

GM-CSF and IL-4 for 24 hours, then the DCs were washed with PBS three times, and resuspended in PBS followed by s.c. injection into mice. To exclude potential HSP70 or MCC toxicity on DCs, day 6 DCs were plated in 96-well flat-bottom plates (50,000 cells/well) in the presence of increasing concentrations of 12B1 tumor derived HSP70 or MCC (0-100 μg/ml) for 24 hours. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, stock solution 5 mg/ml, Sigma] at 10 μl per well as added for an additional 4 hours. The supernatant was aspirated and the formazan crystals were solubilized in dimethylsulfoxide, followed by determination of optical densities at 560 nm and 690 nm using a microtiter plate reader. A total of 5×10^5 DCs were injected per mouse subcutaneously on days -14 and -7. In depletion experiments, $CD4^+$, $CD8^+$, or both T cell populations were depleted by i.p. injection of 200μg anti-CD4 (GK1.5) [108] and/or anti-CD8 (2.43) [153], on days –3, -1, +1, and +3. Tumor size was measured every other day with calipers once the tumors became palpable. Tumor volume was calculated using the formula: length × width^2 × $\pi/6$. Differences in mean tumor volume between groups were compared using an unpaired *t* test. In other experiments the Kaplan-Meier product-limit method was used to plot the time to tumor appearance and the log-rank statistic to test differences between groups [128, 154]. Mice with tumor were enthusiazed at the end point listed. Tumor free mice were kept for rechallenge experiments. In rechallenge experiments, 10^3 live 12B1 (LD_{100}) cells were injected into the right groin 80 to 125 days after the first challenge, whereas 10^6 A20 leukemia cells (LD_{100}) were injected into the left groin.

In pre-established tumor model, mice were injected with 10^3 viable 12B1 cells at right groin on day 0. On day +2, mice were vaccinated as indicated by subcutaneous injection into the left groin. Tumor volume was measured at indicated time points.

A2.4 RESULTS

FS-IEF yields tumor lysate fractions that are enriched for multiple chaperone proteins. We have developed a novel enrichment procedure for heat shock proteins by using FS-IEF, a relatively simple and rapid method that separates soluble protein components of a mixture by their isoelectric points (pI). Proteins were separated into 20 fractions covering a pH gradient as determined by Rotolyte choice. Following FS-IEF of 12B1 tumor lysate, we identified fractions of interest by SDS-PAGE and Western blotting. SDS-PAGE (Figure AII-1, top) and Western blot (Figure AII-1, bottom) results indicated that following FS-IEF, several fractions ranging from pH 5.1 to pH 6.0 (lanes 8-12) contained HSP70, HSP90, GRP94/gp96, and CRT within them. Fractions selected to be pooled for vaccines were those that contained all four of the above HSPs. FS-IEF utilizes small amounts of starting material to yield relatively large amounts of tumor-derived chaperone proteins. In general, 1g of tumor can yield 1000 μg MCC vaccine while from the same amount of tumor, only 30-50 μg of individual chaperone protein such as HSP70 can be generated using conventional purification strategies.

Immunization with 12B1 tumor derived MCC provides tumor specific immunity against autologous tumor challenge. Dose escalation experiments were performed in order to determine the optimal amount of 12B1 MCC needed to immunize the animals. BALB/c mice received 5, 10, 20, or 50 μg of MCC subcutaneously on days –14 and –7, and were challenged on the opposite side with an LD_{100} dose of 12B1 cells on day 0. Tumor volume measurements demonstrated that immunization of mice with 20 μg of 12B1 derived MCC provided optimal protection against tumor challenge (Figure AII-2). This vaccine dose was consistent with our previous studies using other tumor models[46].

To demonstrate the specificity of chaperone protein-stimulated immune responses, mice were vaccinated with MCC isolated from A20 B-cell leukemia/lymphoma or 12B1 bcr-abl^+ leukemia. A20-derived MCC did not generate protection against 12B1 challenge confirming that the immunity elicited is tumor specific (Figure AII-3). Additionally, immunization of animals with tumor lysate generated no protection (Figure AII-3) as was demonstrated previously for A20 B-cell leukemia[46]. These data indicate that unfractionated 12B1 tumor lysate itself is not an effective immunogen, and that the MCC enrichment is necessary to enhance its immunogenicity.

Purified tumor derived HSP70 has been reported to be an effective vaccine[149, 152]. Tumor derived MCC contain HSP70 and at least three other of the reported immunogenic chaperones[46]. To compare the immunogenicity of purified 12B1 HSP70 to that of

MCC, mice were immunized with 20 μg of purified HSP70 or MCC. The protective effects of HSP70 and MCC were comparable with both preparations significantly delaying 12B1 tumor growth (Figure AII-3).

DCs pulsed with MCC have increased MHC class II and costimulatory molecule expression, IL-12 production and immunostimulatory capacity *in vitro*. Since DCs have been reported to express receptors for exogenous HSPs[155] and since they are being used as APCs in clinical vaccine trials, we examined the effect of tumor derived chaperone proteins on bone marrow derived DCs. Day 6 DCs were cultured with 12B1 derived MCC or unfractionated tumor lysate or purified HSP70 for 24 hours. About 80% of these DCs are $CD11c^+$ as assessed by flow cytometry (data not shown). The percentage of $CD11c^+$ gated cells that express CD40, 80, 86, and MHC-II was further determined. Tumor derived MCC and HSP70 had comparable effects in inducing expression of MHC class II and CD80/86 on DCs (Figure AII-4A). In contrast, only MCC preparations significantly increased expression of CD40 on DCs (> than 5-fold increase).

We also evaluated IL-12 secretion by DCs after stimulation with tumor-derived lysate, HSP70 or MCC. Day 6 bone marrow derived DCs were cultured with 50 μg/ml 12B1 lysate, HSP70 or MCC in the presence of 10 ng/ml GM-CSF and IL-4 for 24 hours.

Compared to lysate and HSP70, MCC stimulated DCs clearly had more pronounced IL-12 production as assessed by ELISPOT (Figure AII-4B).

We then examined the effect of 12B1 derived MCC on DC immunostimulatory function in a mixed leukocyte reaction *in vitro*. BALB/c DCs were cultured with 50 μg/ml 12B1 HSP70, or MCC, or tumor lysate for 24 hr. The DCs were treated with 50 μg/ml Mitocycin-C for 20 minutes, then washed and added at different concentrations to plates containing 10^5 viable C57BL/6 splenocytes per well and co-cultured for 4 days. [^{3}H]-thymidine was added to the plates and the cells co-cultured for additional 18 hours. DCs exposed to with 12B1 MCC were more potent stimulators of allogeneic responses in these mixed leukocyte reactions. (Figure AII-4C).

