A STUDY OF THE IMMUNOMODULATORY CHARACTERISTICS OF FIP-FVE PROTEIN AND ITS ADJUVANT EFFECTS IN TUMOR IMMUNOTHERAPY

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SUMMARY

Fve is a 12.7 kDa fungal protein isolated from the *Flammulina velutipes* mushroom and it has previously been reported to trigger immunological responses in both mouse and human lymphocytes. In the present study, the immunomodulatory effects of Fve on the T cells and dendritic cells (DCs) were investigated. In addition, the potential application as an adjuvant for tumor immunotherapy was explored. *In vitro* cell culture experiments showed that Fve stimulated full activation of both purified $CD4^+$ and $CD8^+$ T cells to proliferate and secrete high levels of IL-2, IFN-γ, and IL-6 accompanied by up-regulation of CD69, OX-40 and 4-1BB in the presence of accessory cells such as DCs and B cells. Trans-well studies showed that accessory cell-T cell direct interaction was important for T cell's full activation. Moreover, *in vitro* experiments showed that Fve failed to drive bone marrow-derived dendritic cell's (BM-DC) phenotypic maturation. In contrast, *in vivo* studies revealed that intraveneously injected Fve could drive splenic DC phenotypic and functional maturation as indicated by the up-regulation of MHC class II (MHC II) molecules and CD86 expression on DCs and the DC's capabilities of priming both the antigen-specific Th1-skewed $CD4^+$ cells and $CD8^+$ T cells. Notably, it was found that Fve-activated T cells could provide accessory help to induce phenotypic maturation of DC in cell contact-dependent manner. Taken together, these data demonstrated that Fve was capable of driving enhanced Th1-skewed polarization and $CD8^+$ T cells activity in antigen-specific manner. In view of this, it was hypothesized that Fve could act as a vaccine adjuvant to enhance the

immunogenicity of co-administered antigens. The proof of concept *in vitro* and *in vivo* studies were carried out with HPV type 16 E7 protein as a model antigen in tumor animal model induced by the cervical cancer related E7-expressing TC-1 tumor cells.

The results revealed that mice co-immunized with HPV-16 E7 and Fve showed increased production of HPV-16 E7-specific antibodies as well as enhanced expansion of HPV-16 E7-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells as compared to mice immunized with HPV-16 E7 alone. Tumor protection assays showed that 60% as compared to 20% of mice co-immunized with HPV-16 E7 plus Fve or immunized with HPV-16 E7 respectively remained tumor free for up to 167 days after the tumor cells challenge. Tumor therapeutic assays showed that HPV-16 E7 plus Fve treatments significantly prolonged the survival of tumor bearing mice as compared to those treated by HPV-16 E7. *In vivo* cell depletion and adoptive T cell transfer assays illustrated that $CD4^+$, $CD8^+$ T cells and IFN-γ played critical roles in conferring the anti-tumor effects. Therefore, I conclude that the pleiotropic immunostimulatory effects of Fve on innate and adaptive immune cells leading to enhanced polarization of antigen –specific $CD4^+$ and $CD8^+$ cells can be exploited to develop effective adjuvant for anti-cancer and anti-viral vaccines.

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LIST OF FIGURES AND TABLES

LIST OF PUBLICATIONS

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ABBREVIATIONS

ABTS	2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACs	accessory cells
ACT	Adoptive T cell immunotherapy
Ag	Antigen
α-GalCer	α-galactosylceramide
AIM	activation inducer molecule
alum	aluminum-based salt
AP	ExtraAvidine Alkaline Phosphatase
APC	Antigen Presenting Cell
BCG	Bacillus Calmette-Guérin
Blimp-1	B lymphocyte maturation protein-1
bp	Base Pair
BSA	bovine serum albumin
CD	Cluster of Differentiation
CD137L	CD137 ligand
cDNA	Complementary Deoxyribonucleic Acid
CFA	complete Freund's adjuvant
CFA	complete Freund's adjuvant
Con A	Concanavalin A
cpm	Counts Per Minute
CTL	Cytotoxic T Lymphocytes
CWS	cell-wall skeleton
DC	dendritic cell
EA-1	early activation antigen
EDTA	ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	fetal bovine serum

