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雞熱休克蛋白 70 之 DNA 異種疫苗可抑制犬腫瘤生長

Chicken HSP70 DNA xenogeneic vaccine inhibits tumor growth in a canine cancer

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本論文係游文瑛君（學號 R96629020）在國立臺灣大學獸醫學系、所完成之碩士學位論文，於民國 99 年 4 月 28 日承下列考試委員審查通過及口試及格，特此證明

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文瑛

中文摘要

接種異種動物之去氧核醣核酸 (xenogeneic DNA) 被認為是一種有效治療腫瘤的方式，此種疫苗可使免疫系統針對表現於腫瘤之自我抗原，產生特異性抗體及毒殺型 T 細胞，克服腫瘤免疫耐受性。熱休克蛋白 70 (HSP70) 表現於許多腫瘤，且被認為與腫瘤生長有密切的關係。本研究乃接種雞 (chicken HSP70, *chHSP70*) DNA 疫苗至具傳染性花柳性腫瘤犬隻，作為異種動物 DNA 以引發對抗自我抗原的免疫反應。實驗共分三組：第一組 (G1) 於接種腫瘤前施打此 DNA 疫苗，用以評估預防效果。第二組 (G2) 於接種腫瘤後施打疫苗，用以評估此 DNA 疫苗之治療效果。第三組 (G3)的免疫計劃與第一組相同，僅於第三劑給予方式由肌肉電衝改為經皮注射。另有 4 隻未經治療的腫瘤接種犬 (No treatment, NT) 做為控制組。除此之外，每組疫苗組皆另有一隻腫瘤接種犬隻注射空白載體做為質體控制組。結果顯示，本疫苗於 G1 有明顯抑制腫瘤生長的效果 (第 9 周)，其腫瘤也比第 G2 (第 18 周)、G3 (第 12 周)和 NT 組 (第 14 周) 較早消退。在腫瘤消退期，G1 之 $CD4^+$腫瘤浸潤淋巴球也顯著的高於 G2 及 NT (56.77% vs. 23.56% and 22.73%)。週邊血液單核細胞對腫瘤細胞的毒殺能力在三組疫苗組中皆顯著性上升。ELISpot 試驗顯示，於腫瘤消退期，G1 之犬 HSP70 特異性 IFN-γ 分泌淋巴球亦顯著高於 G2 及 NT (90.68 cells vs. 19.82 and 20.67 cells)。然而，在三組疫苗組中，自然殺手細胞的毒殺能力皆無顯著差異。

綜合以上結果顯示，疫苗引發免疫系統對抗腫瘤的能力會依免疫計劃及疫苗給予方式而有不同。在犬傳染性花柳性腫瘤模式中，預防性的給予異種 *chHSP70* 去氧核醣核酸疫苗， 產生抗犬 HSP70 之免疫反應，可成功克服腫瘤免疫耐受性，抑制腫瘤的生長。然而需更進一步的實驗證明異種 *chHSP70* 可應用於其他腫瘤的治療。

關鍵詞： 異種去氧核醣核酸疫苗、熱休克蛋白 70、犬傳染性花柳性腫瘤、經皮注射

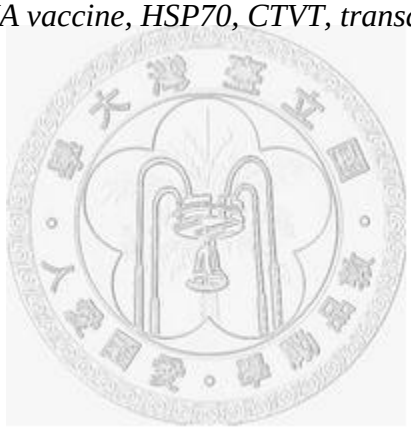
Abstract

Immunization with xenogeneic DNA is a promising cancer treatment, as it generates autoantibodies and cytotoxic T cells to break the tumor tolerance against self-antigens. Heat shock protein 70 (HSP70) is overexpressed in many kinds of tumors and is believed to be heavily involved in tumor progression. This study employed a xenogeneic chicken HSP70 (*chHSP70*) DNA vaccine in a canine transmissible venereal tumor (CTVT) model in beagles to break the tumor tolerance by inducing immune responses towards canine HSP70 self-antigens. In this study, three vaccination groups were created: the first (G1) was designed to evaluate the prophylactic efficiency of the *chHSP70* DNA vaccine by delivering the vaccine prior to tumor inoculation; the second (G2) was designed to evaluate the therapeutic efficacy in developed tumors by vaccinating the dogs after tumor inoculation; and the third (G3) consisted of the same vaccination schedule as that of G1, with the exception that the intramuscular injection/electroporation method used to administer the third vaccination in G1 was replaced with a transdermal injection. Four CTVT-bearing dogs that received no treatment (NT) served as controls, and one dog in each vaccination group immunized with empty vector served as a vector control. Tumor growth was notably inhibited only in the G1 dogs, in which the vaccination program triggered tumor regression much sooner (beginning in week 9) than in the G2 (week 18), G3 (week 12) and NT (week 14) dogs. The $CD4^+$ subpopulation of tumor-infiltrating lymphocytes was significantly increased during tumor regression in the G1 dogs as compared with the G2 and NT dogs (56.77% *vs*. 23.56% and 22.73%, respectively) and was similar to that of G3. The tumor-specific cytotoxicity of peripheral blood mononuclear cells (PBMCs) in all dogs in the three vaccination groups was dramatically enhanced, and ELISpot assay indicated that canine HSP70-specific IFN-γ-secreting

lymphocytes were much more abundant in G1 animals than in G2 or G3 during tumor regression (90.68 cells *vs*. 19.82 and 20.67 cells, respectively). However, there was no significant difference in NK cytotoxicity between the experimental groups.

In summary, the antitumor activity varied according to the vaccination strategy. Prophylactic administration of the xenogeneic *chHSP70* DNA vaccine following delivery of a boost via electroporation successively broke the tumor tolerance to canine HSP70 and effectively inhibited tumor growth. Xenogeneic *chHSP70* can thus be considered a potential tumor vaccine, and further research regarding this application is warranted.

Key words: Xenogeneic DNA vaccine, HSP70, CTVT, transdermal injection



Abbreviation

TCR	T cell receptor
Treg	Regulatory T cell
IL	Interleukin
TGF-β	Transforming growth factor beta
CTL	cytotoxicity T lymphocyte
MHC	Major Histocompatibility Complex
RNAi	RNA interference
SiRNA	Small interfering RNA
APC	Antigen presenting cell
HSP	Heat shock protein
VV	Vaccinia virus
LC	Langerhans cell
DC	Dendritic cell
TD NF	Transdermal needle-free
IFN- γ	Interferon-gamma
TIL	Tumor infiltrating lymphocyte
PEI	polyethyleneimine
TLR	Toll-like receptor
IDO	Indoleamine 2,3-dioxygenase
CMM	Canine malignant melanoma
KP MST	Kaplan-Meier median survival time

Contents

Chapter 1. Background and Literatures review

1.1 The immunological tolerance

The immunological tolerance is defined as “unresponsiveness” to an antigen that is induced by previous exposure to the self antigens during the immature lymphocytes development at thymus or bone marrow. Tolerance to self antigens, also called self tolerance, is a fundamental property of the normal immune system. Notably, the immunological tolerance is essential for discrimination between self and non-self [1, 2].

The self tolerance is acquired and maintained by a combination of central tolerance and peripheral tolerance [1, 2]. Central tolerance occurs at the primary sites of lymphocytes development, the thymus for developing T cells and the bone marrow for developing B cells [1, 3]. The affinity of TCRs on progenitors to self-peptide-MHC complex is crucial parameter that drives the fate of developemental outcome. Progenitors with “high” affinity to self-peptide-MHC complex are eliminated by negative selection. The majority process of negative selection is clonal deletion. Recent studies indicate that B cells with high affinity to self-peptide-MHC complex can edit their antigen receptor, and change antigen specificity in the bone marrow and known as receptor editing. This receptor editing generated less self-reactive lymphocytes that might be selected to the periphrery [4, 5]. All the three processes such as clonal deletion, receptor editing and anergy are thought to be negative selection. In contrast, progenitors-bearing TCRs with “low” affinity to self-peptide-MHC complex expressed on epithelial cells of the thymic cortex are survived from programmed apoptosis and then are positively selected into the periphery [1, 3]. These “weakly” self-reactive progenitors then mature and populate the lymphoid organs to participate in immune responses toward foreign antigens [1].

Meanwhile, the $CD4^+$ lymphocytes with low affinity to self-peptide-MHC complex differentiate into regulatory T cells (Tregs) that are entered the periphery and play an important role in mataining the peripheral tolerance. The overall consequence of these processes of central tolerance are the emergence in the periphery of a T cell repertoire that weakly recognize self MHC/self peptide complex but has strong reactivity for recognizing self MHC/foreign peptide complex [3].

Although most self reactive lymphocytes are elimated during central tolerance, they can not eliminate all of the self-reactive lymphocytes because not all self antigens are expressed at the lymphocyte developmental site [1, 6]. To control the activation of autoreactive lymphocytes in the periphery, a number of supressive mechanisms known as peripheral tolerance, such as deletion, anergy and suppressive regulation by Treg cells to prevent the autoimmunity [2, 6].

1.2 Tumor escaping mechanism

There are mechanisms that tumors may actively evade or suppress immune system as following: 1. Tumor cells escape cytotoxicity T lymphocyte (CTL) recognition by loss of expression of antigens or MHC molecules [7]. 2. Deletion of immune effector cells by expression of the apoptosis-inducing Fas ligands [8-10]. 3. Direct tolerization of tumor-reactive lymphocytes by suppressive cytokine, such as interleukin-10 (IL-10) or transforming growth factor-beta (TGF-β) to inhibit the activities of antigen presenting cells [7, 11]. 4. Suppression of tumor-reactive T cells by Treg cells in the tumor site [3, 12]. 5. Tolerization of host T cells by tumor-derived antigens [3]. However, the major reason that limiting immune recognition of tumor cells is that tumors arise from the host own tissue and express self antigens to which the individual's T cells have been tolerized [3]. Interestingly, numbers of researches indicated that the immune system contains autoreactive lymphocytes that can cause destruction host tissue or rejection of tumors by

both cellular and humoral responses if activated them properly [13].

1.3 Tumor antigens

Based on the antigen expression pattern of tumors, the following two groups of tumor antigens can be classified: tumor-specific antigens (TSA) and tumor-associated antigens (TAA) [3, 14]. TSA are the antigens whose expression restricted only to the tumors but not expressed by normal cells. These TSA are resulting from the somatic mutation, internal deletions, chromosomal translocation, normal immunoglobulin (Ig) or TCR recombination events peculiar to leukemia B or T cell idotypes [14]. In contrast, tumor antigens that are not only expressed on tumors but also expressed on normal cells are TAA. These antigens are normal cell constituents whose expression are aberrant or disregulated in tumors, but are usually expressed at lower levels in normal tissues. The classifications of tumor antigens are showed in Table 1. Researches indicate that TAA overexpression of tumor cells can served as targeted for immune therapy [11]. In recent years, the strategies including genetic modified tumors, dendritic cells either pulsed or transduced with TAA, recombinant viruses encoding TAA, naked plasmid DNA encoding genes of TAA are used to induce specific immune response to reject tumors [15-22].

Table 1 Identified tumor antigens in human

Antigen type	Antigen	Tumoe type	Reference
Universal tumor antigen	telomerase, hTERT, mdm-2	all	[23], [24], [25], [26]
Mutated gene products	CDK-4	renal	[27]
	β-catenin	colon	[28]
	p53	Esophagus, bladder	[29], [30], [31]
	k-ras	colon, pancreas, gastric, breast	[32], [33], [34], [35]
	bcr-abl	leukemia	[36]
Overexpressed self antigen	Her2/neu	breast, lung, overian	[37], [38], [39], [40]
	proteinase-3	leukemia	[41]
	MUC-1	pancreas, grastic ,breast, lung,colorectal, lymphoma, bladder	[42], [43] , [44], [45], [46], [47], [48]
	WT-1	leukemia, ovarian	[49], [50]
	MART-1/melan-A	melanoma	[51]
	Mesothelin	pancreas, ovarian, mesothelioma	[52], [53], [54]
Tissue-specific differentiation antigen	PSA	prostate, breast	[55], [56]
	Tyrosinase, MART-1/melan-A, gp100, TRP-1,TRP-2	Melanoma	[57], [58], [59], [60]
Cancer testis antigen	MAGE,BAGE,RAGE, GAGE, NY-ESO-1	melanoma, renal, ovarian ,breast, prostate, testicular, bladder ,gastric, esophageal, hepatocellular, lung, head and neck	[57], [61], [62], [63], [64], [65], [66], [67], [68], [69], [70], [71], [72]
Idiotype	Ig, TCR	Lymphocyte	[73]

1.4 Heat shock protein (HSP)

Heat shock proteins (HSPs) were first discovered in 1962 and whose expressions can be inducted in kinds of stresses [74-77]. It is highly conserved protein in both prokaryotes and eukaryotes [74, 76]. HSP can be devide into different families according to their molecular weight [78]. The mammalian HSPs are classified into five families: HSP100, HSP90, HSP70, HSP60 and the small HSPs [74]. HSP90 is constitutively abundant expression in the normal cells. HSP70 and HSP27 are induced via different stresses such as heat, oxidative stress, ethanol, oxidative reagents, chemotherapy, heavy metals, inflammation and anoxia [74, 77]. In normal cells, HSP70 and HSP27 are not expressed or at the very low level. In contrast, the high expression of HSP70 and HSP27 in the cells or tissues are observed in kinds of tumors [74, 78, 79]. HSPs have dual functions depending on their location. Intracellular HSPs have a protective function. They allow cells to survive from lethal damage. The release of HSPs from cells to the blood stream is found in the pathological conditions. These extracellular HSPs mediate immunological functions by both innate and adaptive immunity [74, 76, 79, 80]. The previous experiments demonstrate that B16 melanoma cells were transfected with a heterologous HSP65 dramatically increases the levels of MHC class I molecules on their surface and were efficively lysed by alloreactive T lymphocytes [81, 82].

Intracellular HSP70 Intracellular form of HSP70 has a protective function to cells. They allow the cells to survival from lethal death via the anti-apoptosis mechanism [74, 79]. HSP70 can act at multiple points as following in the apoptotic pathways: 1. at a premitochondrial stage by inhibiting the stress-inducing signals. 2. at the mitochondrial stage by preventing mitochondrial membrane permeabilization through the blockage the Bax translocation and then inhibit the mitochondrial release of proapoptotic protein. 3. at postmitochondrial stage by interacting with apoptosis-inducing factor (AIF) or apoptosis

protease-activating factor-1 (Apaf-1) to inhibit the AIF-inducing chromatin condensation and prevent the caspase activation [74]. Therefore, HSP70 play an important role in cell or tumor viability. Intracellular HSP70 also act as molecular chaperones supporting folding, misfolding and transport protins or polypeptides to membrance under the stress or in normal conditions. Peptides of 8 to 26 amino acids are found preferentially interact with the HSP70-peptide binding pockets. And peptides of this lengh are particularly suited as precursors of MHC presentation after subsequent processing [83].

Extracellular or membrane bound HSP70 The release of HSP70 from cells is triggered by physical trauma and stresses. The release of HSP70 occurs both through physiological secretion mechanisms and during necrosis of the cells. After release of HSP70 into the excellular fluid, HSP70 binds to the surfaces of the adjacent cells. Then the signal transduction cascades and the antigenic peptides transporation are initiated. Besides, HSP70 are also able to enter the bloodstream and act at distant sites through the bodies [76]. Research indicates there is no leading sequence in HSP70 [79, 80]. As a chaperone, the mechanism of anchorage and export HSP70 remains to be elucidated. Because the experiment showed that high salt and pH changes did not affect the membrane expression of HSP70, the anchorage of HSP70 in the plasma membrane is though to be associated with lipid protein such as exosome rather than a receptor protein interaction [79].