DCs pulsed with 12B1 tumor derived MCC induce potent T cell dependent anti-tumor immune responses. Given the *in vitro* stimulatory activities of MCC on DCs, *in vivo* experiments exploring whether the immunizing effects of MCC may be augmented by DCs were then conducted. Using MTT assays we first evaluated whether increasing concentrations of 12B1 MCC or HSP70 or unfractionated lysate impaired DC viability. No changes in DC viability were observed with up to 100 □g/ml of tumor-derived proteins (data not shown). Day 6 immature bone marrow derived DCs were then incubated with 50 μg/ml of 12B1 derived MCC or the same concentration of purified 12B1 HSP70 or unfractionated 12B1 tumor lysate for 24 hours. The DCs were washed

and resuspended in PBS and then injected to mice subcutaneously (5×10^5 per mouse). Immunization with 12B1 MCC pulsed DCs significantly inhibited tumor growth with over 80% of mice remaining tumor free (Figure AII-5). This compares favorably to vaccination with 12B1 MCC without DCs resulting in rejection rates of 50% (data not shown). DC pulsed with HSP70 were less effective albeit also significantly better than DCs alone with about 30% protection (Figure AII-5). This was similar to the protection achieved with purified HSP70 without DCs (data not shown). Vaccination of mice with 12B1 lysate pulsed DCs had only a minimal protective effect (Figure AII-5). These data indicate that while tumor derived HSP70 or MCC vaccines yield comparable protective immunity, this can be augmented by *ex vivo* pulsing of DCs only in the case of MCC. These findings suggest that tumor derived MCC may have better capacity to activate DCs compared to unfractionated tumor lysate or purified HSPs which correlate well with our *in vitro* DC studies (Figure AII-4).

To define the roles of $CD4^+$ and $CD8^+$ T cells in the protective immunity induced by MCC loaded DC, mice were depleted of $CD4^+$, $CD8^+$, or both T cell populations. Immunity was partially abrogated when either $CD4^+$ or $CD8^+$ T cells were depleted. Complete loss of protection was observed in mice that were depleted of both T cell populations indicating that $CD4^+$ and $CD8^+$ T cells contribute to the anti-tumor immunity generated by MCC loaded DCs (Figure AII-6).

Eighty to 125 days after initial tumor challenge, surviving and naïve mice, of the same ages as controls, were rechallenged with live 12B1 cells (10^3; LD_{100}) in the right groin and A20 cells (10^6; LD_{100}) in the left. Surviving mice from the group vaccinated with DCs pulsed with MCC had retarded 12B1 growth, with 6/11 (55%) mice rejecting the rechallenge of 12B1. The group that received 12B1 MCC vaccine (without DCs) also had delayed tumor growth, albeit less impressive than that achieved with DC+MCC, with 1 in 4 rejecting their 12B1 rechallenge. In contrast, all naïve mice developed 12B1 tumors (Figure AII-7). Moreover, all of the immune and naive mice that were challenged with A20 leukemia cells in the opposite groin developed tumors with similar growth rates, confirming the specificity of the anti-tumor response generated by 12B1 MCC immunizations (Figure AII-7).

MCC pulsed DCs are effective therapeutic vaccines. To examine the potency of MCC loaded DC vaccine in a preestablished tumor setting, we first induced 12B1 tumor followed by a single subcutaneous immunization with chaperone-pulsed DCs. Specifically BALB/c mice received a lethal dose (LD_{100}) of 12B1 s.c. on day 0 and were immunized in the opposite groin on day +2 with DCs (5×10^5 per mouse) pulsed with HSP70 or MCC. The group receiving HSP70 loaded DCs showed a delay in tumor growth when compared to saline controls but this was not significantly different from mice receiving unpulsed DCs. However, MCC loaded DCs significantly delayed tumor

development when compared to all other groups. Moreover, this therapeutic vaccine resulted in eradication of tumor growth in 12/16 (75%) of the mice (Figure AII-8).

A2.5 DISCUSSION

Chaperone proteins are the most abundant soluble intracellular molecules. Under physiological conditions, their ability to stimulate immune responses is not expected, since the immune system normally does not attack self. However, when presented to cells extracellularly, HSPs or chaperone proteins may act as danger signals alerting the immune system to tissue injury[76, 77, 85]. Individual chaperone proteins, such as HSP70 and GRP94/gp96 have been shown to deliver partial maturation signals to DCs and to stimulate pro-inflammatory cytokine expression thus inducing Th1-type immune responses[86, 96, 156-158]. Stress proteins are known to perform their intracellular functions as multi-protein complexes consisting of chaperones, co-chaperones, and substrate molecules. In our previous studies, we have demonstrated that MCC separate in complexes[46], and therefore may more accurately re-create danger signals, consequently activating APCs more efficiently than individual purified chaperone proteins.

DCs are professional APCs, 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[62, 63, 97]. In the present study, we show that immunization with DCs pulsed with 12B1 derived MCC

significantly delays or prevents tumor growth *in vivo*, indicating that MCC associated peptides are presented by DCs. 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[113, 159-162]. Recently, receptors for HSP70 and GRP94/gp96 have been identified[115, 155, 163]. Tumor derived MCC are made up of multiple chaperone proteins, therefore additional potential chaperone receptors on the surface of APCs may be involved in the endocytosis of these complexes. Following uptake the chaperone protein-escorted peptides are processed and re-presented on MHC molecules generating antigen-specific T cell responses. In addition to HSP70, HSP90, GRP94/gp96, and CRT, other chaperone protein members are also present in the MCC fractions, such as BiP/grp 78, grp75, small amounts of HSP72, HSP 40, and other unidentified proteins[8]. It is possible that other proteins in the MCC may contribute to the superior immunogenicity demonstrated in these studies. Identifying the role of each of these proteins is critical and may help in the discovery of additional immunogenic chaperone proteins.

Tumor lysate has been used as a source of antigen for loading DCs in preclinical and clinical studies[164, 165]. In the 12B1 model and in the numerous other tumor models we have studied, such as A20 lymphoma[8], B16 melanoma, C1300 neuroblastoma, Sa1 fibrosarcoma, and 4T1 mammary carcinoma (data not shown), tumor lysate did not stimulate a measurable immune response when used alone, while some protection was

achieved when they were loaded onto DCs. Moreover, when incubated with DCs, unfractionated tumor lysate did not change the DC phenotype or enhance their immunostimulatory function in an MLR. This lack of protective immunity with unfractionated tumor lysate may be due to the presence of immunosuppressive factors in the lysate and/or to insufficient concentration of chaperone proteins and the peptides they carry (manuscript in preparation). In contrast and consistent with our previous study[8], the FS-IEF-enriched MCC from tumor lysate are very immunogenic indicating that the FS-IEF step is essential to enhance the immunogenicity of tumor lysate preparations.

It has been shown by a variety of studies that the immunogenicity of tumor derived chaperones results from the antigenic peptides escorted by the chaperone proteins[151, 152, 166]. If different chaperone proteins preferentially escort distinct peptides, MCC may have a broader antigenic repertoire and therefore more tumor specific epitopes than those escorted by individual chaperone proteins, such as HSP70. We have also examined the immunogenicity of individual MCC fractions (of those that are pooled to constitute the vaccine) by comparing fractions, distinct in overall protein profile but still containing four of the known immunogenic chaperones HSP70, HSP90, GRP94/gp96, and CRT. We found that these fractions had comparable activity in prolonging survival of mice[46]. Importantly, irrelevant FS-IEF fractions (i.e., containing little or no detectable chaperone proteins) provided no protective benefit. Thus, the protective effect of MCC vaccination is not an artifact of the isoelectric focusing procedure. 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.

Using the A20 B-cell leukemia model, we reported that HSP70 preparations induced more potent tumor specific immunity than any of the other known immunogenic chaperones isolated from the same tumor[45]. In this study, we directly compared the immunogenicity of 12B1 MCC to that of purified HSP70. We found that MCC alone provided equivalent protection against tumor challenge when compared with 12B1 HSP70 immunizations. However, when these chaperones where incubated with DCs and utilized as a cellular vaccine, MCC pulsed DCs showed distinctly better anti-tumor immunity than HSP70-pulsed DCs in both a prophylactic and therapeutic setting. This suggests that MCC may deliver additional signals important in activation of DCs and/or provide additional tumor specific peptides to these APCs. Since 12B1 is a very aggressive tumor, with an LD_{100} of 10^3 cells by subcutaneous injection, achieving cures in a preestablished tumor setting by MCC loaded DCs attests to the effectiveness of this approach.