FCS	fetal calf serum
Fips	fungal immunomodulatory proteins
FNIII	Fibronectin Type III
FPLC	Fast Performance Liquid Chromatography
Fve	*Flammulina velutipes*
G418	Geneticin® selective antibiotic
GM-CSF	granulocyte macrophage colony-stimulating factor
GST	Glutathione S-Transferase
GST	glutathione S-transferase
HBSS	Hanks balanced salt solution
hPBMCs	human peripheral mononuclear cells
HPV	Human papillomavirus
HSP	Heat shock protein
IACUC	Institutional Animal Care & Use Committee
ICAM-1	Intercellular Adhesion Molecule-1
IDO	indoleamine 2,3 dioxygenase
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IgE	Immunoglobulin Epsilon
IgG	Immunoglobulin Gamma
IgSF	Immunoglobulin Superfamily
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-15	Interleukin-15
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IP-10	IFN-g-inducible 10-kDa protein
IP-10	IFN-g-inducible 10-kDa protein

IPTG	isopropyl-β-D-thiogalactopyranoside
IRF-1	Interferon Regulatory Factor-1
kb	Kilo Base Pairs
kDa	Kilo Dalton
KS	Kaposi's sarcoma
LAL	Limulus Amebocyte Lysate
LB	Luria-Bertani
LPS	Lipopolysaccharide
mAb	monoclonal antibody
MAGE-1	melanoma antigen-1
MHC	Major Histocompatibility Complex
MHC	major histocompatibility complex
MICA/B	MHC class I chain-related proteins A and B
min	minutes
MIP-1	monocyte inflammatory protein-1
MIP-1	monocyte inflammatory protein-1
MMC	Mammary carcinoma cells
MMC	mammary carcinoma cells
neu-Tg	neu-transgenic
NK	Natural Killer
NUS	National University of Singapore
ODNs	Oligodeoxynucleotides
PAP	prostate-specific acid phosphatase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PEG	Polyethylene Glycol
PerCP	Peridinin Chlorophyll Protein

PHA	phytohaemagglutinin
pNPP	ExtraAvidin-Peroxidase conjugate, para-nitrophenyl phosphate
PSA	prostate-specific antigen
RAG	recombinase activating gene
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
scFv	single-chain Fv fragments
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
STAT	signal transducer and activator of transcription
TAA	tumor associated antigen
T-bet	T-box Transcription Factor
Tc1	T Cytotoxic Type I
TCR	T-cell receptor
TCR	T cell receptor
Th1	T Helper Type I
Th2	T helper-2
TILs	tumor infiltrating lymphocytes
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor-alpha
TNFR	tumor necrosis factor receptor
Tregs	regulatory T-cells
µg	Microgram
µL	Microliter
MFI	mean fluorescence intensity

Chapter 1

Literature review

1.1 Introduction

The immune system can discriminate a range of stimuli, allowing some to provoke immune responses, which lead to immunity, or preventing some from doing so, which we call tolerance. In tumor immunology, tumor immunity or tumor tolerance refers to the success or failure of the immune system against tumors, respectively.

The origin of tumor immunology dates to 1863, when Rudolf Virchow observed leukocyte infiltration of tumors and for the first time suggested a possible relationship between inflammatory infiltrates and malignant growth. In 1909, Paul Ehrhich predicted that the immune system could repress the growth of carcinoma[1]. However, the hypothesis could not be tested experimentally because of a lack of quatititative *in vitro* techniques and the limited availability of molecular tools. Fifty years later, Burnet and Thomas proposed a new concept: "immune surveillance." They believed that tumor cell-specific antigens could provoke an effective immunologic reaction that would eliminate developing cancers[2,3]. Despite subsequent challenges to this hypothesis over the next several decades[4-9], cancer immunosurveillance was validated in a series of studies[10-12]. These studies found that antibodies and immune T lymphocytes can be detected in patients with

tumors[13-22]; tumors that have severe lymphocyte infiltration have a better prognosis than those that do not[23-38]; immunodeficient patients have an increased incidence of primary and secondary malignancies[39-47]; and tumor immunity can be demonstrated in experimental animal models[48,49].

However, spontaneous tumor eradication was rare. It originally was thought that inefficiency of tumor-associated antigen (TAA) specific immunity is due only to intrinsic cause: tumors do not represent enough tumor associated antigens; tumor antigens have low immunogenecities; antigen-presenting-cells (APCs) do not have sufficient stimulatory capacity; or there are not enough effective T cells or B cells.