Both extracellular or membrane bound HSP70 mediate immunological functions. They can elicit immune responses by the adaptive and innate immunity [74]. The chaperone function of HSP70 generates HSP70-peptide complex on the tumors were taken up by APCs via the receptor-mediated endocytosis and cross-presented as classical antigens for $CD8^+$ cytotoxic T cells on the MHC class I molecules. Several HSP70 receptors including CD40, CD91 and Toll-like receptor family (TLR-2 and TLR-4) alone

or combination with CD14, the LPS receptor are thought to be mediate binding and uptake of HSP70 peptide complex [84-87]. Recently, human APC have been found to interact with HSP70 via scavenger receptor LOX-1 and SRA-1 [88, 89]. HSP70-peptide complex can enhance maturation of DCs and release proinflammatory cytokines such as TNF-α, IL-1β, IL-12, IL-6 and GM-CSF. These cytokines cause nonspecific resposes of the innate immunity [74-76, 80]. Even in the absence of immunogeneic peptide, the soluble HSP70 or transmembrane HSP70 provide immunostimulatory activity as "danger signals" to NK cells [80, 90]. The 14-mer human HSP70 peptide TKDNNLLGRFELSG($aa^{450-463}$) termed "TKD" provides the minimal sequence with the capacity binding to NK cells and enhance NK cells cytotoxicity against HSP70 membrane-positive tumor cells through granzyme B mediated apoptosis[90-93]. HSP70 also promotes mouse NK cells cytotoxicity against tumors that express inducible NKG2D ligands [94].

Tumor-associated HSP70 Up to date, unmutated HSP70 are found that overexpressing in kinds of tumors, such as breast, endometrial, prostate cancer, gliomas and so on [95-98]. HSP70 have been found on the membrane on the malignant cells [99]. Studies indicate HSP70 facilitates tumorigenicity of cells, including the ability to form tumors in nude mice, formation of foci and the tumor formation in mice xenografts [100, 101]. In addition, overexpression of recombinant HSP70 of human breast cell line MCF-7 led to a strong acceleration of cell growth by shortening the G0/G1 phase [102]. Depletion of HSP70 in tumor cells cause apoptosis-like death in various cell lines such as HSC-2, MCF-7, Molf-4, PC-3 snd human oral carcinoma cells [103-106]. In contrast to the untransformed cells, such as primary firbroblasts and tumor cells isolated from human primary oral carcinoma did not lost viability after the HSP70 depletion. Importantly, senescence of tumor cells have been found by depleting the HSP70 results in strong

activation of tumor suppressor genes p53 and cyclin dependent kinase inhibitor p21 [107, 108]. These indicate HSP70 plays an important role in tumor viability and proliferation.

Because of the immunostimulatory functions of HSP70, mycobacterial HSP70 are usually used as adjuvant to kinds of vaccines [109, 110]. The concepts of tumor-derived HSP70 can elicit tumor-specific responses are first found in mouse models [111, 112]. In recent years, tumor-derived HSP70-protein or HSP70-peptide vaccines serve as multivalent or individual vaccines for caner therapy. The HSP70-based DNA vaccines which are fused with DNA of HSP70 and tumor proteins against syngeneic tumors are under investigated for a long times [112-115]. Although HSP70 genes form tumors and normal tissues reveal no differences in the amino acide sequences. Interestingly, HSP70 derived form normal tissue do not elicit immune responses against tumors [112]. The novelty in this study is that we use chicken HSP70 DNA "alone" as vaccines to immunize canine. There is 83.5% high degree of homology between chicken and canine HSP70. The small differences in amino acid sequence are supposed to break tumor tolerance and elicit the cross-react immune responses to canine HSP70 proteins.

1.5 The strategies to break tumor tolerance

Despite the mechanisms by which tumor can evade the immune system, numbers of strategies to enhance antitumor immunity to break tumor tolerance are under investigated as following:

1.5.1 Tumor antigens overexpression

The overexpression of proteins in tumor cells may increase the self peptides presented by MHC molecules, therefore overcoming the tumor tolerance and activating of T lymphocytes [11]. When mice immunized with PSA DNA plasmid, the strong humoral and cellular immunity were induced and the immune responses were found to be skewed toward Th1 by high levels of IFN-γ and IL-2 production. The inducing immune

response was sufficient to protect mice from challenge with PSA-expressing tumor cells. In addition, plasmid PSA vaccination inducing PSA-specific antibody titers in cynomolus monkeys which expressing a closely related genes with the humans [116]. The results indicate the TAA plasmid DNA vaccination can break tolerance.

1.5.2 Difference in HLA molecules needed to present shared antigens

The graft-versus-leukemia (GVL) effect is important for eradication of the malignant cells. In leukemia patients, administration of alloreactive T cells with hematopoietic stem cell transplants cause eradication of the tumors [117, 118]. It shows that the immunomodulation is possible in acute myeloid leukemia [119]. To berak self-tolerance, allorestricted CTL against self-antigens such as CD19, CD20, CD33 and CD45 were generated [120, 121]. The strategy with the disparity in HLA molecules to present the shared antigens rather than relying on a difference in antigen expression between host and donor.

1.5.3 Inhibition of immunosuppressive mechanisms

The tumor-derived suppressive cytokines such as IL-10 and TGF-β were neutralized with antibodies for caner therapy [122, 123]. Treg cells, which play an essential role in mantaining peripheral tolerance and induce apoptosis of APCs or inhibit their activation and function by TGF-β, IL-10, perforin and granzyme [3, 11, 124]. Therefore, depleteion of Treg cells as a strategy to augment the potential of immunotherapy and have been found to cause tumor regression [125-128].

1.5.4. Co-encapsulation of dual siRNAs and tumour antigens

Indoleamine 2, 3-dioxygenase (IDO), an enzyme that degrades tryptophan, is a negative immune regulatory molecule to DCs. IDO-expressing DCs suppress T cell responses and are in the immunosuppressive condition. Tryptopan catabolism also affects naïve T cells proliferation and memory $CD8^+$ T cells generation [129]. IDO also plays an

important role in immune escape in cancer development [130-132]. Therefore, silencing the IDO expression in DCs served as a new method to elicit antitumor immunity [11, 133]. RNA interference **(**RNAi) was used as a cellular gene silencing mechanism that can be applied to the development of new drugs [134]. Small interfering RNA (siRNA), the main effector of RNAi, is thought to be one of the promising anticancer tools [135]. Besides, siRNA can be used alone or combined with other immunotherapies [136]. Knockdown the expression of the pathological proteins such as IDO through the RNAi is applicable to all classes of molecular targets. The resent research indicated administration of IDO siRNA to skin can inhibit tumor growth and increase the survival time in murine models. Besides, the antitumor effect is abolished by depletion of $CD8^+$ T cells [133]. With the same concept, genes of TAAs may serve as ideal targets with for cancer therapies.

1.5.5 Xenogeneic antigens

Nowadays, almost all of identified tumor antigens are also expressed in normal cells and are not altered self antigens [137-139]. Thus it is essential to break tolerance for cancer therapy. Using the "altered self" form of self antigens to overcome tumor tolerance arise from the studies of SK-MEL19, a $gp75^+$ human melanoma cell line [140]. When mice were immunized with "human" melanoma lysates, autoantibodies that recognized "mouse" gp75 were produced. In contrast, immunization with murine B16 melanoma produced no antibody response, even when potent adjuvants were included.

The concept of xenogeneic DNA vaccine is the minor differences in epitopes of homologous xenogeneic protein between species improving the recognition of the MHC class I or II restricated peptide on transfected cells to TCR and then generating the cross-reactive immune responses agsinst self proteins on tumors [137, 141-147]. However, the precise mechanisms that overcome immune tolerance are not completely

understood [148]. The xenogeneic DNA vaccines are extensively investigated in preclinical models, especially in melanoma. The B57BL/6J mice are immunized five times with humn gp75 DNA plamid by gene gun delivery. Almost all mice immunized with human gp75 develop autoantibody to syngeneic gp75 (69 of 71 mice). No autoantibodies are detected in mice immunized with mice gp75 (0 of 39 mice). The autoantibodies aginst mice gp75 are weakly after the third vaccinations but peaked after the fourth and fifth vaccinations. Autoimmunity was also characterized by coat depigmentation. In addition, depletion of $CD4^+$ or $NK1.1^+$ cells but not $CD8^+$ cells can partically reversed the rejection of the lung metastases in immunized mice. This indicates $CD4^+$ or $NK1.1^+$ cells played critical roles in antitumor activity in melanoma model [147]. In other studies, mice are immunized with xenegeneic human gp100 DNA showed CD4 independent T cell antitumor immunity [146].

1.6 The delivery system for DNA vaccines

Gene therapy was transferring genetic material into the cells to prevent or treat diseases. The introduction of exogeneous DNA into cells or tissue can be carried out through various viral vectors and non-viral approaches.

1.6.1 Viral vector

A large of different recombinant viral vectors system had been evaluated in preclinical models. Administeration of recombinant viruses not only induces immune responses against proteins of transgene itself but also the viral vector proteins. Besides, pre-existing T cells and antibody-mediated immunity to the viral vector can negative influence in the administration of recombinant viruses or to boost [149]. Thus, pre-existing vector immunity must to be avoided in using viral vector that humans have not been exposed to the viruses before. Repeat immunization with the same virus vectors can also lead to the induction of vector immunity. Because of the previously mentioned

problems, only a small proportion of DNA vaccines via virus vectors have been evaluated in clinical studies [150-152].

1.6.2 Non-viral vector

Although the virus vectors have been extensively explored to vaccines delivery, they have many side effects such as host immune rejection, viral toxicity, high risk of insertional mutagenesis and so on [153-155]. Studies showed the adenoviral capsid proteins stimulated the innate immunity led to the inflammation and substantial loss of vector DNA in the first 24 hours [156]. For these reasons, non-viral vectors gene delivery has been recently become an attractive alternative method. To develop a successful protein therapy, effective DNA delivery is required to induce high and substantial level of protein production and long-lasting immune responses. The non-viral vectors are divided into two categories. One is the chemical methods. Cationic lipids and polymers such as liposomes have been extensively ecplored as a vehicle for gene therapy. Liposomes have been used to protect DNA from degradation, enhance the entrance of the plasma membranes and improve the uptake by APCs. Cationic polymeric materials such as polylysine and polyethyleneimine (PEI) have been shown to compact the DNA and form nanoparticulate complex, which can very in large and size thus they are suitable for both systemic and mucosal delivery. PEI also has enhanced ability to condense DNA to small particles and enter the cells by the adsorptive endocytosis [157]. The others are mechanical methods such as jet injection, electroporation, gene guns. The method of gene gun delivery involves coating micrometer sized colloidal gold particles with plasmid and propelled the beads into the epidermis or the dermis in the skin using the compressed helium. Less of the DNA is required for immunization with gene gun. There have been a number of reports of gene gun delivery in non-rodent system including cats, chickens, pigs, sheep, horse, cattle, dogs, non-human primates and humans [158-164].

Electroporation and jet injection are chosen to use in this study. The detail mechanisms are described as following.

1.6.2.1 Electroporation

Electroporation is a mean that cause cellular membrane are transiently destabilized by localized cotrolled electric fields, facilitating the entry of foreign molecules into cells [165-168]. The delivery of plasmid DNA with electroporation is dramatically elvated the transfection efficiency in muscle tissue rangeing from 10 to 1000-fold [169-171]. Electroporation has been used for nucleic acids transferation from 1980s [165, 166]. This technique has been applied to a wide variety of tissues, including muscle [172, 173], skin [174, 175], liver [176, 177], lung [178], artery [179], kidney [180], brain [181, 182] and tumors [183, 184]. The actural mechanism that nucleic acids transferation via electroporation has not been elucidated. However, the hypothesis that the permeability results from the formation of hydrophilic pores was proposed. During the electroporation, the cells act like a capacitor and may be a dielectric consisting of non-conducting material (the bilayer membrane) between two conductors (the extracellular fluid and cytoplasm of the cells). In the presence of an external applied electric field, a transmembeane potential could be created. Once the potential has been achieved the membrane discharges, the membrane could be porated and the extracellular materials moved into the cells. This critical potential is the minimum voltage that was required to aid the nucleic acids into the cells. This is so-called “reversible electrical breakdown” because the membrane pores will immedicately reseal in several milliseconds [165, 185]. After the formation of the membrane pores, there is tremendous influx of the extracellular DNA into the cells. Although the casue of the molecular movement is unclear [165]. There are two hypothesis related with the DNA movement. First, the electrical current acts on the nucleic acids by electrophoretic-like effect and the nucleic acids move across

the cell membranes [165, 186]. Second, the research demostrate that the short duration high voltage (HV) pulses promote the formation of membrane pores. On the contrary, the long duration low voltage (LV) pulses promote the movement of DNA into the cells. Further experiment demonstrate that only in the combination with HV and LV can achieve the highest transfection efficiency [187]. The successful electroporation required both the steps of " poration formation on transmembrane" and "electrophoresis effect for DNA movement into cells".

There sre several factors can increase the efficiency of electroporation. **1. The size, shape and morphology of the transfected cells :** these affect the voltage required to porate the membrane. Studies demonstrate the relationship between the transmembrane potential (ΔVm) and the cell redius as $\Delta Vm=\delta E_{ext}$ rcosφ, where r=cell redius, φ= the polar angle with the applied electrical field. The E_{ext} stands for the applied electric field. It indicate the larger of the size of the cells, the lower the field the strength that is required for portion [188]. **2. The viscosity of extracellular fluid:** the extracellular fluid affects the efficiency of electroporation. Studies demostarte that injection the hyaluronidase into the muscle before the elecrtoporation led to the dramatical elevation the muscle fiber transduction [189]. **3. The divalent cations:** If the divalent cations are present in the culture medium, the electroporation efficiency is increased, probably because they abolish the electrostatic repulsion which was existed between in the DNA and cell membrane [188]. **4. Adjuvant:** the adjuvant such as poly-L-glutamine increase the electroporation efficiency in muscle tissues [190, 191]. Besides, it also thought to prevent degradation of the plasmid by endonucleases for further increase the DNA into

the cells.

Skeletal muscle has the properties that make it as the most suitable for the gene therapy. They are easily accessible, well-vaucularized and have large mass throughout the bodies. These properties of skeletal muscle result in systemic delivery of proteins in animal models. Besides, in tissue where undergoes the more cell division results in the more loss of the plasmid DNA from the cells. The post-mitotic nature of muscle fibers prevent the plasmid DNA from rapid loss, therefore it can provide a long-term source of proteins production. The electroporation of skeletal muscle are applied to human clinical trials, for instance, delivery of vascular endotheial growth factor into the patients with severe limb ischemia [192]. The other clinical trials such as melanoma with cytokine genes are and prostate caner therapy with PSA genes are now onging [193, 194].

1.6.2.2 Needle-free injection

Jet injection is a needle-free delivery system method with a high-speed stream of fluid through the skin and delivers the drugs intradermally, subcutaneously or even intramuscularly without using of needles [195-197]. They have been used to deliver numbers of marcromolecules as well as the small molecules such as imsulin, anesthetics and DNA molecules [198].

The skin divides into two layers, the epidermis and dermis. The epidermis served as a barrier to prevent the external pathogens from entering the body. It is the first line of immune surveillance. The dermis is resbonsible for blood supply to the epidermis and immune surveillance. There are numerous resident APCs populated in the epidermis and dermis: the Langerhans cells (LCs) in the viable epidermis and the dermal dendrocytes (DCs) in the dermis [199, 200]. The protein expressions are observed from transfected cells such as keratinocytes, LCs and DCs. The majority of transfections are presumably through endocytosis or some other undefined mechanism [200]. Nowadays, needle-free

plasmid delivery had been preious demestrated in many species including mice [201], guinea pigs [202], rabbit [203], sheep and cattle [204], canine and humans [141].

Transdermal needle-free (TD NF) plasmid delivery transfers the plasmid DNA into both the dermis and muscle. The safety and efficiency of TD NF are demostrated in veterinary species. The advantages of TD NF are the overall procedure is easily accessible to the clinicians. Besides, the less DNA amounts are required because there are substancial DCs populated in the dermis [200]. Clinical studies indicate the responders with TD NF delivery have the better mean antibody reponses than the delivery with needles [205]. The superiority of TD NF plasmid delivery are under investigated that its potential to elicit more interferon-gamma (IFN-γ) than intramuscular or interdermal administration with xenogeneic DNA vaccine in beagles. However, the humoral response are failed to be detected [206]. Although the jet injection has less efficiency compared to gen gun and electroporation. The jet injection is thought to be a critical and potential technology for cancer therapy and chronic infection diseases no matter in companion animals or in humans [167, 205, 206].