In conclusion, the present study demonstrates that when compared to purified HSP70 or unfractionated lysate, MCC have superior ability to activate/mature DCs and are able to

induce potent, long lasting and tumor specific T-cell-mediated immunity. The FS-IEF technique for enhancing MCC is relatively easy and rapid, yielding sufficient immunogenic material for clinical use. MCC in combination with DCs may provide a practical and effective strategy for the development of a clinically useful anti-cancer vaccine.

A2.6 ACKNOWLEDGEMENTS

The authors wish to thank Luke Whitesell for his helpful comments, and Barbara Carolous for assistance in FACS assay.

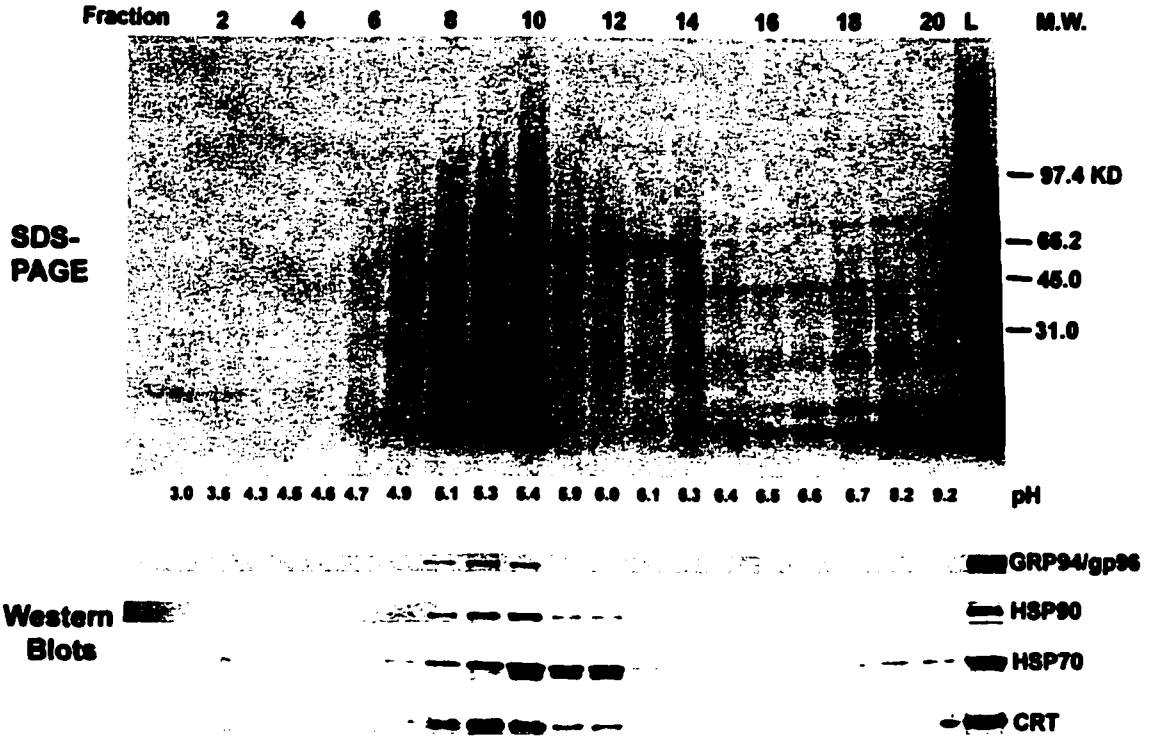

Figure AII-1. SDS-PAGE (*top*) and Western blot *(bottom)* analyses of 12B1 tumor-derived free-solution isoelectric focusing (FS-IEF) fractions. 12B1 tumor was homogenized in lysis buffer and a 100,000g supernatant was obtained. The high-speed supernatant was dialyzed into a low-ionic-strength buffer; this preparation was brought to 6 M urea, Rotolyte (pH3.9-5.6, 4.5-6.1, and 5.1-6.8) and 0.05% in detergents. The sample was filtered through 0.8 μm filter and subjected to FS-IEF in a Bio Rad Rotofor cell for 5 h at 15 W constant power. Twenty fractions were harvested, the pH was determined for each fraction, and each fraction was analyzed by SDS-PAGE and Western blot. *(Top)* Coomassie blue-stained gel of lysate prior to isofocusing and the resultant 20 fractions following isofocusing. The pH of each fractions is listed beneath the respective gel lane. Molecular mass markers are indicated that the right. Following SDS-PAGE, gels were electroblotted to nitrocellulose and probed with specific antibodies for the chaperones HSP70, 90, gp96, and calreticulin (CRT) *(bottom)*.

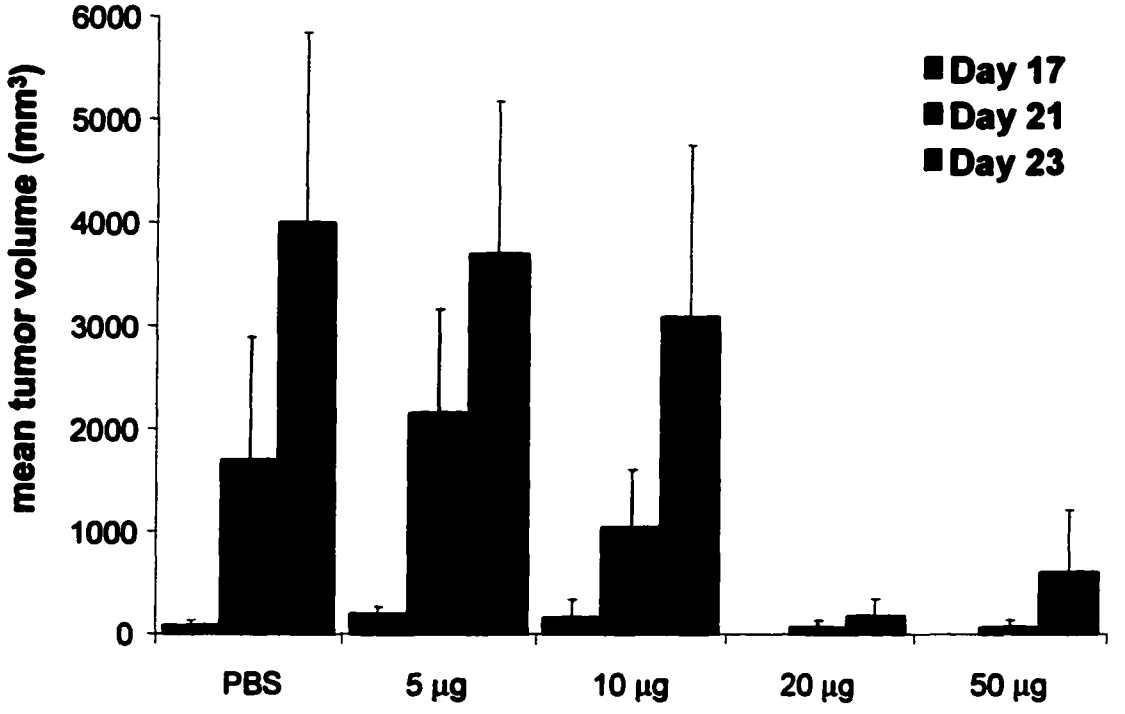

Figure AII-2. Effect of MCC immunizing dose on mean tumor volume of challenged mice. BALB/c mice (n=4/group) were immunized with increasing quantities (5-50 µg) of 12B1 tumor derived FS-IEF vaccine, or with saline on days −14 and −7, and were challenged in the opposite groin with 12B1 leukemia cells on day 0.