Recent work recognized that pathological interactions between cancer cells and host immune cells in the tumor environment can create an immune suppressive network that promotes tumor growth, protect tumor from immune attack, and thus attenuate immunotherapeutic efficacy[11,50,51]. In the tumor-associated antigen-based immunotherapy, poor antigen-specific immunity is not due simply to the failure of TAAs passively recognized by adaptive immunity. There is an active process of "tolerization" taking place in the tumor microenvironment. These finding have led to the development of the cancer immunoediting hypothesis, a refinement of immunosurveillance that takes a broader view of immune system–tumor interaction that compass both potential host-protecting and tumor-sculpting actions of the immune systems throughout tumor development.

Therefore, successful tumor immunotherapy aims to enhance the TAA's specific

immune response not only by boosting components of the immune system that produce an effective immune response intrinsically but also by inhibiting components that may induce tolerance. Besides, tumorogenesis is a slow process that can occur over several years. Thus, how to generate an immune memory to provide long-term protection against tumors also is a key point in tumor immunotherapy.

In this chapter, I first provide evidences to support the existence of the tumor immunosurveillance as it occurs in mice and humans and three phases of immunoediting process, elimination, equilibriation, and escape. Secondly, I summarize recent work on tumor immunotherapy, including vaccination and T cell adoptive transfer. Thirdly, I review some adjuvants used in clinic trial, and finally, I summarize the objective and significance of my thesis study.

1.2 Tumor immunology

1.2.1 Historical Perspective of tumor immunosurveillance

The validity of the tumor immunosurveillance hypothesis has emerged only recently after proposed by Macfarlane Burnet and Lewis Thomas. In 1957, Burnet stated[52] that small accumulations of tumor cells may develop and provoke an effective immunological reaction with regression of the tumor. Almost at the same time, Thomas suggested that the primary function of cellular immunity was, in fact, to protect from neoplastic disease and maintain tissue homeostasis in a complex

multicellular organism[2]. Later on, several groups of investigators demonstrated: a) that the immune system of inbred mice and rats can recognize antigens expressed by tumor cells induced by chemical carcinogens; b) that such recognition results in rejection of a subsequent challenge of the same tumor in previously immunized animals; and c) that immune cells but not antibodies can mediate this reaction[53-55]. Based on these findings, Burnet defined the concept of tumor immunosurveillance in 1970 as follows[3]. In large, long-lived animals, like most of the warm-blooded vertebrates, inheritable genetic changes must be common in somatic cells and a proportion of these changes will present a step toward malignancy. It is an evolutionary necessity that there should be some mechanisms for eliminating or inactivating such potentially mutant cells and it is postulated that one of the mechanisms is of immunological character.

The proposal of the immunosurveillance hypothesis quickly was challenged by subsequent experimental tests using athymic nude mice[4,9]. They found that CBA/H strain nude mice did not form more spontaneous or chemically induced tumors, nor did they show a shortened tumor latency period compared with wild type control[5-8,56]. For example, in an experiment by Stutman, nude mice or control were injected subcutaneously with 0.1 mg of the chemical carcinogen MCA at birth and were monitored for tumor incidence[7]. After 120 days, five of 27 nude mice formed tumors at the injection site with a mean time to tumor appearance of 90 days. Of the control mice tested, seven of 39 formed tumors with a mean time to tumor appearance of 95 days. There wase no significant difference between nude mice

and their counterpart control when using different doses of carcinogen or mice with different age[8]. Moreover, these observations were supported by studies of Rygaard that showed no differences in spontaneous tumor formation in 10,800 nude mice over a study period of three to seven months[5,6]. Due to the limited understanding of immunologic defects in the nude mice at that time, these results were highly convincing and thus resulted in the abandonment of the immunosurveillence hypothesis.

We know now that there are several important caveats to these experiments that could not have been appreciated at the time. Firstly, nude mice have natural killer (NK) cells that may provide some tumor immunosurveillance capacity[57]. Secondly, the nude mouse now is recognized to be an imperfect model of immunodeficiency. These mice produce low but detectable numbers of functional populations of αβ T cells and therefore can manifest at least some degree of protective immunity[58,59]. Thirdly, the CBA/H strain mice used in Stutman's MCA carcinogenesis experiments express the highly active isoform of the aryl hydroxylase enzyme that is required to metabolize MCA into its carcinogenic form[60,61]. Therefore, it is conceivable that MCA-induced cellular transformation in CBA/H strain mice occurred so efficiently that it masked any protective effect that immunity could provide. Nevertheless, the Stutman experiments were considered to be so convincing at that time.