1.7 Current progresses of xenogeneic DNA vaccine in treating cancers in canine and humans

Human vascular endothelial growth factor plasmid DNA was used to treat the canine soft tissue scrcoma. Autoantibies were found in two of the three patients receiving all the six times vaccination procedure. The decrease of tumor microvessel density and the phenomenon of IgG deposition in tumor site were also detected and no adverse effects such as coagulopathty [144].

In phase I trial of canine malignant melanoma (CMM) was conducted in 2003, three cohort of CMM dogs (WTO stage II, III and IV) were immunized with human tyrosinase four times biweekly by Biojetor 2000. No signs of autoimmunity were found. One dog

with stage IV disease had a complete clinical response in multiple lung metastases for 329 days. Two dogs with stage IV disease had a long-term survivals (421 and 588 days) in the face of metastatic disease, and two other dogs with locally control stage II/III disease had long term survivals (501 and 496 days) with no evidence of melanoma on necropsy. The Kaplan-Meier median survival time (KM MST) for all nine dogs was 389 days compared with CMM dogs without local tumor control had survival time ranging from 54 to 126 days [207].

CMM dogs with stage II to IV disease were immunized with human tyrosinase four times biweekly by Biojetor 2000 in 2006. The KM MST for all stage II-IV dogs treated with human tyrosinase, mouse tyrosinase and mouse gp75 were 389, 224 and 153 days compare with the standardized therapies 1 to 5 months. Autoantibodies were found 2 to 5 times increasion compared with the pre-immune sera in 3 out of 9 immunized dogs [145]. Another thirty-three CMM dogs fit the stage II to III disease with local reginally controlled CMM across the xenogeneic vaccine studies. The KM MST for these dogs was 569 days in 25 out of 33 dogs which were still alive [145]. Meanwhile, allogeneic whole-cell CMM tumor vaccine expressing human xenegeneic gp100 was developed to treat stage II to IV CMM dogs at the same year. Objective evidence of tumor regression (one complete response and five partial responses) was observed in six of the thirty-four dogs (17.6% objective response rate). The overall survival times for complete or partial regression dogs in this study were 417 days compared with the 153 days in all dogs. The PBMC cytotoxicity towards autologous tumor targets was found in 8 of the 12 dogs. Also, anti-human gp100 antibodies were elicited in the 9 of the 16 tested dogs. However, this not correlated with the clinical responses [208].

Because there are many the sucessful studies in developing the xenogeneic DNA vaccine in experiment mice models and large animals. The first of human clinical tiral

have been tested in the 2007. In phase I of clinical trial, human and mouse tyrosinase DNA were vaccinated in stage III/IV melanoma patients. Eighteen human leukocyte antigen (HLA)-A*0201^{+} melanoma patients were randomized as follows : one group recevied three vaccination of mouse tyrosinase DNA injections followed by three human tyrosinase DNA injection ; other group received the same vaccines in the opposite sequence. The study was conducted at three dosage level : 100, 500 and 1500μg DNA injection administered intramuscularly with Biojector every three weeks. No toxicity was found in immunized patients. There was found no relationship with the does, assigned schedule and T cell response. In the tetramer assay, the peak response was found to be 2.5 -fold to 8.6-fold greater than the respective pre-treatment values, while the intrcellular IFN-γ staining was 2.1-fold to 4.5-fold increase. The phenotype of responding CD8^{+} T cells from two of tetramer positive patients were CD45RO^{high} and CD62L^{high} indicating a central memory phenotype. The phenotype of responding CD8^{+} T cells from another of tetramer positive patients showed the CD62^{low} population indicating the effector memory phenotype. No immunoglobulin G antibodies against tyrosinase were detected in pre- or post- vaccination sera. Although at a median of 42 months follow-up, median survival has nor been reached, the mouse and human tyrosinase DNA vaccines were found safe and induced CD8^{+} T cell response in 7 of 18 patients [141]. In 2009, the mouse and human gp100 DNA were immunized melanoma patients with the same strategy. However, the similar results were found with the previuos research [209].

1.8 Canine transmissible venereal tumor (CTVT) model and vaccine development

Canine transmissible venereal tumor (CTVT) is a naturally occurring contagious round cell neoplasm which are located mainly on the external genitalia and are transmitted by the transplantation of viable tumor cells with such behaviors like social smelling and licking [210, 211]. CTVT consists of solitary or multiple tumor cell nodules

divided by fine connective tissues into small islets. CTVT cells are round to ovid in shape with a large nucleolus and prominent vacuolated cytoplasma [212]. It is histiocytic origin and can be transplanted as an allograft with intact viable cells across MHC barriers within the canine population [210, 212-215]. A transposable elements sequence that is a long interspersed nuclear element insertion near the c-myc can be used as a specific maker to diagonose the CTVT [216, 217].

In experimental CTVT-bearing dogs, CTVT cells are progressive growth (P phase) for three to five months, and then stable growth (S phase) for two to thress weeks, finally are spontaneously regressed (SR phase) in four to eight weeks [218]. During the P phase, CTVT dogs show signs of severely suppressed immune responses, much like those observed in humans with malignant tumors [219]. There are several mechanisms that CTVT invade and suppress the immune surveilance. On the one hand, the extremely low expression of MHC class I molecules on the CTVT cells during the P phase [220, 221]. On the other hand, high concentration of CTVT-derived TGF-β at P phase suppresses the immune activities of DCs and NK cells to create an immune tolerance microenvironment [222-224]. At SR phase, a large number of tumor infiltrating lymphocytes (TILs) secrete IL-6, which antagonizes the suppressive effects of TGF-β and may work synergistically with MHC stimulating factor IFN-γ to promote the MHC molecules expression and restores the NK cells cytotoxicity [224, 225]. Besides, the dogs are genetically and physiologically much more related to human [226]. CTVT is considered an ideal large animal model to study host/cancer interaction and preclinical cancer immunotherapy [227, 228]. Electroporation-meiated human IL-12 gene therapy has been demonstrated successfully in treating CTVT. The gene therapy attracted significantly more $CD4^+$ and $CD8^+$ TILs. It offers more clinical relevance is required for evulating human cancer immunotherapy [228].

Up to date, there is only one research about CTVT and HSP expresssion. The expressions of HSP60, HSP70 and HSP90 are investigated in CTVT at P phase and SR phase. Immunohistochemistry staining indiacate that HSP60 and HSP90 are found aggregated or granulated in the cytoplasma in CTVT, whereas HSP70 are dispersed in the cytoplasma. The expressions of HSP60 at SR phase are significantly higher than P phase and serve as maker for SR phase of CTVT. However, the serological experiments show that there are no anti-HSP antibodies found in the serum although the detection of HSP expression at both P and SR phase [81]. Their chaperone function may play an important role in presenting the CTVT antigens to break the tolerance and led to tumor regression. However, the relationship between HSP70 and CTVT need to further investigated.

1.9 Objectives of this study

(a) Design three kinds of chicken HSP70 DNA vaccination schedules and find out the best one in CTVT model.

(b) Prove the protective or inhibitory effect of chicken HSP70 DNA vaccine by immunological experiments.

Chapter 2. Introduction

The immune system guards the host against pathogenic attacks by microorganisms and other foreign/non-self particles; it does not react against cells expressing self-antigens due to negative and positive selection during the development of immature lymphocytes [11]. Tumors usually arise from one's own tissue (self), and almost all of the tumor antigens characterized to date are unmutated self-antigens [137]. It has previously been supposed that self-antigens on tumor cells do not elicit immune responses or activate lymphocytes against themselves; however, recent studies have demonstrated that immunity to self-antigens on tumor cells does exist, and is merely constrained by tumor tolerance [116]. Therefore, the development of a method by which tumor tolerance can be overcome may present a potential cancer treatment.

In fact, several studies in preclinical animal models have indicated that tumor tolerance to self-antigens can be overcome by immunization with a xenogeneic DNA vaccine, the mechanism of which involves improvement of T cell recognition, NK cell activation and autoantibody production [143, 229]. In addition, tiny discrepancies in epitopes of homologous xenogeneic proteins between two species are sufficient to activate self-reactive $CD4^+$ or $CD8^+$ T lymphocytes and generate self-reactive antibodies [143]. DNA vaccines have many advantages over conventional vaccines such as attenuated live or killed pathogens, including induction of a long-term immune response, a higher stability, and a simple method of preparation in large quantities [230].

Heat shock protein 70 (HSP70) is a member of a family of highly-conserved molecules that maintain the function of crucial cellular pathways during stress [231], and

performs a variety of chaperoning functions depending on its subcellular location [74, 232]. Intracellular HSP70 serves as a powerful anti-apoptotic protein and protects cells from lethal threats. Overexpression of HSP70 has been observed in various tumors including breast cancer, endometrial cancer, lung cancer, prostate cancer and other types of cancer [78]. The expression level of HSP70 is known to be correlated with cell proliferation, lymph node metastasis, poor response to chemotherapy and poor survival [79, 99]. Apart from intracellular locations, HSP70 has also been found on the cell membrane of malignantly-transformed cells, on virally-/bacterially-infected cells, and in the extracellular space [74], and extracellular or membrane-bound HSP70 can elicit an immune response either by adaptive or innate immunity [74]. In recent years, tumor-derived HSP70–peptide vaccines have served as multivalent or individual vaccines [77], and the use of HSP70-based DNA vaccines fused with DNA tumor proteins against syngeneic tumors is under investigation by several research groups [114].

Canine transmissible venereal tumor (CTVT) is a naturally-occurring contagious round-cell neoplasm of possible histiocytic origin and is transmitted across the major histocompatibility complex (MHC) barriers within the canine population by the transplantation of viable tumor cells [210, 211]. CTVT overexpresses HSP70 (caHSP70) molecules [81]. Experimentally-transplanted CTVT in dogs exhibits a predictable growth pattern that includes progressive (P), stable (S) and spontaneous regression (SR) phases [218]. During the P phase, the CTVT employs several mechanisms to suppress immune surveillance, including the extremely low expression of MHC class I molecules on CTVT cells to avoid immune system recognition [220, 221] and the high concentration of CTVT-derived TGF-β to suppress immune activities such as those of dendritic cells (DCs) and NK cells. Via these mechanisms, the CTVT creates an immune tolerance microenvironment suitable for its growth [222, 224]. During the SR phase,

tumor-infiltrating lymphocytes (TILs) secrete IL-6, which antagonizes the suppressive effects of TGF-β and works synergistically with MHC-stimulating factor IFN-γ to promote MHC molecule expression and restore the cytotoxicity of NK cells. It is believed that CTVT is an ideal large animal model for the study of host/cancer interaction and preclinical cancer immunotherapy [227, 228].

In this study, we administered prophylactic (G1 and G3) and treatment vaccination (G2) programs in CTVT-bearing dogs using xenogeneic DNA plasmid-encoded chicken HSP70 (*chHSP70*). The novelty of this study was the employment of *chHSP70* DNA itself as a vaccine, rather than the commonly-used HSP70–peptide complex vaccine in which HSP acts as the chaperone to carry cancer antigen peptides [77, 114]. Our results suggested that the group 1 (G1) vaccination strategy, which consisted of 2 consecutive vaccinations followed by intramuscular (IM) injection/electroporation prior to tumor inoculation, induced the best autoimmune response against caHSP70 and activated the tumor regression process much earlier than in the spontaneous case. The G1 program most effectively inhibited tumor growth among the 3 programs, and T-cell cytotoxicity and immune responses related to IFN-γ production were significantly enhanced. It is believed that the autoimmunity induced by the *HSP70* DNA vaccine could be useful in suppressing the growth of HSP70-producing tumors.

Chapter 3. Material & Method

3.1 Chicken HSP70 xenogeneic DNA vaccine

Both the vector pCG073 and empty vector pLL14 plasmids used in this study were provided by Merial S.A.S. The 1908 nucleotides of the synthetic gene *chHSP70* (Swissprot accession: P08106, GenBank accession: J02579) were assembled from synthetic oligonucleotides (GENEART, US). To construct the *chHSP70* DNA plasmid, designated pCG073, a fragment of synthetic *chHSP70* was subcloned into pLL14-expressing vector using EcoRV and Xbal restriation sites. The final construct was verified by sequencing, and the sequence congruence was 100%. A plasmid map of pCG073 is shown in Fig. 1. Empty vector pLL14 was used as the control vector in this study. The alignment of amino acids demonstrated an 83.5% homology between caHSP70 and chHSP70 (Fig. 2).

3.2 Animals and vaccination schedules

A total of nineteen adult beagles were used in this study. All of the animal experiments were performed in compliance with standard operating procedures issued by the Institutional Animal Care and Use Committee of National Taiwan University. The vaccination procedures were as shown in the diagram of Fig. 3. Twelve dogs were evenly divided into three vaccination groups (G1, G2 and G3); each group also contained one additional dog injected with empty vector as a vector control (G1-c, G2-c and G3-c). Four CTVT-bearing dogs that were not administered any treatment served as non-treatment controls (NT). The aim of the group 1 (G1) experiment was to evaluate the efficiency of the xenogeneic *chHSP70* DNA vaccine in preventing CTVT, and the

treatment protocol consisted of two transdermal vaccinations (two weeks apart) prior to CTVT inoculation and one vaccination followed by electroporation three weeks after inoculation. The aim of the group 2 (G2) study was to evaluate the therapeutic efficiency of the xenogeneic *chHSP70* DNA vaccine on CTVT in the growth stage: G2 dogs were immunized with the vaccine three times, in the first, second and fifth week after CTVT inoculation. The vaccination schedule of the G3 dogs was essentially the same as that of G1, with the exception that the third vaccination followed by electroporation was replaced with vaccination using a transdermal needle-free (TD NF) delivery system. The vaccine dosage was 400μg/ml of *chHSP70* DNA and was administered in the upper part of the left hind leg using Vitajet3, a transdermal needle-free delivery system (Merial S.A.S., France). A total dosage of 800μg/ml of the *chHSP70* DNA vaccination followed by electroporation was administered intramuscularly to the right and left semitendinosus muscles, and electroporation was then performed three weeks (W3) after CTVT inoculation. pCG073 and empty vector pLL14 were delivered via the intramuscular injector, followed by a prototype Sphergen generator (reference G-150; Sphergen, France), the voltage and electric field settings of which were adjusted to 87.5V and 175V/cm, with a frequency of 10Hz, a pulse duration of 20ms, and interpulse intervals of 80ms within one second.

3.3 Sample collection

Details of all samples taken and collection schedules in the experimental groups are presented in Table 1. Peripheral blood mononuclear cells (PBMCs) were collected from all dogs prior to experiment to obtain baseline data. PBMCs, tumor cells and TILs were collected from the dogs five weeks (W5, as P phase) after CTVT inoculation, and then when the CTVT begun to regresses (R) after vaccination or entered the SR phase for further immunological experiments. The P phase was defined as a progressive increase in

the average volume of the tumors. When more than half of the tumors in a dog had become smaller in volume, and the average volume of all tumors in the dog had also decreased, the tumor was defined as being in the R phase of tumor suppression in the vaccinated groups or in the spontaneous regression (SR) phase in non-treated animals. To evaluate the vaccine toxicity, canine peripheral blood was collected from G1 and G3 dogs for clinical chemical analysis studies 4 weeks before vaccination (-W4), at CTVT inoculation (W0), at the third vaccination (3 weeks after tumor inoculation, W3), at W5, and upon entering the R phase, while for G2 dogs, blood samples were collected before vaccination (W0) then at W1, W2, W5, and upon entering the R phase. In NT dogs, blood samples were collected at week 0 (W0), W1, W2, W3, W5, and on entering the SR phase after CTVT inoculation.