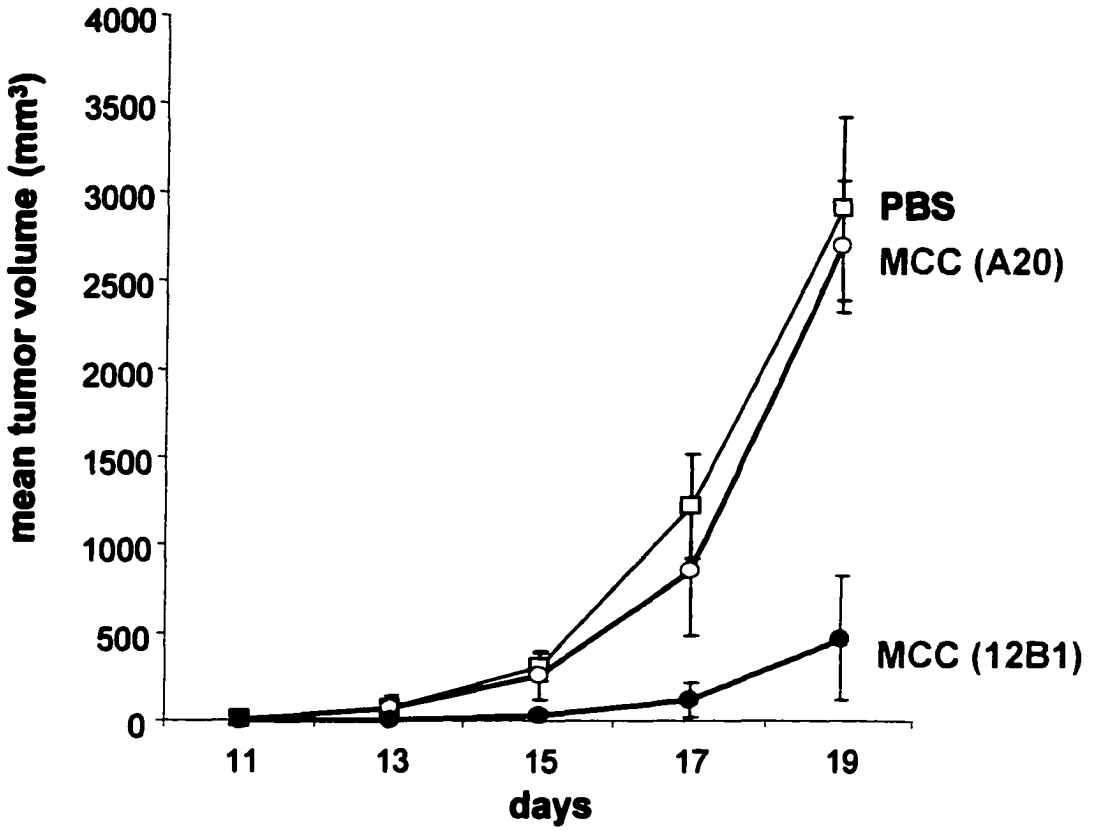

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Figure AII-3. (A) Immunization with 12B1-derived MCC provides specificity protection against 12B1 tumor challenge. BALB/c mice were immunized with 20 µg 12B1 or A 20 tumor derived FS-IEF enriched chaperone proteins, on days –14 and –7 followed by challenge with 10^3 12B1 cells (10^3) in the opposite groin day 0. (saline *versus* MCC (12B1) p <0.05 from day 15 onward; n=11-24 mice per group).

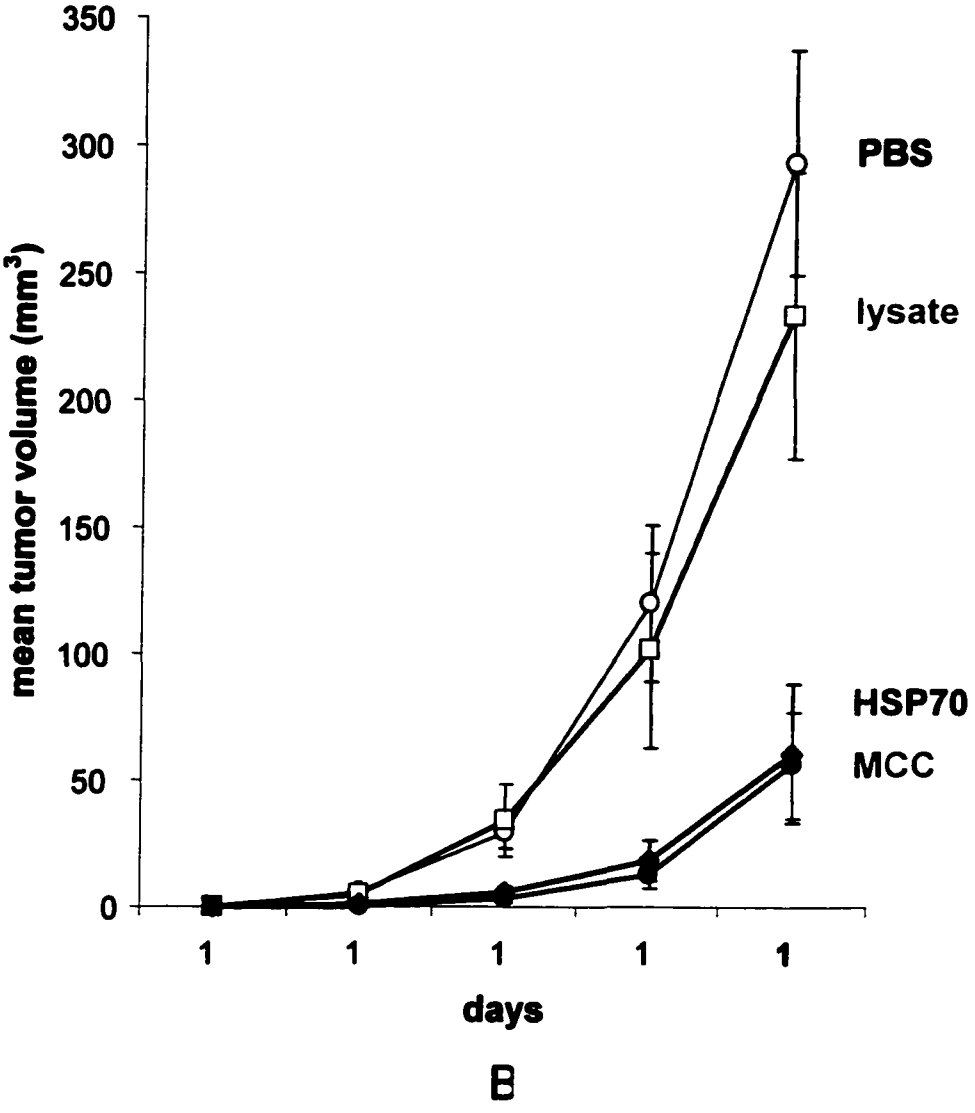

Figure AII-3. (B) Immunization with purified HSP70 or MCC from 12B1 tumor delays tumor growth. Mice were immunized with 20 μg 12B1 tumor derived HSP70 or MCC or unfractionated lysates on days –14 and –7 followed by challenge with 12B1 cells (10^3) in the opposite groin on day 0. (saline *versus* HSP70 or MCC, $p < 0.05$ from day 15 onward; n=8 mice per group).