From the 1990s, several key findings invigorated interest in the process of tumor

immunosurveillence with the development of technologic advances in mouse genetics and monoclonal antibody (mAb) production. Firstly, endogenously produced IFN-γ was shown to protect the host against the growth of transplanted tumors and the formation of primary chemically induced and spontaneous tumors[48,62-67] Secondly, mice lacking perforin ($pfp^{-/-}$) were found to be more susceptible to MCA-induced and spontaneous tumor formation compared with their wild type[64,65,68-71]. Thirdly, gene-targeted mice that lack the recombinase activating gene (RAG)-2 definitely validated that lymphocytes play a key role in the tumor immunosurveillance[48,72]. Moreover, there was evidence showing that the IFN-γ and lymphocyte-dependent extrinsic tumor suppressor mechanisms were heavily overlapping[48].

These data, therefore, showed that components of the immune system were involved in controlling primary tumor development and overwhelmingly support the existence of an effective cancer-immunosurveillance process in mice.

1.2.2 Components of the Immmunosurveillance Network

1.2.2.1 IFN-γ in tumor immunosurveillance

IFN-γ originally was recognized for its capacity to protect naive cells against microorganism infection, but now it is known to have a critical role in protecting the host from the development of neoplasia and thus has been regarded as an obligate component in tumor immunosurveillance. In one work from Dighe,

LPS-induced tumor rejection was abrogated by injecting neutralizing monoclonal antibodies specific for IFN-γ into transplanted Mech A tumors (which are MCA-induced fibrosarcoma cells of BALB/c mice)[62]. In addition, the sarcoma induced by MCA grew more aggressively when transplanted into naive mice that had been treated with neutralizing IFN-γ-specific monoclonal antibodies. In another experiment, a similar result was found in overexpression of dominant-negative IFNGR1 mutant to Meth A tumor cells[63]. These observations then were confirmed by a primary tumor formation model. IFN-γ-insensitive mice 129/Sv mice — lacking either the IFNGR1 subunit of the IFN-γ receptor or the STAT1, which is a transcription factor responsible for mediating much of IFN-γ biological effects on cells — developed MCA-induced sarcomas at a higher rate and three-to-fivefold more frequently than did their wild-type controls[66]. In addition, mice lacking the p53 tumor suppressor gene and either IFNGR1 or STAT1 formed a tumors more rapidly than did IFN-γ-sensitive p53-deficient mice, and these mice also developed a broader range of tumor types[66]. A similar result was found in an independent experiment by using C57BL/6 mice lacking the gene encoding IFN-γ itself[64]. Furthermore, another study showed that mice deficient in GM-CSF, IFN-γ, and IL-3 developed cancer more rapidly than did mice deficient in GM-CSF alone, both GM-CSF and IL-3, or IFN-γ alone. This data indicates that IFN-γ may cooperate with other cytokines to protect the host from tumor formation[67].

Subsequent studies focused on possible mechanisms of the effects of IFN-γ in preventing tumor formation or promoting tumor elimination. In one study, the researchers found that highly immunogenic and poorly tumorigenic sarcoma from RAG2-deficient mice[48] were converted into highly tumorigenic tumor cells with poorly immunogenic when there was overexpression of IFNGR1 in the tumors[73]. Moreover, IFNGR1 deficient sarcomas (poorly immunogenic and highly tumorigenic) can be rendered highly immunogenic and were rejected when their IFN-γ sensitivity was restored by enforced expression of IFNGR1[74,75]. Together, these results indicate that the sensitivity to IFN-γ may affect tumor immunogenicity in antitumor immune response. Additional work from Shankaran's lab showed that the ability of IFN-γ to promote tumor rejection is mediated, at least in part, through its capacity to upregulate the major histocompatibility complex (MHC) class I pathway of antigen processing and presentation in tumor cells. The researchers chose IFN-γ-insensitive sarcomas (RAD.gR.28) derived from IFNGR1$^{-/-}$ 129/SvEv mice for their studies. These tumor cell lines that express low but detectable amounts of TAP1 and H-2K^b protein (H-2K^b) were stably transfected with expression plasmids encoding the 129/SvEv haplotypes of TAP1 or H-2K^b, and clones were selected that expressed high protein levels comparable to those expressed in IFN-γ-treated, IFN-γ-responsive cells. Parental RAD.gR28 cells, empty vector-transfected RAD.gR28.neo cells, and 2/2 clones of H-2K^b-transfected RAD.gR28.Kb cells grew progressively in immunocompetent mice. In contrast, TAP1-transfected RAD.gR28.TAP1 cells formed small subcutaneous masses that