3.4 CTVT inoculation

Freshly-collected viable tumor cells obtained from beagles during progressive tumor growth were used for experimental transplantation. Ten million tumor cells suspended in Hanks' balanced salt solution was inoculated subcutaneously at eight sites were evenly devided on the each of left and right lateral back of the beagles. All procedures were performed under general anesthesia with isoflurane. Tumor volumes were measured every week with calipers and calculated by a formula: π× length × width × thickness/4 (cm^3).

3.5 Peripheral blood mononuclear cell preparation

PBMCs were isolated using the Ficoll-Hypaque method (density: 1.077; GE Healthcare Bio-Sciences, Uppsala, Sweden) at different time points. A 4ml blood sample was layered over 3ml of Ficoll gradient and centrifuged at 4°C for 20min at 420×*g*. After centrifugation, PBMCs deposited at the interface were harvested and washed three times with buffer (FAB; 1% bovine serum albumin and 0.2% sodium azide in

phosphate-buffered saline (PBS), pH 7.2) in preparation for further use.

3.6 CTVT samples and TILs isolation

One tumor mass was excised at each assigned time point, as mentioned above. For H&E and IHC staining, tumor masses were immersed in 10% neutral formalin solution for 24h then embedded in paraffin, and 4-μm sections were obtained for staining. To isolate TILs, the tumor mass was first minced in RPMI 1640 containing antibiotics and 10% fetal bovine serum (Gibco), and a single-cell suspension was then obtained as described previously by mechanically crushing the minced tumor mass with the aid of a stainless steel mesh and passing it through a 3-layer metal filter (pore size: 190μm) [222, 233]. The cell suspension was layered on 42% Percoll™ (Pharmacia Biotech, USA) gradient and centrifuged at 4°C for 25min at 800×*g*, after which TILs that lay on the bottom were harvested and treated with 10ml ACK buffer for 10min at room temperature to avoid contamination with red blood cells. Isolated TILs were subsequently washed three times with staining buffer for flow cytometry studies.

3.7 Flow cytometry analysis

TILs (2×10^5) were incubated with specific mouse monoclonal antibodies against canine CD4 or CD8 (Serotec, UK) for 30 min at 4 °C as previously described [233]. Mouse IgG1 and IgG2a were used as isotype control (Serotec, UK). Cells were then washed and incubated with FITC-conjugated goat $(Fab')^2$ anti-mouse IgG (Serotec, UK) as secondary antibody for 30 min. Finally, all cells were suspended in staining buffer (0.1% sodium azide, 2% fetal bovine serum in PBS) containing 5 mg/ml propidium iodide (Sigma, St Louis, USA). The surface immunofluorescence viable cells were measured with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA, USA). Fluorescence intensities were analyzed with Cell Quest Software (Becton

Dickinson).

3.8 Immunohistochemical staining

A super-sensitive non-biotin horseradish peroxidase detection system (BioGenex Laboratories, San Ramon, CA) was used to detect CD3 T lymphocytes in tumor sections, as described previously [224]. Briefly, series paraffin sections (4-μm-thick) were deparaffinized with xylene for 15min and dehydrated in a graded ethanol series. The sections were then placed in Trilogy™ solution (CellMarque, Hot Springs, AR) and heated at 121°C for 15min in an SA-252F autoclave (Sturdy Industrial, Taipei, Taiwan) for antigen retrieval, followed by immediate transfer to Trilogy™ solution and incubation at 80°C for 10min. A 400x-diluted antibody against CD3 (DakoCytomation, Carpinteria, CA) was applied for 24h at 4°C, followed by Super Enhancer™ (BioGenex, San Ramon, CA) treatment for a further hour at room temperature. Tris-buffered saline (TBS; DakoCytomation, Carpinteria, CA) was used to wash the slides following each staining step. Slides were treated with the substrate, diaminobenzidine tetrahydrochloride (BioGenex, San Ramon, CA), for 1min and then counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for 10min. Rabbit normal serum (Biogenex, San Ramon, CA) replaced CD3 antibody in the same protocol as a negative control. CD3 positive staining of TILs in tumors was evaluated by manual counting; the density was quantified from fifteen randomly-chosen high-power microscopic fields (HPFs, 400x), and the mean TIL value was calculated. The results were analyzed using two-tailed Student's *t*-test.

3.9 CTVT specific cytotoxicity assay

Effector (E) cells were developed by co-culturing PBMCs (3.2×10^6cells/ml) with 15μg/ml mytomycin-C-treated CTVT cells (8×10^5cells/ml) for 6 days in 24-well plates according to a previously-described procedure [224]. Following washing thrice with PBS,

2×10^3 CTVT cells (Target (T) cells) in 50μL medium were placed in 96-well U-bottom plates with effector cells at various E:T ratios (50:1, 25:1, 12.5:1, and 6.25:1) and incubated at 37°C for 6h. After incubation, the supernatants were collected and the cytotoxicity of the effector cells towards CTVT was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA) as the manufacturer's instructions. Lactic dehydrogenase (LDH) production in the cultured medium was measured at an absorbance of 490nm. Percent cytotoxicity was calculated as (Experimental–Effector Spontaneous–Target Spontaneous)/(Target Maximum–Target Spontaneous)×100%. A baseline CTL assay was performed following the same procedure: PBMCs collected at baseline were used as effector cells, and CTVT cells at W5 were used as target cells.

3.10 IFN-γ secreting and HSP70 specific lymphocytes detection

To evaluate the specific activity against chHSP70 and canine HSP70 (caHSP70) molecules, the HSP70-specific IFN-γ production capacity of PBMCs was evaluated using a canine IFN-γ ELISPOT kit (R&D, USA) in nitrocellulose-lined 96-well microplates (Millipore MAHAS45) according to previously-described procedures [227]. Briefly, 50μg/ml CTVT cell lysates and 1μg/ml chHSP70 or caHSP70 peptide pools were mixed with $1x10^5$ CTVT-activated PBMCs (effector cells) and incubated at 37°C for 24h. Each peptide pool was composed of a combination of individual peptides of 15-mers overlapping by 10 amino acids. All C-ter positions were kept unblocked, and when applicable, glutamine in the N-ter position was systematically replaced by the previous amino acid. PBMCs isolated from healthy beagles were treated with conanavalin A (Sigma, St Louis, USA) and used as positive controls. Biotinylated polyclonal antibodies against canine IFN-γ were added and the mixture incubated at 4°C for 24h, followed by incubation with Streptavidin-AP and development in substrate BCIP/NBT Chromogen. Spots were counted manually under dissection microscopy (Olympus, Japan). Baseline

ELISpot assays were performed following the same procedure: similarly, PBMCs at baseline were used as effector cells, and CTVT cells at W5 were used as target cells.

3.11 NK cytotoxicity assay

Canine thyroid adenocarcinoma cells (CTACs) purchased from the European Collection of Cell Cultures (Salisbury, England) were used as target cells (T) for canine NK cytotoxicity assay, which was performed following previously-described procedures [227]. Two-thousand CTACs per 50μl medium were placed in 96-well U-bottom microtiter plates and incubated at 37°C overnight, and PBMCs (effector cells, E) obtained from vaccinated beagles were co-cultured with CTACs at various E:T ratios (50:1, 25:1, 12.5:1, and 6.25:1). NK cytotoxic activity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, USA) as the manufacturer's instructions. LDH production in the culture medium was measured at an absorbance of 490nm. Percent cytotoxicity was calculated as (Experimental–Effector Spontaneous–Target Spontaneous)/(Target Maximum–Target Spontaneous)×100%. Baseline ELISpot assays were performed following the same procedure, using PBMCs collected at baseline as effector cells.

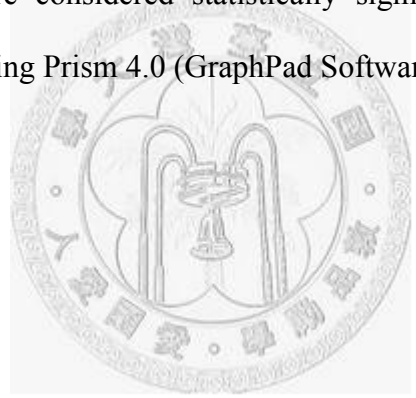
3.12 ELISA

Serum antibodies titer was analyzed by ELISA. The caHSP70 or *chHSP70* peptide pools were resuspended in PBS to a final concentration of 1mg/ml and incubated in each of the ELISA wells overnight at 4°C. Plates were then rinsed with washing buffer (0.45% NaCl in deionized water containing 0.05% Tween-20) and blocked with blocking buffer (5% bovine serum albumin in washing buffer) for 2h at 37°C. Serum samples were obtained from the NT group at baseline (W0) and from G1-c and G1 at baseline (-4W), CTVT inoculation (W0), after inoculation (W3, W5), and upon entering the R phase, and were diluted 10x in PBS. The diluted serum samples were then incubated in caHSP70- or

chHSP70-peptide-coated wells for 2h at room temperature, followed after washing by further incubation with 2000x diluted rabbit anti-canine immunoglobulin-horseradish peroxidase conjugated in dilution buffer (ICN Biomedicals, Costa Mesa, CA). After extensive washing, the plates were developed with 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB, Clinical Science Products Inc., Mansfield MA, USA) substrate for 30min, and the reaction stopped with 1N HCl. Color development was quantified at 450nm.

3.13 Statistics

Data were presented as mean± SD and were analyzed using the two-tailed Student's *t*-test. The differences were considered statistically significant at $p<0.05$. Statistical analyses were performed using Prism 4.0 (GraphPad Software, Inc.).



Chapter 4. Results

4.1 Vaccinations followed by electroporation prior to tumor inoculation suppressed the tumor growth more efficiently.

In the G1 dogs (Fig. 4A), significant suppression of tumor growth began at W6 after tumor inoculation ($p<0.01$), and complete regression was achieved at W12, while the NT dogs did not exhibit any suppression of tumor growth until W11, and tumors remained large in size (127cm^3) at the end of the experimental period (W18; 82cm^3). Tumors of the G2 dogs (Fig. 4B) grew progressively and exhibited no regression until W17, while those of the G3 dogs (Fig. 4C) entered the regression phase at around W12. The G1 vaccination procedure itself was seen to somehow affect tumor growth, although data were only obtained from one dog (Fig. 4A): this was evidenced by the fact that the G1-c dog exhibited earlier regression than the NT dogs (W11 for G1-c *vs*. W14 for NT). The G2 tumors were smaller in size than those of the NT group at first ($p<0.05$); however, they began to grow progressively thereafter, and no size difference between G2 and NT tumors could be discerned by the end of the experiment.

Because the electroporation procedure required an anesthetic, to achieve better clinical applicability, the protocol for the third vaccination followed by electroporation used in the G1 dogs was replaced with a TD vaccination protocol in the G3 animals. The tumors of the vaccinated G3 dogs were smaller than those of the NT dogs; however, the tumors of the vector control animals (G3-c) were similar in size to those of the G3 dogs (Fig. 4C). The tumor growth curves for all experimental groups are shown in Fig. 4D: these data indicate that tumor growth was suppressed most effectively in the G1 dogs among the three tested groups, and vaccination of the dogs prior to tumor establishment

was the most effective protocol for the inhibition of tumor growth. In addition, vaccination followed by a single electroporation procedure resulted in the most effective suppression of tumor growth.

4.2 Infiltration of tumor masses by many T lymphocytes in G1 tumors began in P phase

After vaccination with *chHSP70* DNA, the tumors of the G1 animals (Fig. 5a) were infiltrated with lymphocytes in greater abundance than those in the other groups in this study (Fig. 5b to 5g). Many necrotic foci were also found in the G1 tumors (Fig. 5h).

To investigate the $CD4^+$ (Fig. 6A) and $CD8^+$ subpopulations (Fig. 6B) of TILs in the experimental groups, TILs purified from tumors of all groups were analyzed by flow cytometry. The percentages of both $CD4^+$ (Fig. 6Aa) and $CD8^+$ (Fig. 6Ba) TILs during progressive tumor growth were significantly higher in the G1 dogs than in the G1-c and NT control dogs (28.90% *vs*. 5.00% and 9.38% for CD4 and 22.45% *vs*. 1.49% and 3.10% for CD8, respectively). However, the percentages of $CD4^+$ and $CD8^+$ TILs in the G2 (18.22% for CD4 and 10.7% for CD8) and G3 dogs (17.87% for CD4 and 12.86% for CD8) were similar to those of the NT group (9.38% for CD4 and 3.10% for CD8). In suppressed tumors in the treated groups or spontaneously-regressed tumors in the controls, the percentage of $CD4^+$ TILs in G1 dogs (56.77%) was also significantly higher than in G2 and NT dogs (G2: 27.90%, G3: 34.42%, NT: 22.74%) (Fig. 6Ab); however, the percentage of $CD8^+$ TILs was similar in all groups (G1: 28.52%, G2:28.33%, G3: 22.47%, NT: 15.94%)(Fig. 6Bb).

Immunohistochemical staining showed that at W5, many $CD3^+$ T lymphocytes had infiltrated the tumors in the G1 (Fig. 7a), G2 (Fig. 7b) and G3 dogs (Fig. 7c), while only small numbers were found scattered in the tumors of the empty vector (Fig. 7d, 7e and 7f) and NT controls (Fig. 7g). The serial sections shown in Fig. 7b were stained with normal

rabbit serum as a negative control (Fig. 7h). CD3 positive staining of TILs in P phase (Fig. 7Ba) and SR/R phase (Fig. 7Bb) tumors was evaluated by manual counting in fifteen randomly-chosen HPFs, and the number of CD3 positive lymphocytes infiltrating the G1 tumors was significantly higher than those infiltrating the G2, G3 and NT tumors during both the P phase and the SR/R phase.

These data imply that the vaccination program administered to the G1 dogs promoted more efficient infiltration of TILs, including CD4 and CD8 cells, which coincided with tumor growth inhibition; however, the difficulties in staining CD4 and CD8 surface markers using our tested antibodies prevented us from obtaining related results from tissue sections (Table 2).

4.3 Cytotoxicity toward CTVT was higher in G1 dogs.

We further sought to compare the tumor-specific cytotoxicity between the different vaccination groups. No differences in cytotoxicity were observed at baseline before vaccination between groups (Fig. 8A). However, the cytotoxicity of PBMCs from G1 and G3 dogs was significantly higher than that of the control groups during the P and R phases (Fig. 8B and 8C), and again, cytotoxicity was highest in the G1 dogs than in any other groups in both the P phase and the R phase.

4.4 Xenogeneic chHSP70 DNA vaccination induced caHSP70-specific Th1 response.

To further investigate whether administration of the xenogeneic *chHSP70* DNA vaccine elicited specific immune responses against caHSP70, canine and chicken HSP70-specific IFN-γ-producing cells were enumerated in PBMCs incubated with caHSP70 or chHSP70 peptides by ELISpot assay. No differences in the baseline data before vaccination were observed between groups (Fig. 9A). Generally, scores in the SR/R phase (Fig. 9C) were higher than those in the P phase (Fig. 9B), and in both the P and R phases, caHSP70, chHSP70, and tumor-specific IFN-γ-secreting cells were most

abundant in G1 among all groups. As the tumors regressed, the caHSP70 peptide-specific IFN-γ-secreting cells were present in significantly greater numbers in G3 as compared with G2 and NT dogs (Fig. 9C). Thus, prophylactic administration with one electroporation session induced the best reaction in terms of enhancing the canine HSP70-specific Th1 responses.

4.5 NK cytotoxicity may not contribution to the tumor inhibition.

T No differences in NK cytotoxicity before treatment were observed between groups (Fig. 10A). The NK cytotoxicity of the G1 dogs (P phase: 22.96% and R phase: 30.40% in a 50:1 E/T ratio) was slightly higher than that of the other groups at P phase (Fig. 10B) and upon entering the SR/R phase (Fig. 10C), but this was not statistically significant, which indicated that NK cells may not play a major role in tumor regression in this vaccination program.

4.6 Humoral response against canine and chHSP7*0* did not differ between groups.

To investigate the humoral response in *chHSP70* vaccination, antibodies against caHSP70 and chHSP70 after vaccination were evaluated by ELISA. The levels of both caHSP70 (Fig. 11A) and chHSP70 antibodies (Fig. 11B) were low in serum from immunized G1 dogs, and no significant variation was observed between samples collected at different time points among the groups. It was interesting to find that chHSP70 antibodies were present prior to treatment in the dogs, and the differences between the NT, G1-c and G1 dogs were significant ($p<0.05$) (Fig. 11C). The lack of antibody responses implied that humoral immunity did not make a sizable contribution to the inhibition of tumor growth under the *chHSP70* vaccination strategy.