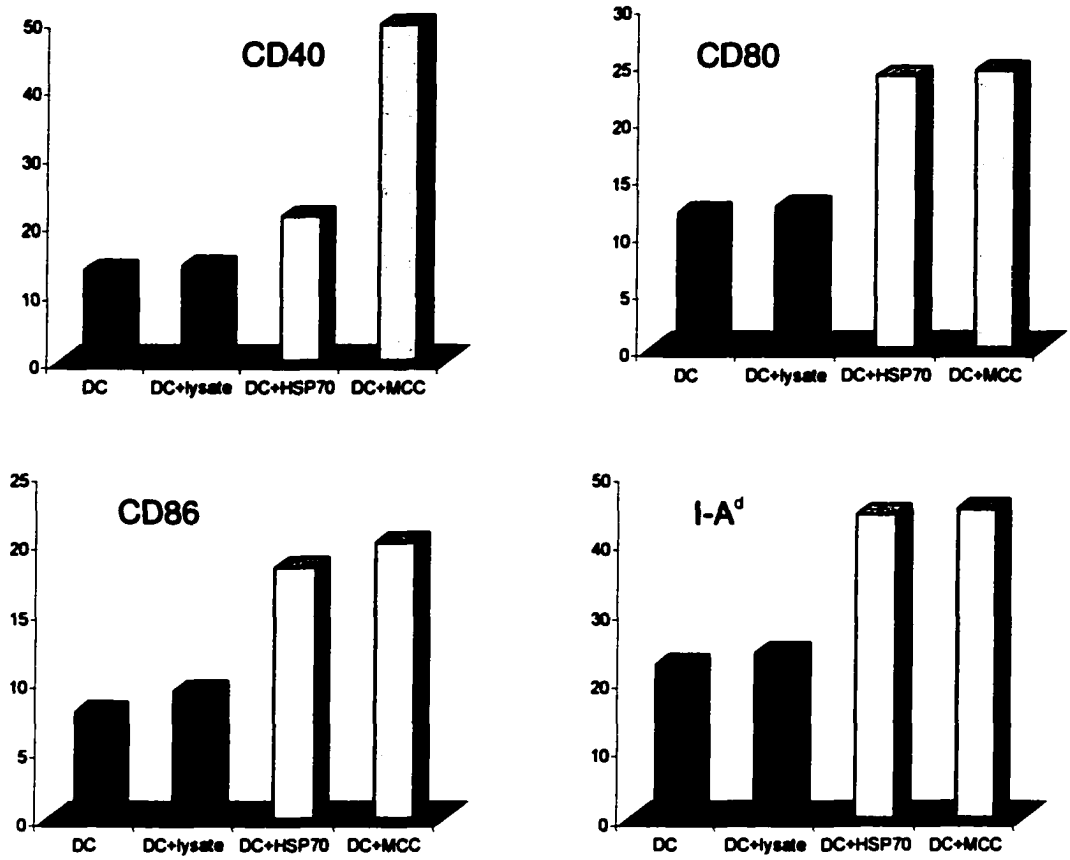

Figure AII-4. (A) 12B1 derived chaperone proteins stimulate DCs to express MHC class II and co-stimulatory molecules. Bone marrow-derived DCs were exposed to 50 μg/ml 12B1 HSP70, FS-IEF complexes, or unfractionated lysates, for 24 hours, harvested and analyzed by flow cytometry for expression of the cell surface markers indicated. The numbers in the ordinate represent the percentage of positive cells in the $CD11c^+$ gated population. Representative data from three experiments are shown.

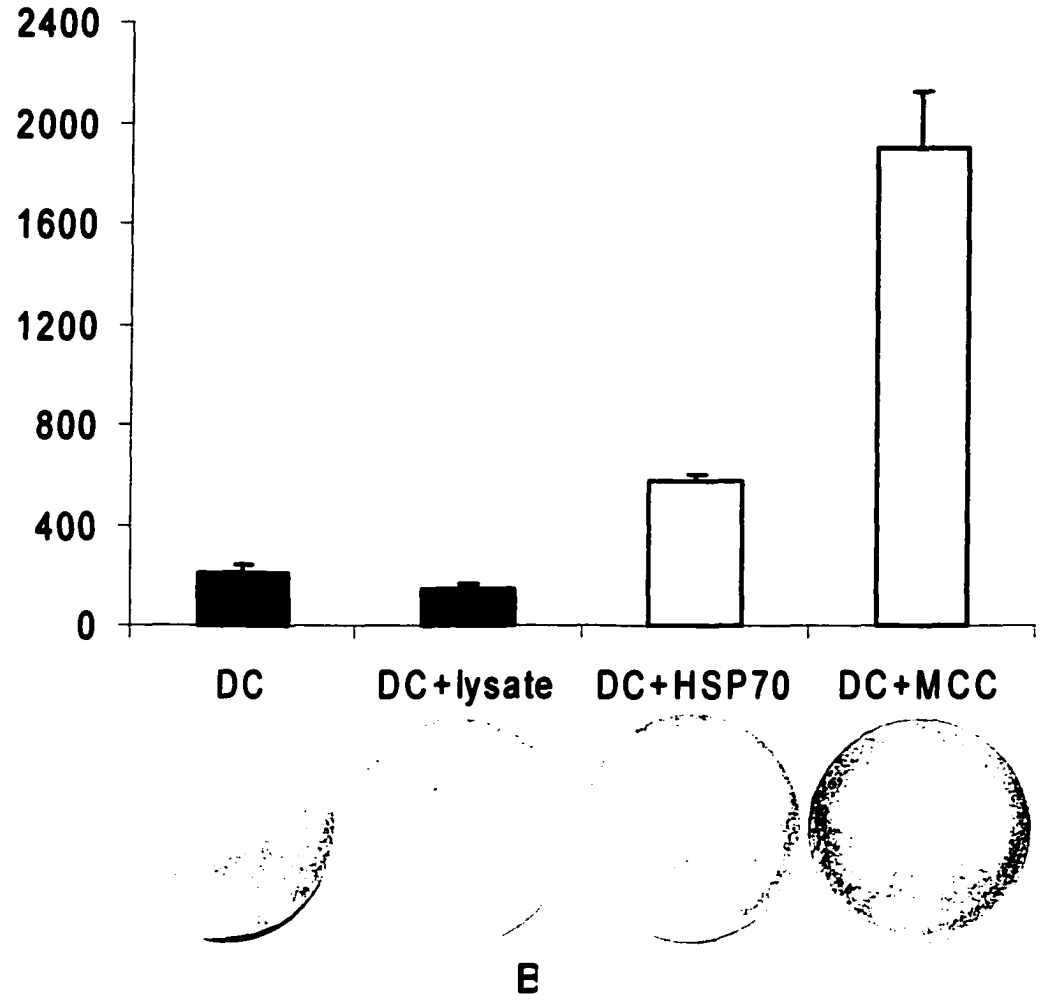

Figure AII-4.. (B) DCs pulsed with MCC have increased IL-12 production. ELISPOT assays were performed to measure IL-12 production by DCs. 3×10^5 day 6 DCs were cultured with 50 μg/ml 12B1-derived lysate, HSP70 or MCC in the presence of 10 ng/ml GM-CSF and IL-4 for 24 hours. (DC+MCC or DC+HSP70 *versus* DC or DC+lysate: $p< 0.05$; DC+MCC *versus* DC or DC+HSP70: $p< 0.05$, representative data from three experiments are shown).