expanded for the first 5–10 days but then disappeared two weeks after inoculation. Subsequent work showed that selective overexpression of TAP1 or H2-D^b increased the susceptibility of RAD–gR.28 cells to *in vitro* killing by RAD–gR.28-specific CTLs[73]. These findings indicate that augmented expression of components of the MHC class I pathway is sufficient to induce the rejection of an otherwise IFN-γ-insensitive tumor.

1.2.2.2 Perforin in tumor immunosurveillance

Perforin is a component of the cytolytic granules of cytotoxic T cells and NK cells that play an important role in mediating lymphocyte-dependent killing[49]. It is the second key factor in the renaissance of the concept of tumor immunosurveillance. In 1994, Kagi et al. found that perforin-deficient (perforin$^{-/-}$) mice have a reduced ability to control the growth of synthetic MC57G fibrosarcoma tumor cells *in vitro*[68]. Subsequent work showed that perforin$^{-/-}$ C57BL/6 mice were more prone to MCA-induced and spontaneous tumor formation compared with wild-type mice *in vivo*[64,69-71]. Moreover, BALB/c mice lacking perforin also showed a higher incidence of spontaneous lung adenocarcinomas, which was not observed in wild-type mice[65].

Taken together, these observations demonstrated that deficiencies in key immunologic molecules enhanced host susceptibility to both chemically induced and spontanesous tumors. Thus, the next question is: what immune cells protect the host from tumor development?

1.2.2.3 Effector cells in tumor immunosurveillance

The definitive studies supporting the existence of a tumor immunosurveillance process came through the use of gene-targeted mice lacking the recombinase activating gene-2 (RAG-2)[48]. RAG-2 expression is limited to cells of the lymphoid system, and its major function is to repair the breaks of double-stranded DNA. Mice lacking RAG-2 cannot rearrange lymphocytes antigen receptors and thus cannot produce peripheral T, B and NK T cells[72]. In contrast, the absence of RAG-2 does not affect DNA damage repair pathways in nonimmune cells. Thus, RAG-$2^{-/-}$ mice provide an appropriate model to exclusively study the effects of host lymphocyte on tumor development.

In the MCA-induced tumor system, 129/SvEv and C57BL/6 RAG-$2^{-/-}$ mice developed sarcoma more rapidly than stain matched wild-type mice[48,76]. In addition, helicobacter-negative RAG-$2^{-/-}$ 129/SvEv mice developed significantly more spontateous epithelial tumors than did wild type control. Specifically, 26/26 RAG-2-/- mice ranging in age from 13–24 months developed spontaneous neoplasia whereas only 5/20 wild-type mice developed spontaneous neoplasia, which was predominately benign. Thus, lymphocytes protect mice against both chemically-induced and spontaneous tumor formation.

Subsequent works have extended these findings by identifying which of the possible subsets of lymphocytes are involved in host antitumor defense. Girardi et al. found in 2001 that lack of either αβ T cells or γδ T cells increase susceptibility

to MCA-induced tumor formation in comparison to strain match wild type control[77-79]. Using a carcinogenesis model involving initiation with DMBA and TPA, 67 percent of T cell recepter (TCR)$\delta^{-/-}$ mice were tumor-bearing versus 16 percent of wild-type mice at seven weeks. By contrast, TCR$\beta^{-/-}$ mice and wild-type mice were equally susceptible to DMBA/TPA carcinogenesis[77]. Interestingly, TCR$\beta^{-/-} \times \delta^{-/-}$ mice were more susceptible to DMBA/TPA induced tumor formation than TCR$\beta^{-/-}$ mice, indicating host-protective role of αβ T cells in the setting of γδ T cells[79]. Thus, different subsets of T cells make distinct contributions to the regulation of tumor growth.