4.7 The toxicity of chHSP70 vaccination.

Blood samples were collected from animals of all groups at different time points and processed in order to evaluate the chemical parameters of the vaccine by routine clinical

chemical laboratory techniques using standard equipment (Table 3). The results from the NT, G1, G2 and G3 dogs were similar, and no detectable adverse effects, including inflammation at the injection site, were observed in any of the animals.

Chapter 5. Discussion

The importance of using "altered self-antigens" to overcome tumor tolerance was highlighted in studies using lysates of SK-MEL19, the $gp75^+$ human melanoma cell line [140]. When mice were immunized with "human" melanoma lysates, autoantibodies were produced, which recognized "mouse" gp75, while immunization of mice with "mouse" B16 melanoma elicited no antibody response. The results of this and other studies support the idea that ignorance of or tolerance to self-antigens could be overcome by homologous xenogeneic proteins. Development of the xenogeneic DNA vaccine was based on the notion that small differences in epitopes of homologous xenogeneic proteins between two species improve the recognition of MHC class I or class II molecules on transfected cells by T cell receptors (TCRs), leading to the generation of cross-reactive responses towards homogeneous self-proteins and inducing autoimmune-like tumor rejection. The production of cross-reactive antibodies and Th1-associated cytokine IFN-γ is also involved in this type of immunity [143, 145, 229]. $CD4^+$ T cells and $CD8^+$ cells have also been proven to play important roles in this kind of tumor rejection process in a mouse melanoma model [146, 147]. In addition, the efficacy of xenogeneic DNA vaccines other than HSPs for the treatment of human melanoma has also recently been tested in phase I clinical trials [141, 209].

In this study, we used chicken "xenogeneic" HSP70 DNA "alone", rather than as a tumor-derived HSP70–peptide complex, as a vaccine to elicit cross-species specific immune responses against tumor-associated HSP70 in dogs. The homology of this molecule in chickens and canines was 83.5% (Fig. 2), which is in accordance with the notion that small differences in amino acid sequences between species can overcome

immune tolerance and further elicit tumor immunity. The significant increase in *caHSP70*-specific IFN-γ-producing cells in the G1 dogs proved that *chHSP70* administration prior to tumor inoculation activated cross-reactive immune responses against self-antigens. The increase in the CD8 T-cell population in TILs began in the P phase in G1 dogs, and the enhanced cytotoxicity indicated that the vaccination program was effective in activating what was most likely MHC class I-dependent CD8 T-cell-associated immune responses and CTVT-specific cytotoxicity. Pre-vaccination of *chHSP70*, as was administered in the G1 animals, exhibited a high efficiency in suppressing tumor growth: this effect was dramatically decreased in the G2 dogs in which vaccination was initiated after the tumor had already become established in the tissue. Together, prophylactic vaccination with *chHSP70* DNA plus IM vaccination/electroporation activated significant autoimmune responses, mainly cellular immunity against caHSP70, and initiated early regression of the tumor.

It is generally believed that the establishment of a tumor-favorable microenvironment is essential for progressive growth of the tumor [12], this microenvironment usually facilitating neovasculization, better nutrient access, and immunosuppression/tolerance [234]. Within the tumor microenvironment, accessory cells work through cell–cell contact and crosstalk between functional molecules to develop conditions suitable for the survival and propagation of tumor cells. Thus, once the tumor has built up its microenvironment, its foundation for survival in the tissue, it becomes more resistant to enhanced immune responses. This was the case in the tumors of the G2 dogs, which were vaccinated after the tumor had been allowed to grow for two weeks – these animals were more resistant to the enhanced immune responses elicited by the vaccination program than the G1 dogs, and therefore the G2 tumors grew continuously for a significantly longer period of time than those of the G1 animals before entering the

regression phase. In G1, the enhanced immune responses elicited by vaccination before tumor inoculation, i.e., before the establishment of a microenvironment favorable to the survival of cancer cells, began to exert an impact immediately upon inoculation. This impact was apparently potent in inhibiting tumor growth, and therefore prophylactic xenogeneic vaccination was found to be an important step in breaking the tumor tolerance. Further, it could be useful to decrease the size of the tumor before initiating a vaccination program in order to weaken the foundation of the tumor microenvironment – in other words, it is very important to set up an immune-enhancement program as early as possible.

Antitumor research has been focused on eliciting tumor-specific $CD8^+$ CTL responses [235]; however, tumor immunity that is considered more akin to autoimmunity has previously been demonstrated to require the assistance of $CD4^+$ T cells [147, 236]. In addition, $CD4^+$ T cells are indispensable to the primary CTL response and facilitate the persistence of the CTL effector function [237-239]. More and more studies have suggested that Th1 $CD4^+$ cells are capable of protecting experimental animals against tumors without the participation of a $CD8^+$ population [240, 241], and some studies have shown that $CD4^+$ cells can be more efficient at tumor rejection than $CD8^+$ cells [242]. We found that in the early stage post-tumor inoculation of G1 dogs, both the $CD4^+$ and $CD8^+$ subpopulations were increased in TILs; however, only $CD4^+$ cells significantly increased in number during tumor regression. Together, these results suggested that $CD4^+$ cells play an important role in HSP70-associated immune suppressive responses.

For better clinical applicability of the xenogeneic *chHSP70* DNA vaccine, the third vaccination followed by electroporation was replaced with administration via the Vitajet3 system in the G3 dogs. Therefore, administration of this vaccine to tumor patients need not require anesthetization, which would be of particular benefit in patients with cancer

of an advance stage. However, a lesser protective effect and lower inhibition of tumor growth was observed in the G3 dogs treated with electroporation. In addition, although the PBMC cytotoxicity to CTVT was significantly greater in the G3 dogs as compared with the NT dogs, the $CD4^+$ and $CD8^+$ lymphocyte populations recruited to the tumor site were small, and were no different to those in the G2 and NT dogs, the 2 groups in which no inhibition of tumor growth was seen. The efficiency of various DNA delivery systems, including the gene gun (GG), intradermal injection (ID), and intramuscular injection with or without electroporation (IM+E or IM), against HER2/neu was investigated in mice [243], and IM+E was found to result in the best antitumor effect and generation of a Th1-type immune response. This Th1 immune response has also been observed in other studies in which vaccination was conducted via electroporation [244, 245], and accordingly, electroporation followed by IM vaccination was an important protocol to examine in our vaccination study. However, further study in a larger number of animals is necessary in order to confirm this conclusion.

The administration of a boost with syngeneic tumor antigen is important. In a murine melanoma model, xenogeneic human gp75 (hgp75) and syngeneic mouse gp75 (mgp75) DNA vaccines were administered to C57BL/6J mice [147], with the result that the tumor tolerance was broken in hgp75-immunized mice and autoantibodies against mgp75 were generated, further inhibiting distal lung metastases. Interestingly, the authors replaced the third vaccine in the hgp75 group with syngeneic mgp75 in the hgp75-immunized group, and immunoprecipitation analyses indicated that the injection of a boost of “syngeneic” gp75 augmented the immune response to mgp75 antibody production from 50% to 87%. However, cellular immunity towards mgp75 was not investigated in this study. In comparison with the G2 dogs, prophylactic administration of the *chHSP70* DNA vaccine in the G1 dogs caused potent immune responses that inhibited

tumor growth. CTVT inoculation following two *chHSP70* vaccinations may also act as a "syngeneic antigen boost" and provide the host immune system with syngeneic canine HSP70 antigens, which may be beneficial in terms of further augmenting the clonal expansion of HSP70 cross-reactive lymphocytes.

Regarding the humoral response, recombinant caHSP70 and chHSP70 peptide pools were used to investigate the variation in caHSP70 and chHSP70 antibody titers via ELISA. No significant variation at different time points were observed in any of the immunized dogs (Fig. 10A and Fig. 10B), and the broad range of baseline chHSP70 antibody titers observed in dogs of all groups (Fig. 10C) could be one of the reasons for this lack of variation. Other studies have shown anti-HSP70 antibodies to be present not only in autoimmune-disease patients but also in 60% of healthy human sera [246, 247]. In addition, anti-syngeneic HSP70 antibodies have been evaluated in unimmunized healthy BALB/cJ, C3H-HeJ and C57BL/6 mice, and it has been shown that the titer of HSP70 autoantibody differed between strains in mice, and even in individuals of the same strain [248]. It is generally believed that microorganism infection could lead to the presence of HSP70 antibodies, which cross-react with syngeneic HSP70 [249]. This is one explanation for the broad range of baseline titers found in our experimental dogs. In addition, in studies of xenogeneic DNA vaccines, humoral antibodies have not always been found in immunized animals with different vaccination schedules [143, 145], and different genetic backgrounds can cause varying responses to xenogeneic HSP70 immunization. MHC presentation of antigens is also important in shaping antibody responses [229], and because the beagles in our study were outbred, differences in MHC alleles between dogs must be taken into consideration with regards to the high baseline HSP70 antibody levels.

HSP70 induces IL-6 production, which triggers CD4- and CD8-dependent

progressive T helper 17 (Th17)-mediated autoimmunity [250]. The presence of TGF-β is required for the differentiation of both Treg and Th17 subsets in mice, and IL-6 acts as a switch to direct naïve T cells towards Th17 and away from the Treg lineage [251]. The major task of Treg is thought to be the maintenance of immune tolerance to prevent the occurrence of autoimmunity in healthy individuals [3, 11]. On the other hand, the presence of Treg cells within a tumor was found to suppress T-cell responses against the tumor [3]. In the CTVT model, the high concentration of CTVT-derived TGF-β in the P phase suppresses the immune activities of DCs and NK cells to create an immune tolerance microenvironment [222, 224]. During the SR phase, TILs secrete IL-6, which antagonizes the suppressive effects of TGF-β to promote expression of MHC molecules and restore the cytotoxicity of NK cells [224, 225]. Thus, it would be interesting to employ the CTVT model to study the role of immunocyte interactions in relation to self-tolerance which were overcome by *chHSP70* DNA vaccination, such as the measurement of balance arm in the decrease of Treg and Th17-related cytokine gene expression after vaccination,

In conclusion, only prophylactic administration of the xenogeneic *chHSP70* vaccine induced significant host immune responses specifically against caHSP70 and caused tumor regression in a canine cancer model. No adverse effects were observed in immunized dogs. This protocol has the potential to be a promising novel prophylactic strategy to inhibit the growth of tumors expressing HSP70. However, additional research is still required to ensure that these data can be translated into a commercial product.

Figures

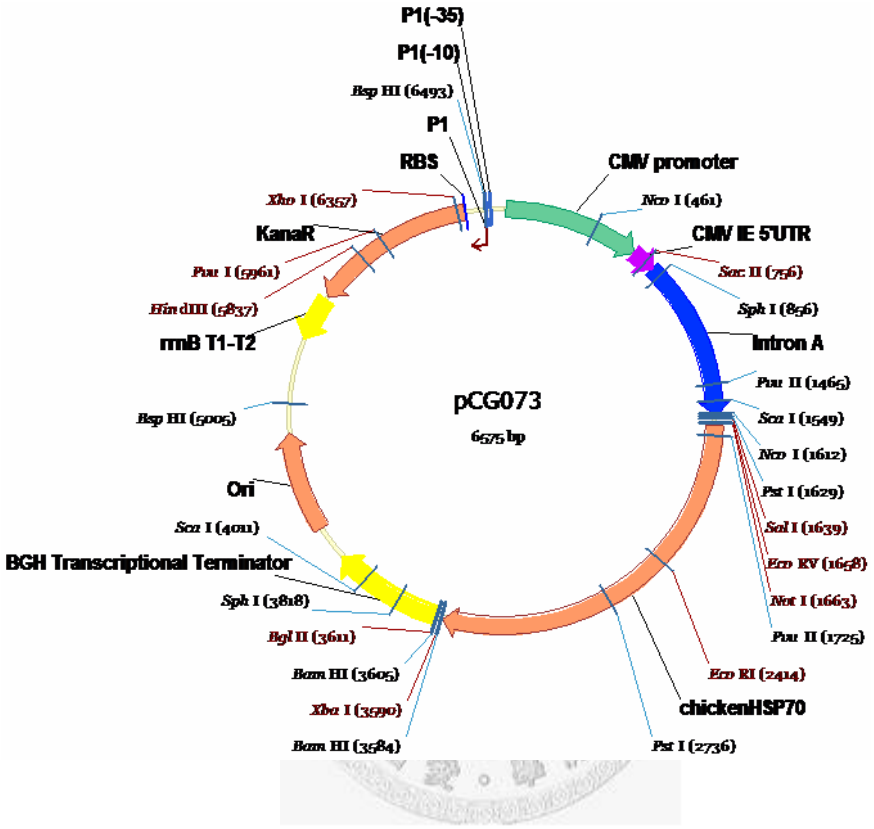


Figure 1 Schematic map of the pCG073 plasmid map of which encoded chicken HSP70 sequence.

The fragment of synthetic *chHSP70* was subcloned into pLL14 expressing vector using EcoRV and Xbal restriation sites and named pCG073. The *chHSP70* sequence is under the control of cytomegalovirus immediate promoter (CMV). Ori: origen of replication; kanaR: kanamycin resistence gene.

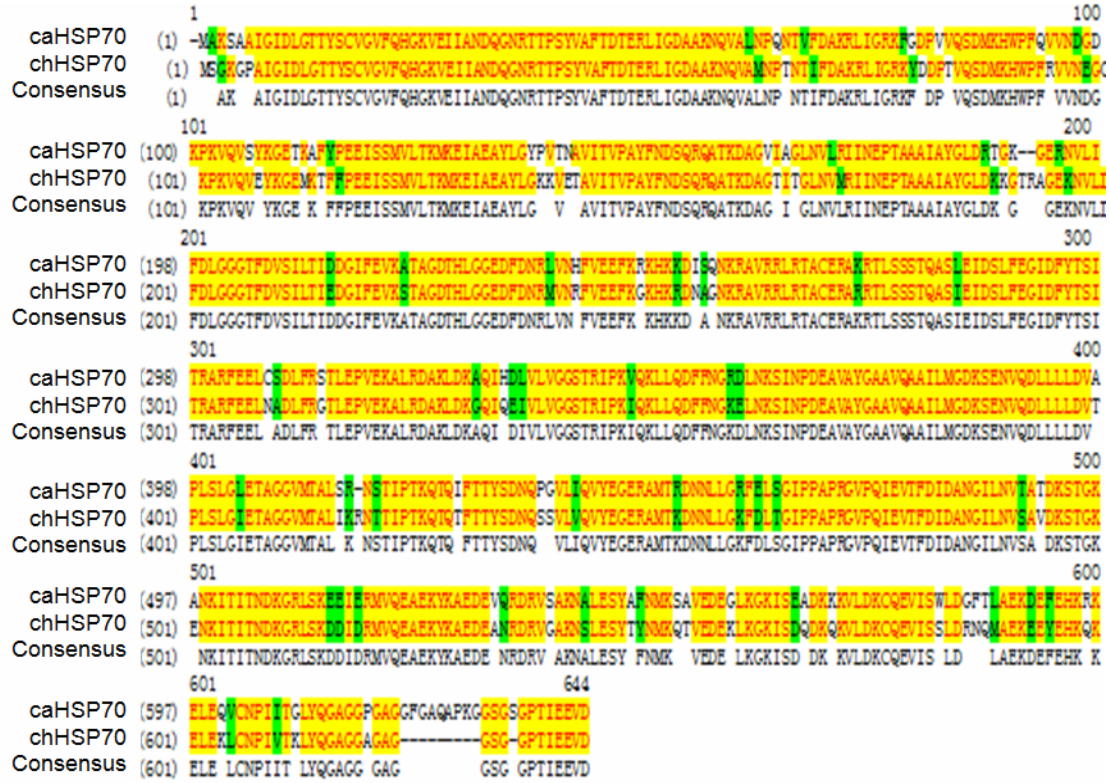


Figure 2 The homology between canine and chicken HSP70.