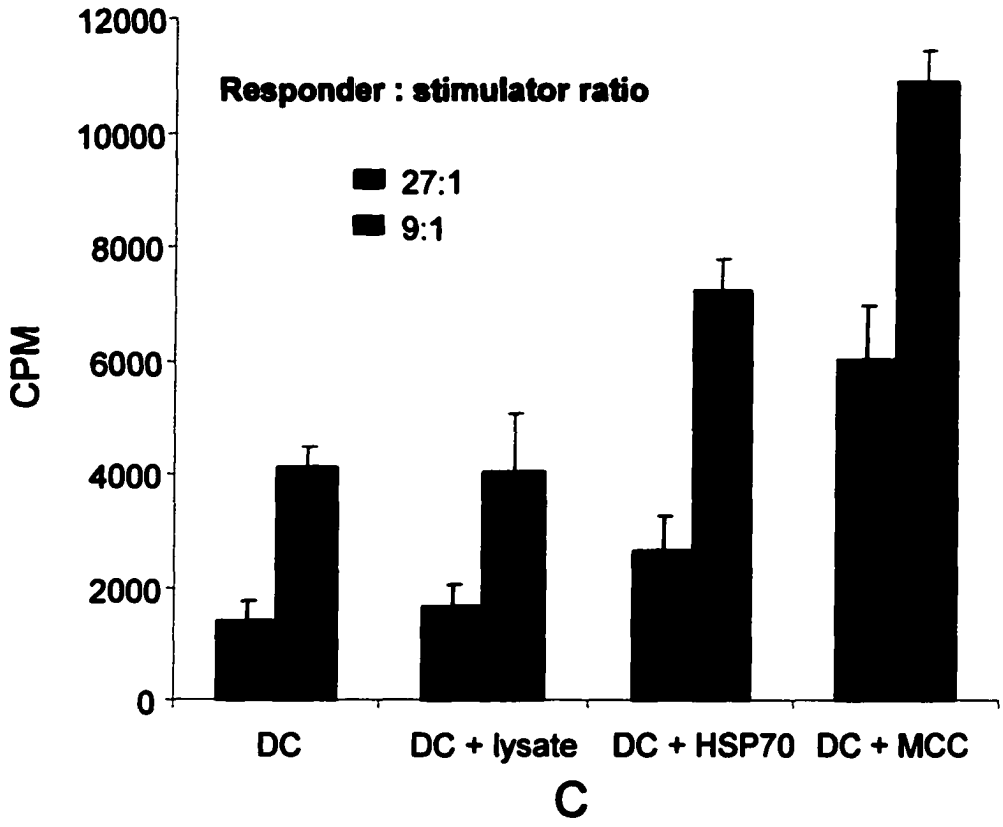

Figure AII-4. (A) 12B1 derived chaperone proteins stimulate DCs to express MHC class II and co-stimulatory molecules. Bone marrow-derived DCs were exposed to 50 μg/ml 12B1 HSP70, FS-IEF complexes, or unfractionated lysates, for 24 hours, harvested and analyzed by flow cytometry for expression of the cell surface markers indicated. The numbers in the ordinate represent the percentage of positive cells in the CD11c+ gated population. **(B) DCs pulsed with MCC have increased IL-12 production.** ELISPOT assays were performed to measure the IL-12 secretion by DCs. 3×10^5 day 6 DCs were cultured with 50 μg/ml 12B1-derived lysate, HSP70 or MCC in the presence of 10 ng/ml GM-CSF and IL-4 for 24 hours. Representative data from 3 experiments is shown. **(C) 12B1 derived MCC increase DCs capacity to stimulate allogeneic splenocyte proliferation.** DCs were cultured as in materials and methods, harvested, treated with Mitomycin C and washed. 10^5 splenocytes from C57BL6 mice were added per well and cultured with indicated ratio of pretreated BALB/c DCs for 4 days. [^{3}H]- thymidine was added and the cells cultured for additional 18 hours before the incorporated radioactivity was counted.

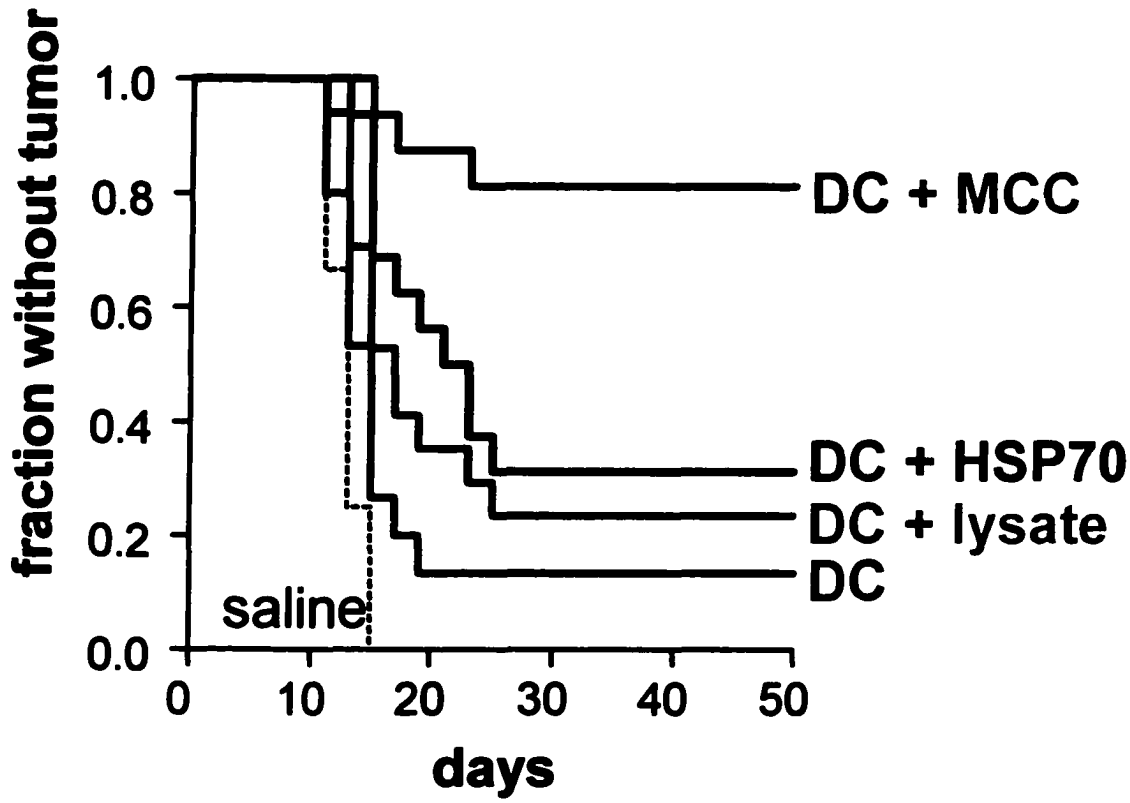

Figure AII-5. Immunization with DCs pulsed with 12B1 derived MCC provides significant protection to 12B1 tumor challenge. DCs were incubated with 50 μg/ml of 12B1 derived MCC or the same concentration of purified HSP70 or unfractionated lysate protein for 24 hours. DCs (5×10^5) were injected s.c. into the groin on days –14 and –7 and the mice were challenged with 12B1 cells (10^3) in the opposite groin on day 0. (saline *versus* DC ns; DC *versus* DC+lysate ns; DC *versus* DC+HSP70 $p < 0.05$; DC *versus* DC+MCC $p < 0.0001$; DC+HSP70 *versus* DC+MCC $p < 0.005$; pooled data from two experiments, n=12 to 16 mice per group).