NK and NK T cells also participated in the tumor immunosurveillance. NK cells first were shown to effectively eliminate tumor cells from the circulation of mice[80,81]. Subsequent studies showed that NK cells protected the host against the initiation and metastasis of MHC-I-deficient tumor cells in an IL-12 and T cell independent manner[70,82-86]. The direct evidence that NK cells protect mice against tumors was that NK1.1 depleted mice were more susceptible to MCA-induced tumor formation than wild type mice. The roles for NK T cells in the tumor immunosurveillance were shown when Jα281$^{-/-}$ mice, which lack a large population of Vα14Jα281-expressing invariant NK T cells, were found to develop MCA-induced sarcomas at a higher incidence than their wild-type control in an IL-12 dependent manner[70]. Moreover, mice treated with the NK T cell-activating ligand α-galactosylceramide (α-GalCer) exhibited a reduced incidence of MCA-induced tumors and displayed a longer latency period to tumor formation

than control mice[87].

Additional study also showed the overlap between the IFN-γ- and lymphocyte-dependent tumor suppressor pathways. Shankaran et al. found that four lines of gene-targeted mice formed three times more similar tumors than syngeneic wild-type control when injected with a single 100 ug of MCA by comparing tumor formation in 129/SvEv mice lacking either IFN-γ responsiveness ($IFNGR1^{-/-}$ or $STAT1^{-/-}$ mice), lymphocytes ($RAG\text{-}2^{-/-}$ mice), or both RAG-2 and STAT[$RAG\text{-}2^{-/-}$ X $STAT1^{-/-}$ RkSk mice][48]. No significant differences were detected among any of the gene-targeted mice. This indicates that the IFN-γ /STAT1 and lymphocyte-dependent tumor suppressor mechanisms overlapped heavily. However, RkSk mice also developed spontaneous breast tumors that were not observed in wild-type or $RAG\text{-}2^{-/-}$ mice, therefore demonstrating that the overlap between the two pathways was not complete.

In summary, using a variety of well-characterized gene-targeted mice, specific immune system activators, and blocking monoclonal antibodies highly specific for distinct immunologic components, a large body of work overwhelmingly supports the basic tenets of the tumor immunosurveillance concept and highlights important roles for lymphocytes and cytokines in the tumor immunosuveillance in mice.

1.2.3 Tumor immunosurveillance in human

Given that there is significant evidence supporting the existence of a cancer

immunosurveillance process in mice, does a similar process exist in humans? Three lines of evidence suggest that cancer immunosurveillance indeed occurs in humans: (a) Specific antibodies and T lymphocytes can be detected in patients with tumors; (b) tumors that have severe lymphocyte infiltration have a better prognosis than those that do not; (c) immunodeficient or immunosuppressed patients have an greater incidence of primary and secondary malignancies than age-matched immunocompetent control populations.

1.2.3.1 Tumor Recognition by lymphocytes in humans

In order for the immune system to react against a tumor, the latter must have antigens that are recognized as foreign. Chemically induced or spontaneous tumors in mice, when transplanted from one syngeneic animal to another, express unique tumor-associated antigens. The TAAs of chemically induced tumors are discrete for each tumor, whereas those induced by oncogenic viruses are virus specific. TAAs are defined functionally by their ability to reject a tumor in preimmunized syngeneic mice.

In humans, the presence of Abs to TAAs and of specific[14-19] as well as nonspecific effector cells in the peripheral circulation of patients with cancer has been reported often. This implies that immune cells and Abs potentially capable of tumor rejection exist in these patients. Since the first human tumor antigen was identified in 1991[13], a large array of immunogenic human tumor antigens has been identified[20-22]. These can be classified into six groups: class I HLA-restricted

cancer/testis antigen; class I HLA-restricted differentiation antigens, e.g., melanocyte differentiation antigens, Melan-A/MART-1, tyrosinase, gp-100; class I HLA-restricted over expressed antigen, e.g., HER-2/neu; class I HLA-restricted mutational antigens, e.g., abnormal forms of P53; viral antigens, e.g., EBV and HPV; and Class II HLA-restricted antigens. T lymphocytes ($CD4^+$, $CD8^+$ αβ T cells) expressing a unique TCR recognize tumor epitopes in the context of the MHC molecules. These T cells, together with B cells producing tumor-specific Abs and dendritic cells (DCs) processing and presenting tumor epitopes, are responsible for adaptive immunity against tumors.