Amino acid sequence comparison of canine and chicken HSP70 shows a high degree of homology at amino acid level. The calculated sequence identity by Vector NTI between canine and chicken HSP70 is 83.5%, indicating the high homology of the HSP70. Yellow highlights identical residuals and the green highlights similar residuals.

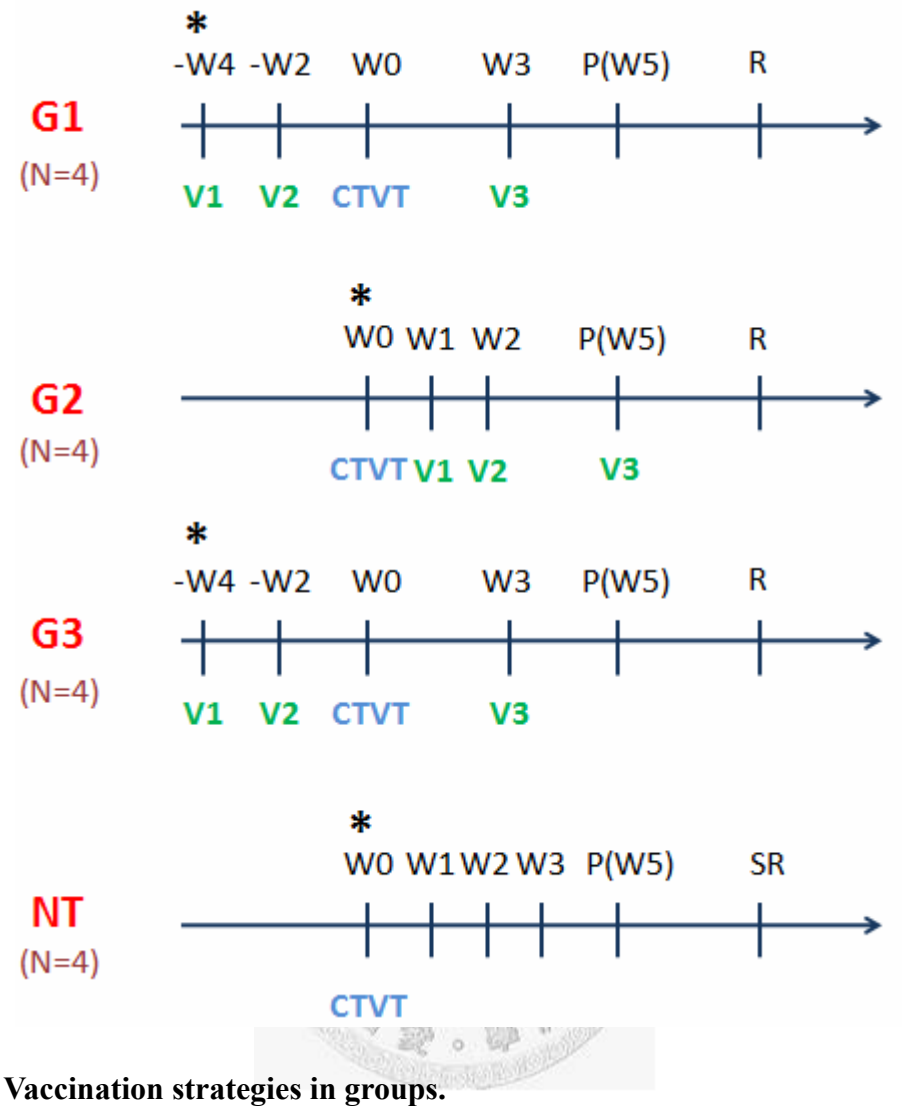


Figure 3 Vaccination strategies in groups.

To evaluate the prophylactic efficiency of xenogeneic chicken (ch) HSP70 DNA vaccine, the G1 dogs (n=4) were immunized twice via transdermal injection using Vitajet3 (Merial, France) at four and two weeks before CTVT inoculation. The third vaccine was administered intramuscularly plus electroporation (Sphergen, France) at three weeks after CTVT inoculation. To evaluate the therapeutic efficiency of *chHSP70* DNA vaccine on growing tumors as in G2, *chHSP70* DNA was administered to the dogs at first, second and fifth weeks after CTVT inoculation via transdermal injection (n=4).

The vaccination schedule of G3 was the same with G1 but the vaccination/electroporation was replaced by TD NF injection at the third vaccination. There was one dog each for each group (G1-c, G2-c and G3-c, respectively) as vector control immunized with empty

vector following the same strategies. NT group (n=4) were dogs inoculated with CTVT without any treatment and were served as tumor normal growth curve. The samples from control groups were also collected at the same time points for further experiments. All the sample collection times were described in Table 1.

V1: first vaccination, V2: second vaccination, V3: third vaccination, CTVT: tumor inoculation.

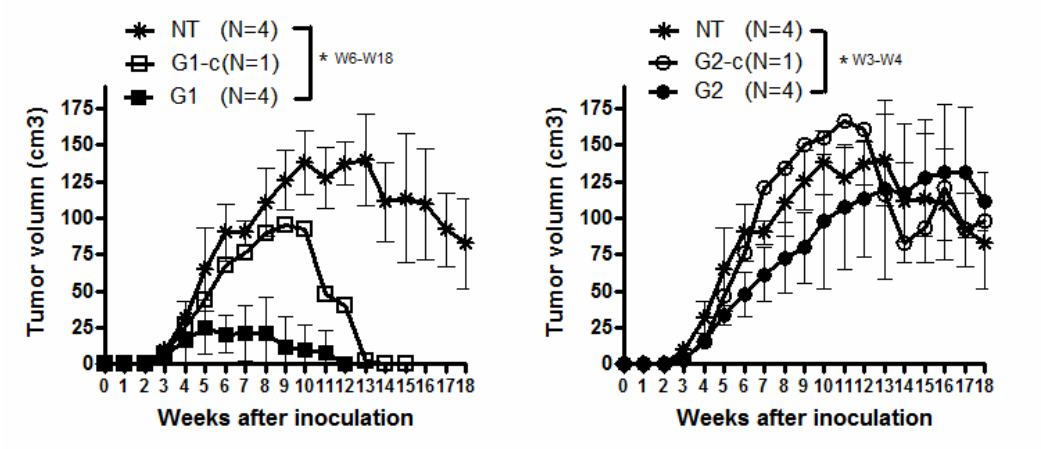
P: tumor progressive phase.

SR: tumor spontaneous regression in normal CTVT-bearing dogs.

R: tumor regression in *chHSP70* DNA vaccinated dogs.

* the time point served as the baseline data.

A. B.



C. D.

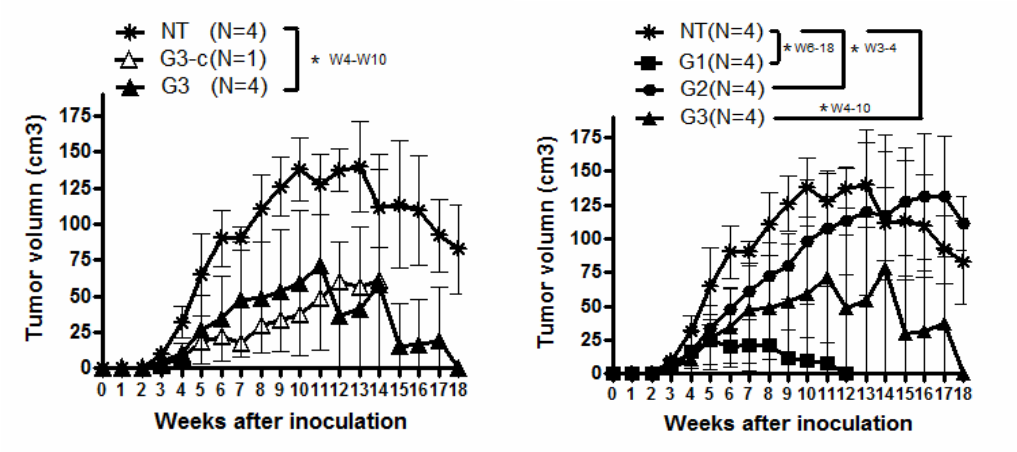


Figure 4 Inhibition of tumor growth after vaccinations.

A. Tumor sizes in G1 and NT dogs were measured weekly after CTVT inoculation. The inhibition of tumor growth from prophylactic administration of *chHSP70* in G1 was significantly effective.

B. Tumor sizes in G2 and NT dogs after CTVT inoculation. The inhibition of tumor growth from G2 dogs were observed only in the early stage of tumor growth.

C. Tumor sizes in G3 and NT dogs after CTVT inoculation. The inhibition of tumor growth from G3 was effective.

D. Tumor growth curves from all of the experimental groups and NT group were compared.

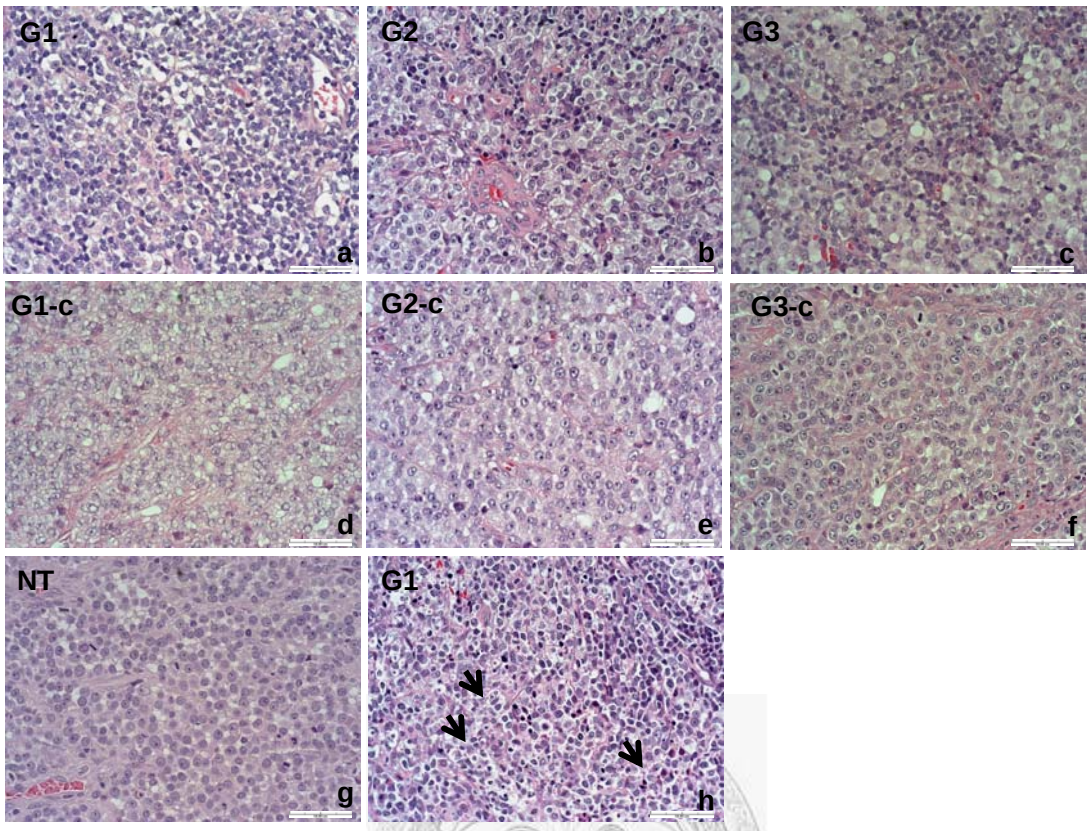


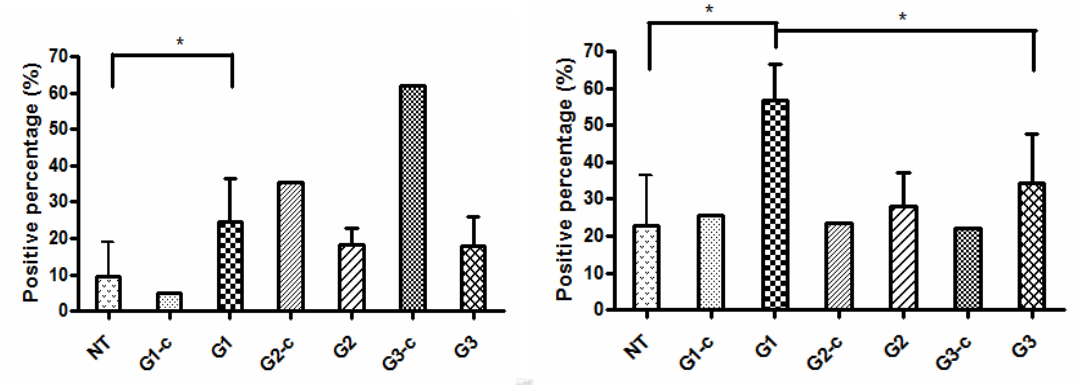
Figure 5 Histopathology of CTVT at P phase.

Abundant lymphocytes infiltrated focally to the tumors in *chHSP70* DNA immunized G1 dogs (a). Fewer or no lymphocytes infiltrations were seen in G2 (b), G3 (c), G1-c (d), G2-c (e), G3-c (f) and NT dogs (g) compared with the G1 dogs. Many necrotic foci (arrow) were also found in G1 tumors (h). H&E stain. Original magnifications, 400X.

A. CD4 TILs

a. P phase

b. SR/R phase



B. CD8 TILs

a. P phase

b. SR/R phase

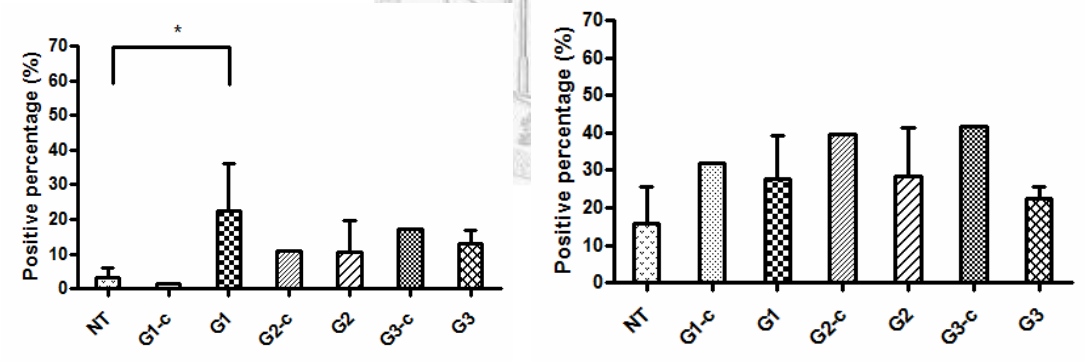


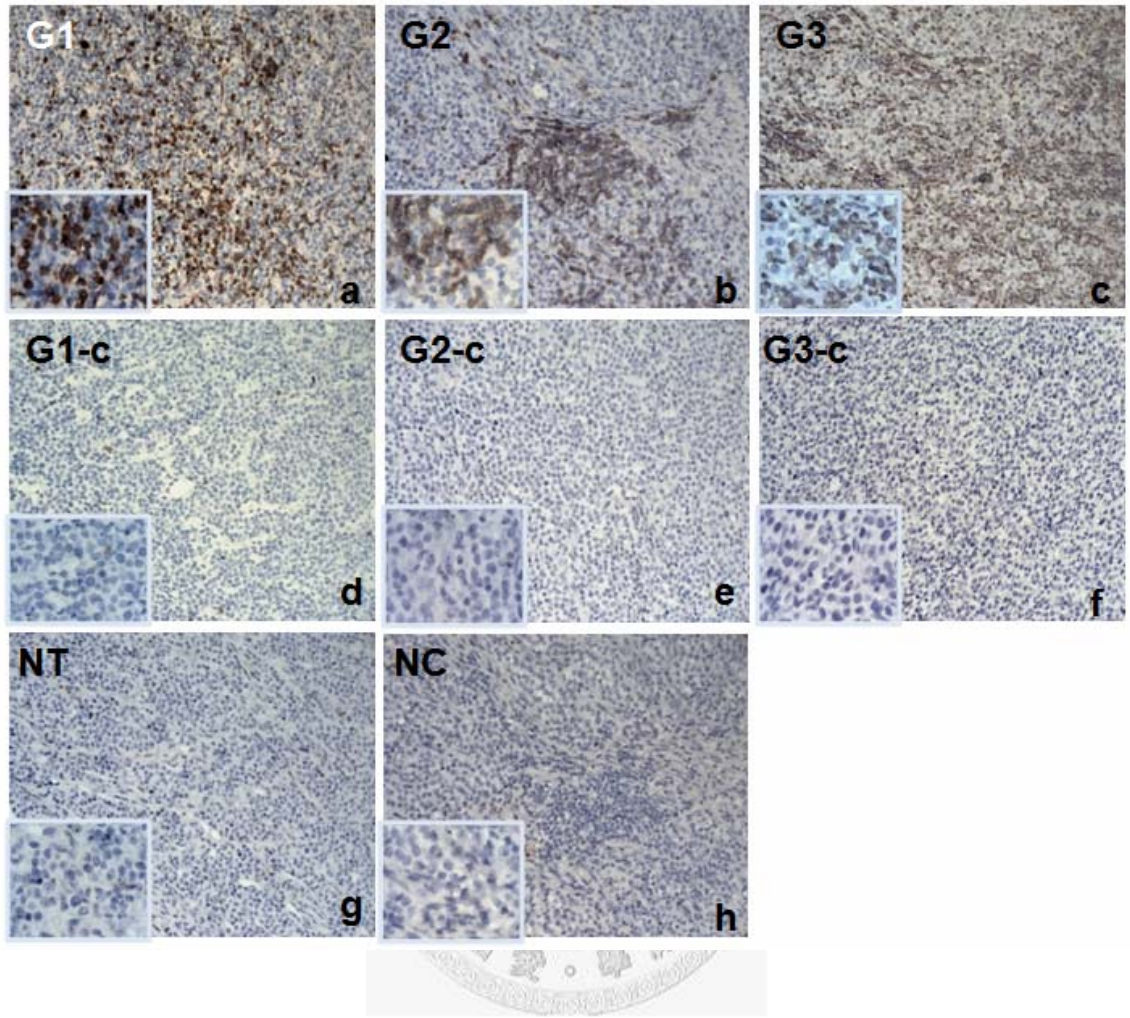
Figure 6 $CD4^+$ and $CD8^+$ TILs subpopulations at P phase and R phase in dogs.