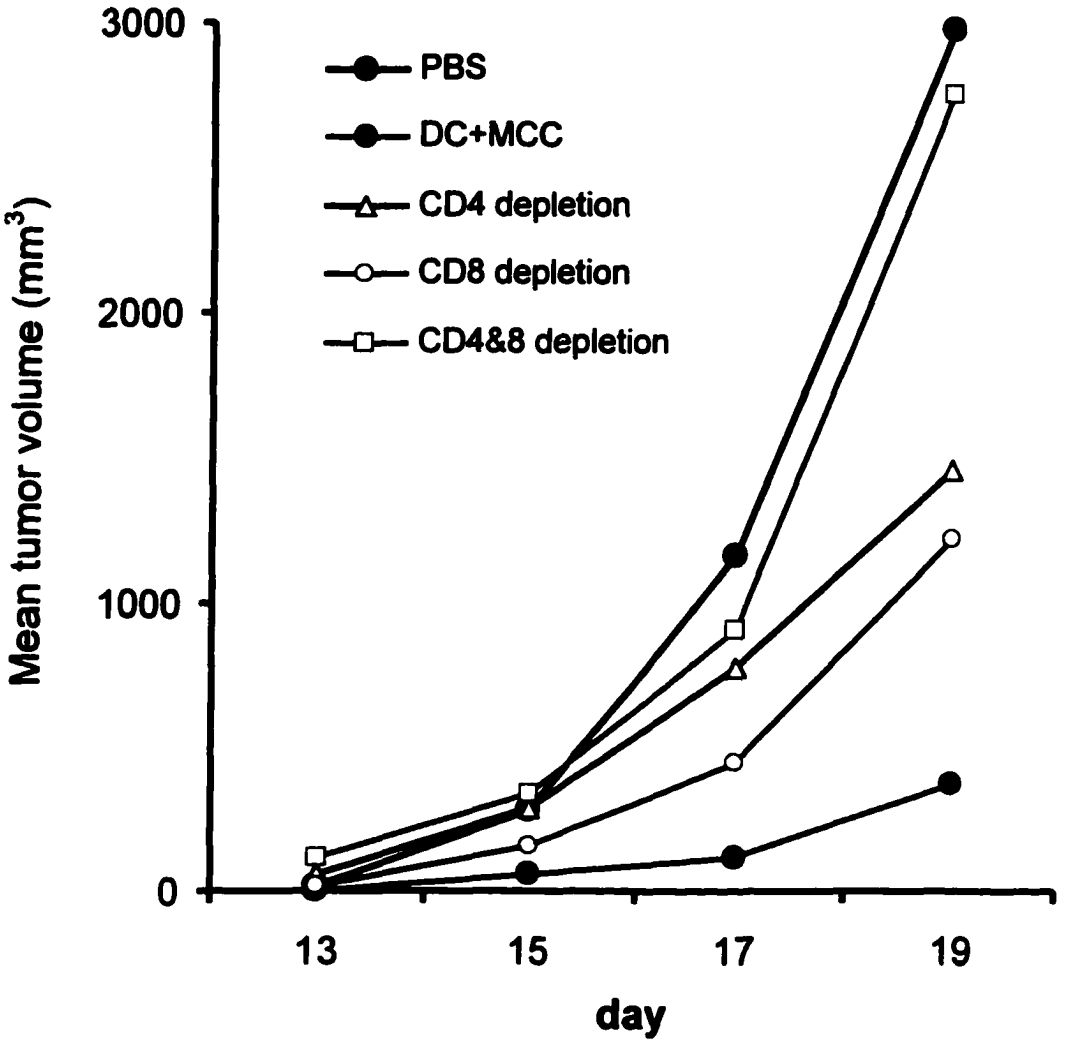

Figure AII-6. Anti-tumor immunity induced by DCs pulsed with 12B1 derived MCC is both CD4+ and CD8+ T-cell-dependent. On days –14 and –7, mice were immunized s.c. with DCs (5×10^5) that had been pre-incubated with 50 μg/ml of 12B1 derived MCC for 24 hours. Groups of mice were injected i.p with 200μg of anti-CD4 and/or anti-CD8 antibodies on days –3, -1, +1, and +3. On day 0, mice were challenged with 12B1 cells (10^3) in the opposite groin (saline *versus* CD4 and CD8 depletion ns; MCC versus CD4 and CD8 depletion: $p< 0.05$; n=4 mice per group, representative data from one of two experiments)

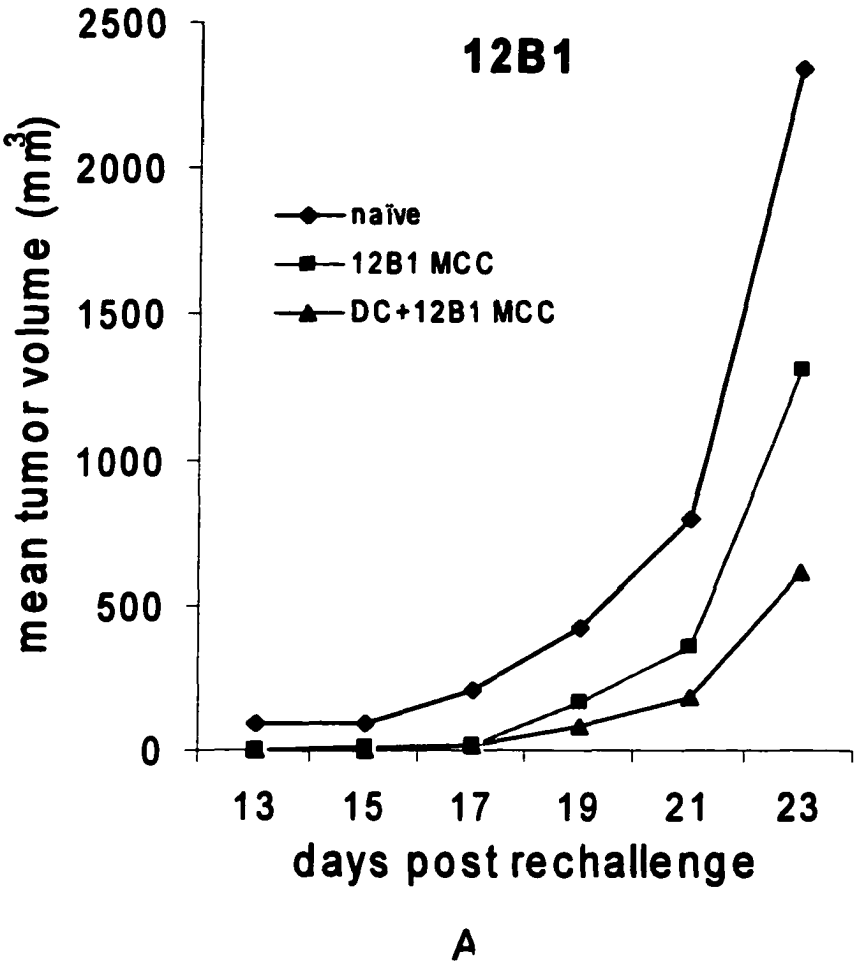

Figure AII-7. DCs pulsed with 12B1 derived MCC induce a long term tumor-specific immune response. **(A)** 11 or 18 weeks after initial challenge, naïve mice (n=3) or surviving mice (n=4 for MCC, 11 for DC pulsed with MCC) were rechallenged with 10^3 live 12B1 cells.

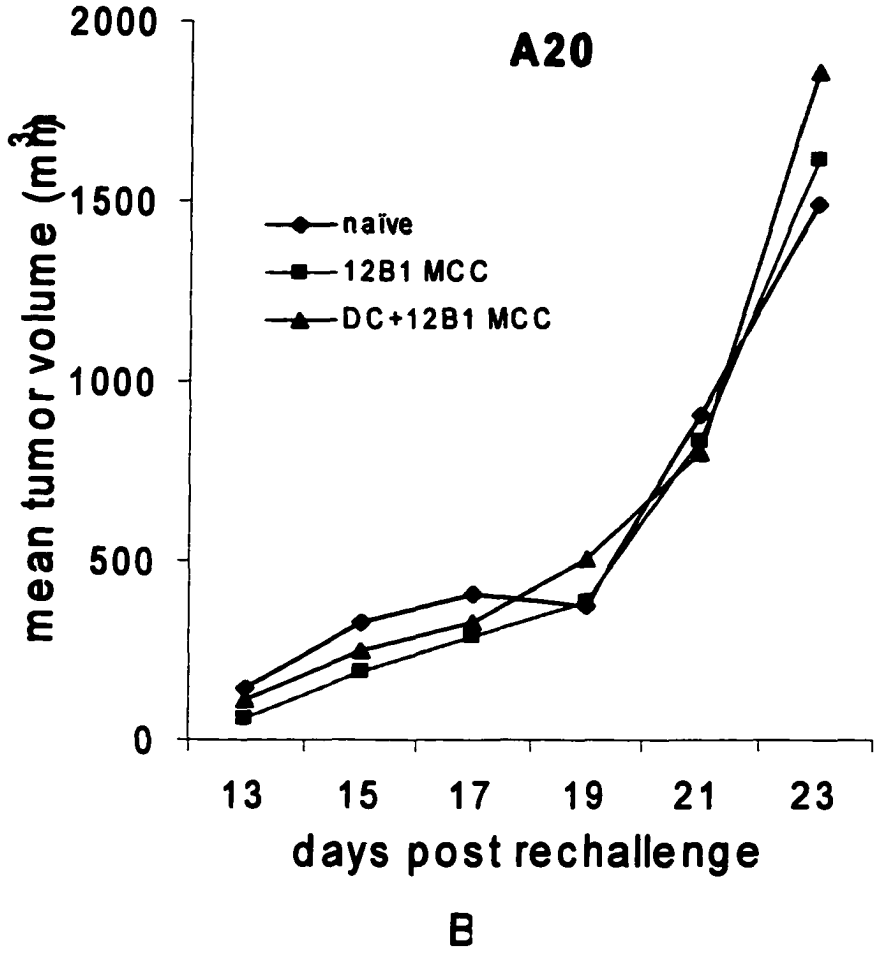

Figure AII-7. DCs pulsed with 12B1 derived MCC induce a long term tumor-specific immune response. (B) The same mice were challenged with 10^6 A20 cells in the right groin.

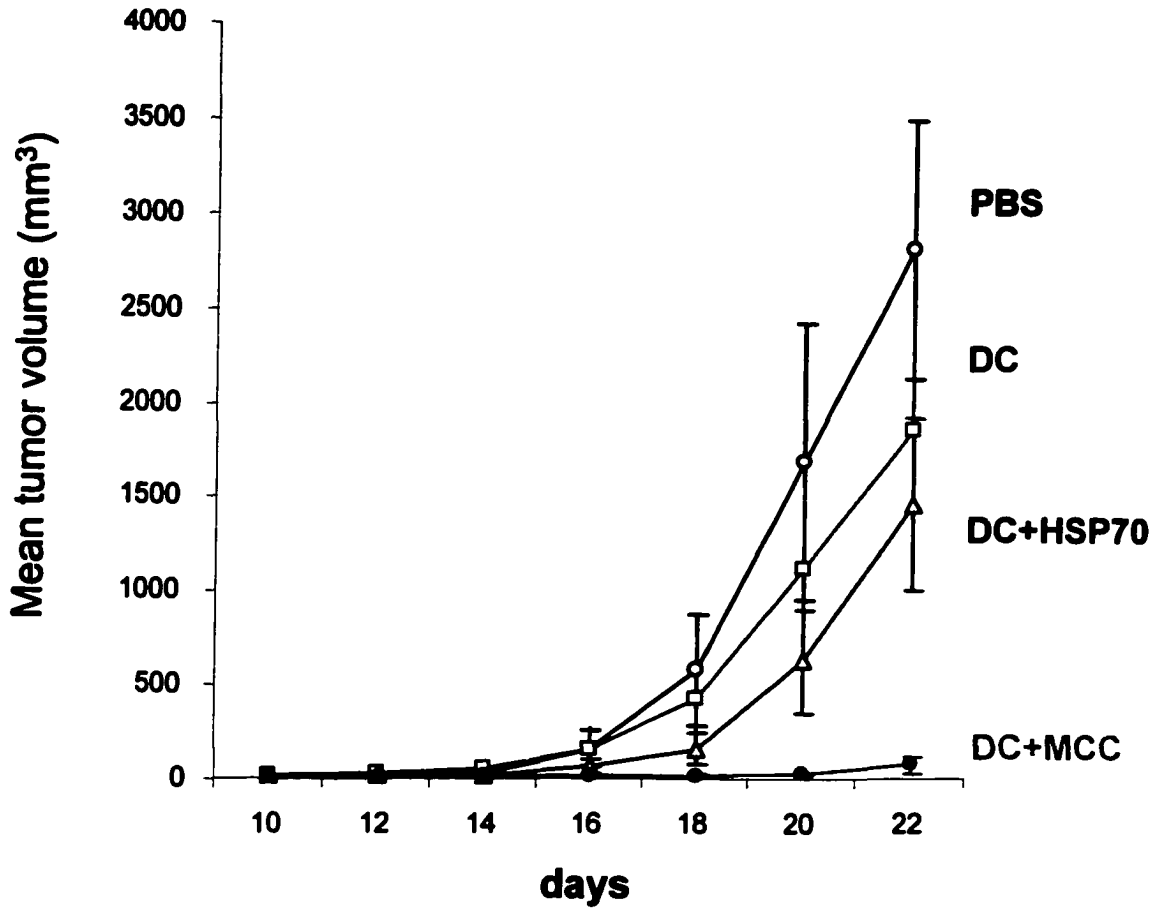

Figure AII-8. DCs pulsed with 12B1 derived MCC are effective therapeutic vaccines in tumor bearing mice. DCs were incubated with 50 μg/ml of 12B1 derived MCC or the same concentration of purified HSP70 for 24 hours. Mice were injected with 12B1 cells (10^3) s.c. in the right groin on day 0 and injected with MCC or HSP70 loaded DCs (5×10^5) in the opposite groin on day 2 (DC *versus* DC+HSP70 ns; DC *versus* DC+MCC $p < 0.05$; DC+HSP70 *versus* DC+MCC $p < 0.005$; pooled data from two experiments, n=8-16 mice per group)

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