The TILs were isolated from CTVT tissue. The percentage of $CD4^+$ (upper panel) and $CD8^+$ (lower panel) subpopulation in TILs at P phase and SR/R phase were analyzed by a FACSCalliber.

A. The percentage of the $CD4^+$ TILs in G1 dogs was statistically higher than groups at P phase (a) and also in SR/R phase (b).

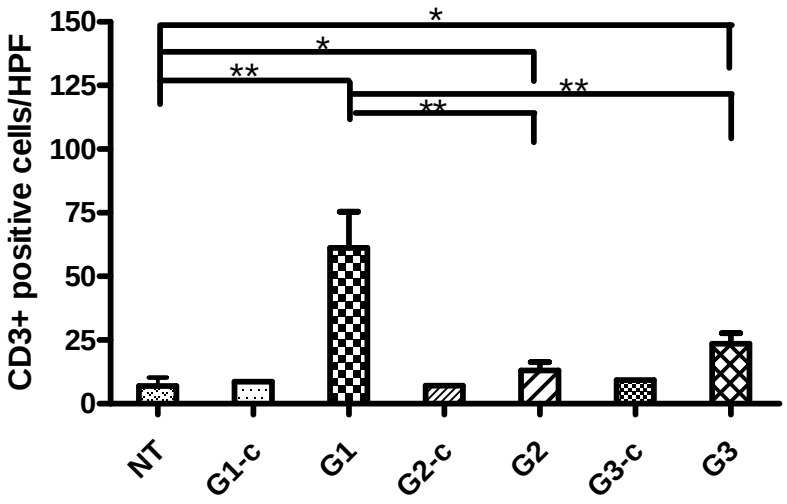
B. The percentage of the $CD8^+$ TILs in G1 dogs (a) was statistically higher than NT dogs at P phase. However, there was no statistical difference among groups at SR /R phase (b).

A.


G1
a
G2
b
G3
c
G1-c
d
G2-c
e
G3-c
f
NT
g
NC
h

B.

a. P phase


CD3+ positive cells/HPF
150
125
100
75
50
25
0
*
*
**
**
**
NT
G1-c
G1
G2-c
G2
G3-c
G3

b. SR/R phase

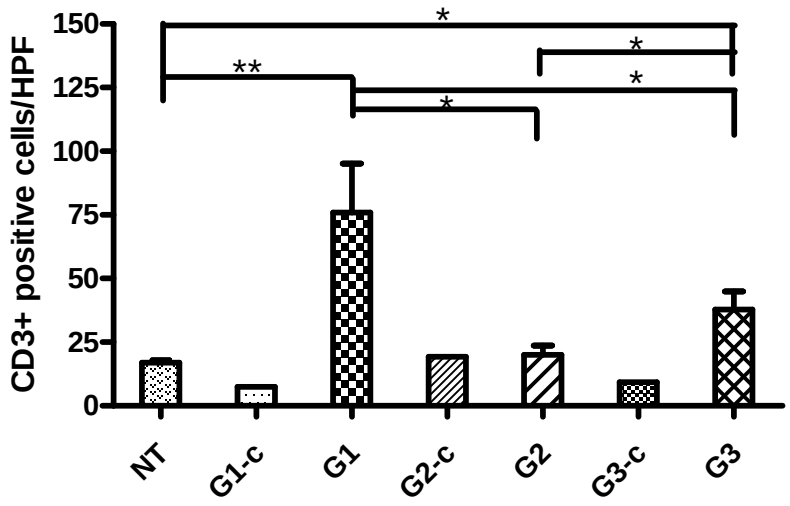
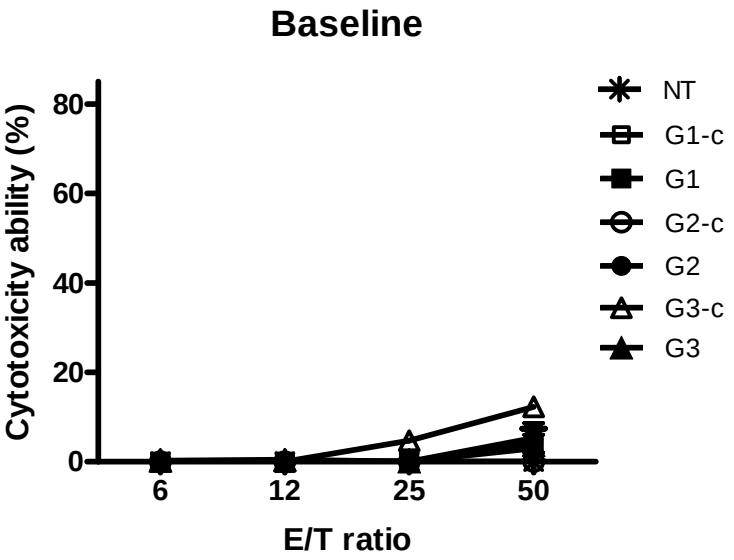


Figure 7 Immunohistochemistry staining for CD3 lymphocytes.

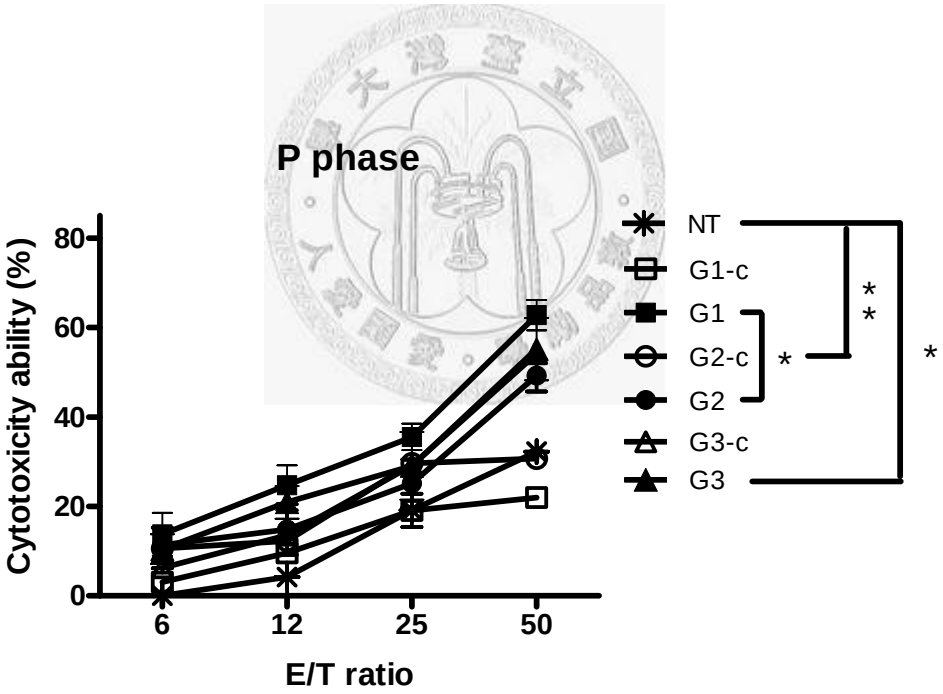
A. The immunohisotochemistry staining for CD3 lymphocytes at P phase showed that there are plenty of $CD3^+$ T lymphocytes infiltrated and clustered in CTVT in G1 (a), G2 (b) and G3 dogs (c). Only small number of $CD3^+$ T lymphocytes are observed but scattered in CTVT of vector control dogs (G1-c, d; G2-c, e; G3-c: f) and NT dogs (g). The rabbit normal serum was used as negative control (h). Original magnification, 200X and 1000X (inset).

B. Evaluation and analysis for Immunohistochemistry staining at P phase (a) and S/SR phase (b).

A.


Baseline
Cytotoxicity ability (%)
80
60
40
20
0
6
12
25
50
E/T ratio
NT
G1-c
G1
G2-c
G2
G3-c
G3

B.


P phase
Cytotoxicity ability (%)
80
60
40
20
0
6
12
25
50
E/T ratio
NT
G1-c
G1
G2-c
G2
G3-c
G3
*
*
*
*

C.

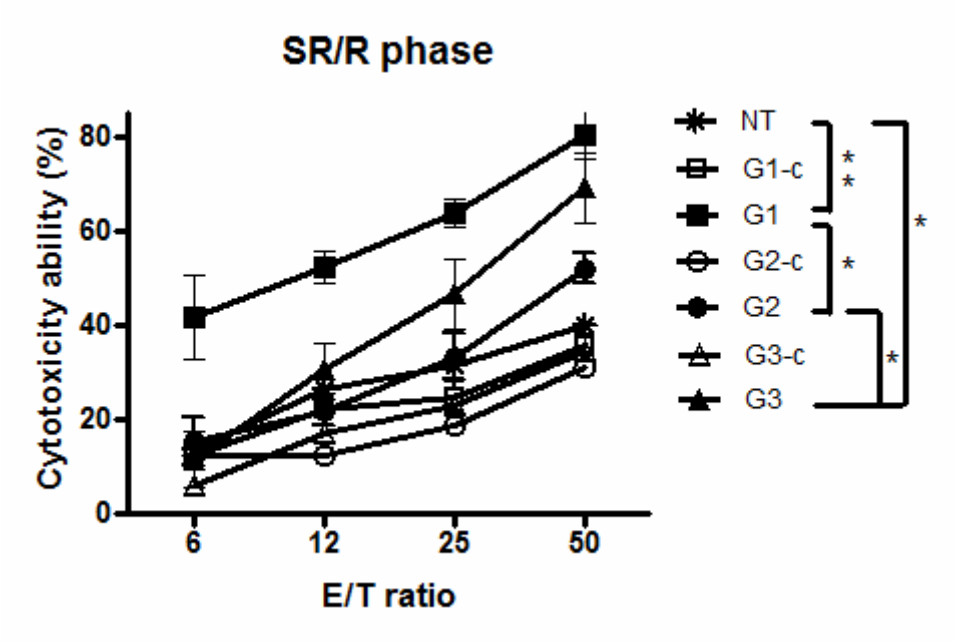


Figure 8 The cytotoxicity of PBMC to CTVT from dogs immunized the xenogeneic chHSP70 DNA vaccine were increased.

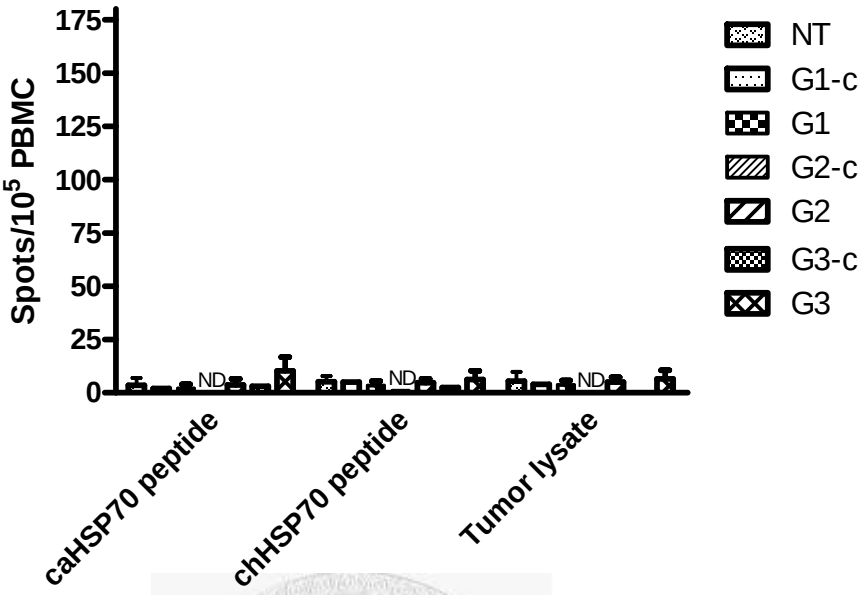
PBMC were collected as effector cells. CTVT cells were served as target cells. A non-radioisotope LDH releasing test (Cytotox 96®, Promega) was used to assay the cytotoxicity of PBMC toward CTVT.

A. PBMC from dogs from groups before experiments were collected and co-cultured with P phase CTVT cells. It indicated the baseline PBMC showed no cytotoxicity towards CTVT before any treatments.

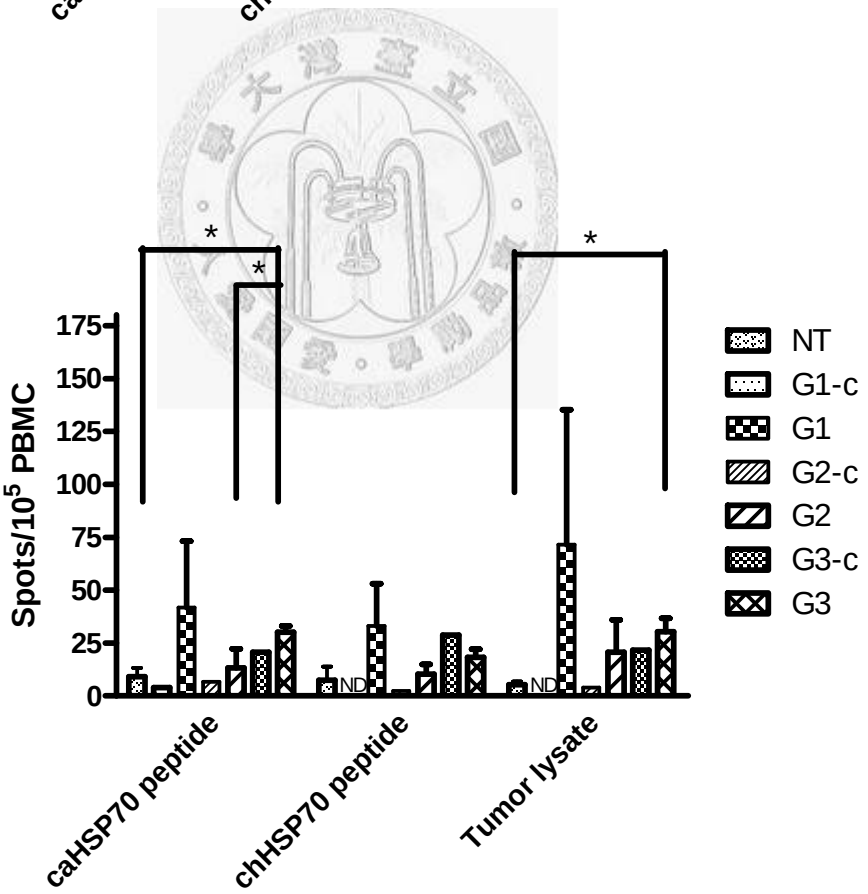
B. PBMC from dogs in each groups at P phase were collected as effector cells and co-cultured with P phase CTVT cells. It indicated that the cytotoxicity of PBMC from G1, G2 and G3 dogs were significantly higher compared with NT dogs, especially in G1 dogs.

C. PBMC from dogs at SR/R phase were collected as effector cells and co-cultured with SR/R phase CTVT cells. The cytotoxicity of PBMC toward CTVT in G1, G2 and G3 dogs were also significantly increased compared with NT dogs. *$p<0.05$, **$p<0.01$.

A. Baseline



B. P phase



C. S/SR phase

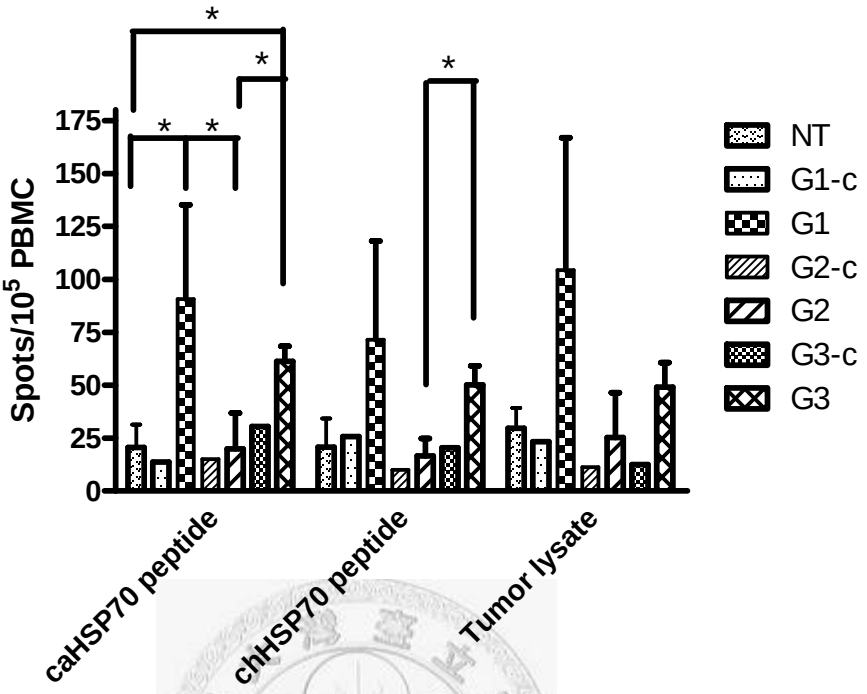


Figure 9 Canine HSP70-specific IFN-γ production at SR or R phase were significantly higher in G1 dogs than other groups.

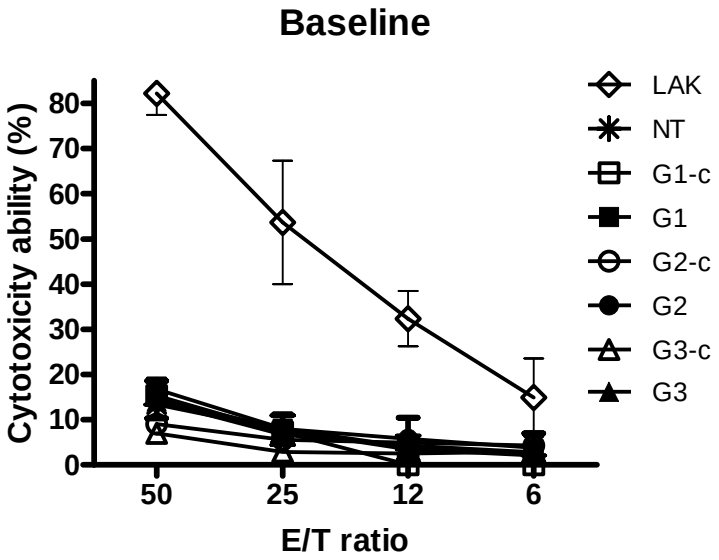
A. PBMC before experiments were collected used to evaluate the caHSP70 and *chHSP70* or tumor lysates specific IFN-γ producing cells with canine IFN-γ ELISpot kit (R&D). The quantity of specific IFN-γ producing cells were no difference between each group dogs and each antigen groups before experiments.

"ND": not detectable.

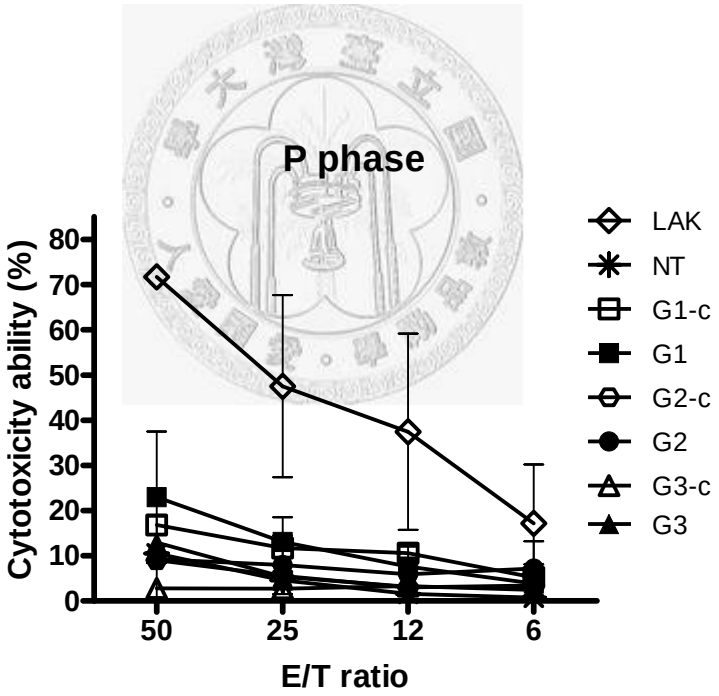
B. PBMC were collected from dogs at P phase were used to evaluate the HSP70-specific IFN-γ producing cells. The caHSP70, *chHSP70* and tumor lysates specific IFN-γ producing cells from G1 dogs at P phase were obviously higher than other groups. "ND": not detectable.

C. The evaluation of specific IFN-γ secreting cells at SR or R phase. The caHSP70 producing cells in G1and G3 dogs were significantly more than G2 and NT groups.

A.


Baseline
Cytotoxicity ability (%)
80
70
60
50
40
30
20
10
0
50
25
12
6
E/T ratio
LAK
NT
G1-c
G1
G2-c
G2
G3-c
G3

B.


P phase
Cytotoxicity ability (%)
80
70
60
50
40
30
20
10
0
50
25
12
6
E/T ratio
LAK
NT
G1-c
G1
G2-c
G2
G3-c
G3

C.

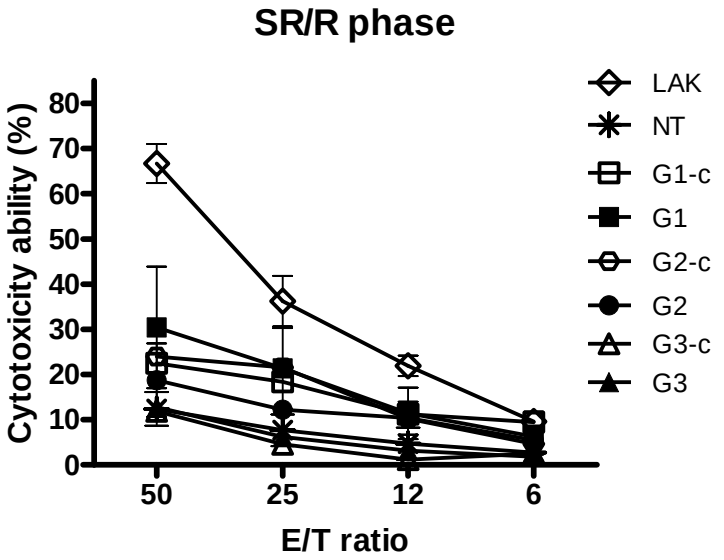


Figure 10 NK cytotoxicity in vaccination groups showed no significantly increased after three times vaccinations.

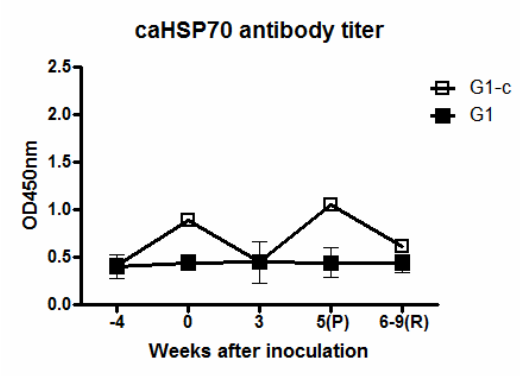
PBMC were collected to evaluate the NK cytotoxicity by a LDH releasing assay (Cytotox96®, Promega). The target of NK cells were canine thyreoid adenocarcinoma (CTAC).

A. PBMC before experiments were collected to evaluate the NK cytotoxicity.The NK cytotoxicity was no difference before experiment.

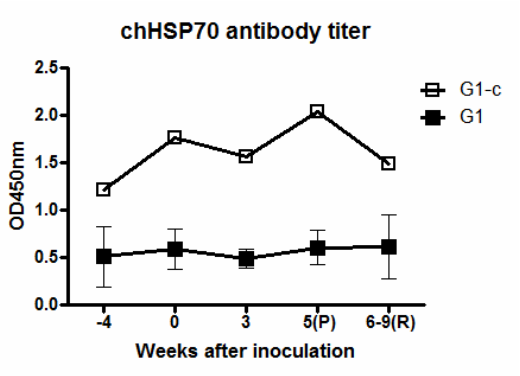
B. PBMC were collected at P phase. The NK cytotoxicity was no significant difference among groups.

C. PBMC were collected from SR or R phase were used to evaluate the NK cytotoxicity. The NK cytotoxicity was also no significant difference among groups at R phase.

A.



B.



C.

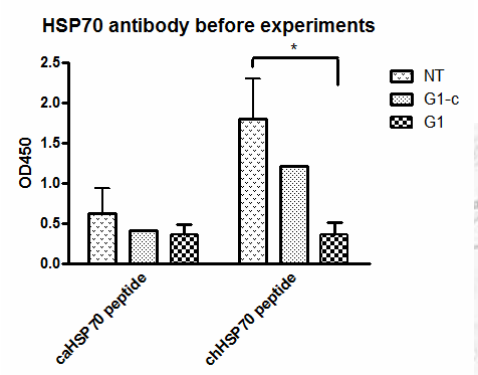


Figure 11 The humoral response against caHSP70 and chHSP70.

The caHSP70 and chHSP70 antibodies in serum from dogs were evaluated by ELISA with recombinant caHSP70 and chHSP70 peptide pools.

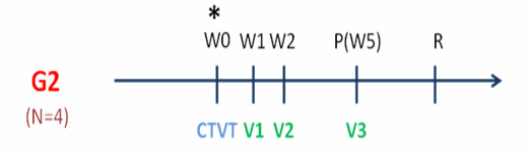
A. Evaluation of caHSP70 antibody in serum from G1-c and immunized G1 dogs at different time points.

B. Evaluation of chHSP70 antibody in serum from G1-c and immunized G1 dogs at different time points.

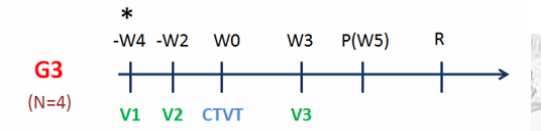
C. Evaluation of caHSP70 and chHSP70 antibodies at baseline in serum from NT, G1-c and G1 dogs.

Tables

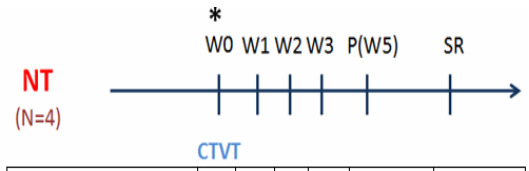
Week(Phase)	-4	-2	0	3	5(P)	(R)
Vaccination approach	TD	TD		IM+E		
PBMCs	v		v	v	v	v
Serum	v		v	v	v	v
Peripheral blood	v		v	v	v	v
Tumor cells					v	v
TILs					v	v



Week(Phase)	0	1	2	5(P)	(R)
Vaccination approach		TD	TD	TD	
PBMCs	v		v	v	v
Serum	v	v	v	v	v
Peripheral blood	v	v	v	v	v
Tumor cells				v	v
TILs				v	v



Week(Phase)	-4	-2	0	3	5(P)	(R)
Vaccination approach	TD	TD		TD		
PBMCs	v		v	v	v	v
Serum	v		v	v	v	v
Peripheral blood	v		v	v	v	v
Tumor cells					v	v
TILs					v	v



Week(Phase)	0	1	2	3	5(P)	(SR)
Vaccination approach		N	N	N		
PBMCs	v				v	v
Serum	v	v	v	v	v	v
Peripheral blood	v	v	v	v	v	v
Tumor cells					v	v
TILs					v	v

Table 1 The samples and collection time points in each experimental group.

This table showed the different samples from four experimental groups and its sampling time points. “TD” means transdermal needle-free injection. “IM+E” means injection intramuscularly followed with electroporation. “N” means non- treatment to dogs.

Antibody source	CD4	CD8
1. UC Davis	CA13.1E4	CA9.JD3
2. SeroTec	CA13.IE4	CA9.JD3
3. CMI		1.140

Table 2 The CD4 and CD8 antinodies haven been tested for immunohisochemistry staining for TILs subpopulations assay.

This table showed the all the tested CD4 and CD8 antibodies for TILs subpopulations in paraffin embedded sections. However, all of these antibodies did not work.

	Reference	NT	G1	G2	G3
RBC (10^6/μL)	5.5~8.5	7.3±1.0	5.8±0.4	5.7±0.9	6.9±0.6
Hb (g/dL)	12~18	16.8±1.9	13.9±1.1	13.4±2.3	16.5±1.4
WBC (/μL)	6000~17000	6600.0±984.9	7266.7±1026.3	6100.0±1389.2	6700±629.8
Platelet (10^3/μL)	200~900	364.5±103.9	235.3±121.6	193.8±70.6	263.0±12.7
Albumin (g/dL)	2.3~3.9	2.8±0.2	2.7±0.5	2.63±0.15	2.6±0.2
ALT (U/L)	3~50	25.3±2.0	25.3±19.9	12.3±8.2	14.0±10.4
AST (U/L)	1~37	38±7.8	38.0±3.4	36.3±3.3	33.0±2.0
BUN (mg/dl)	4.5~30.5	8.7±2.3	10.3±2.2	6.5±1.8	4.7±0.6
Creatinine (mg/dL)	0.5~1.5	0.5±0.1	0.7±0.2	0.6±0.1	0.5±0.0
Glucose (mg/dL)	67~147	87.7±12.6	85.8±21.7	95.5±19.3	115±30.5
Total protein (mg/dL)	4.8~6.6	6.3±0.3	6.3±0.4	5.9±0.3	6.3±0.6

Table 3 Toxicity study of xenogeneic *chHSP70* DNA vaccine.

The blood samples were collected and processed for the evaluation of chemical parameters by routine clinical chemistry laboratory techniques using standard equipment. The mean values obtained from the G1 and G3 dogs at P phase 2 weeks after the third vaccination. And the mean values also obtained from G2 dogs at P phase after the second vaccination.

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