In addition to the tumor antigen presented on MHC molecules, transformed cells may overexpress other molecules that can function as recognition targets in the immunosurveillance process. Several studies have cited the human MHC class I chain-related proteins A and B (MICA/B) that are expressed differentially on tumor cells and function as ligands for two receptors expressed on cells of the innate immune system: NKG2D and the T cell receptor on Vα1 γδ T cells. MICA expression was found only on gastrointestinal epithelium of the stomach and large and small intestines. However, MICA/B gene expression could be induced in certain nontransformed cell lines by heat shock or viral infection[88,89]. Constitutive MICA/B expression has been documented in a high percentage of primary carcinomas of the lung, breast, kidney, ovary, prostate, and colon[90], melanomas[91], and hepatocellular carcinomas. Tumor cells expressing MICA/B are killed by effector cells with functional NKG2D receptors, and lysis can be inhibited by

pretreating the effector cell with blocking NKG2D mAb[92]. Moreover, Vα1 γδ T cells lysed the MICA-expressing target tumor cell by direct MCA binding to the γδ TCR[93].

Taken together, these observations indicate that human cancer patients indeed develop immune responses to the tumors and, in some cases, these responses may eliminate tumor from formation.

1.2.3.2 Tumor-infiltrating lymphocytes correlates with patient prognosis

In addition to the supporting data described above, there is accumulating evidence showing a positive correlation between the presence of tumor infiltrating lymphocytes (TILs) in a cancer patient's tumor and improved clinical outcome.

For example, Clemente et al. reviewed 285 primary cuteneous melanoma patients, which previously have been divided into three groups (brisk, nonbrisk, and absent, according to lymphocytic infiltrates) by Clark[24], to verify the relationship between TILs and survival. The results showed that brisk patients had higher five and, ten-year survival rates than those of nonbrisk as well as absent patients, indicating that tumor infiltrating lymphocytes are significant positive histologic prognostic factors[25]. Similar correlation between the presence of TIL and patient survival also have been made in patients with cancers of the colon[26], breast[27], bladder[28], prostate[29], ovary[30], rectum[31], esophagus[32], and neuroblastoma[23]. Moreover, other cases examined the prognostic significance of individual T-cell subsets that

infiltrate tumors[32-35]. Piras et al. reported that $CD8^+$ T cells considered as independent, favorable prognostic factors in melanoma and $CD4^+$ T cells also had similar distribution[34]. Finally, NK cell has been reported as a positive prognostic factor in breast cancer[33], gastric carcinoma[36], squamous cell lung carcinoma[37], and colorectal cancer[38].

1.2.3.3 Immunodeficient or immunosuppressed patients display increased incidences of malignancies

The third line of evidence that tumor immunosurveillance process exists in human comes from the findings in patients with primary (congenital) or acquired immunodeficiencies, since some of them live long enough to allow tumor development[39]. In some of these patients, the risk of developing cancer is increased up to 100-fold. Also, this group develops neoplasms that mostly involve the lymphoid system, including NHL, leukemia, and Hodgkin's lymphoma. Gastric carcinoma is the most frequent epithelial cancer reported in these patients. In HIV-infected individuals, several types of malignancies may occur and most of them are virus-associtated cancers. Kaposi's sarcoma (KS) and NHL are two most common neoplasms[46,47]. Human papillomavirus (HPV)-related cancers are another type of AIDS-related malignancy. There are likely to be two mechanisms by which papillomaviruses induce neoplasia — by altering the tumor microenvironment, and by directly disrupting cell differentiation, to induce cell proliferation.

Other cases are from organ transplant patients undergoing chronic immunosuppressive therapy to prevent transplant rejection. Israel Penn et al. found that transplant recipients have increased risk in virus-associated neoplasm, in pariticular B-cell derived lymphoma, skin cancer, cervical cancer, and Kaposi's sarcoma[42].

Besides, they and other groups of researchers found that increased relative risk ratios have been observed in these patients for a broad subset of tumors that have no apparent viral origin. Transplant patients were two or three times more likely to develop melanoma or non-Kaposi's sarcomas[43,44]. Furthermore, analysis of 5,692 patients who received renal transplants from 1964–1982 in Finland, Denmark, Norway, and Sweden exhibited an increased cancer incidence ratio for development of a variety of cancers, including those of lung, colon, bladder, kidney, ureter, and endocrine tumors compared to the general population[41]. In Australia and New Zealand, assessment of 925 renal transplant patients from 1965 to 1998 showed increased risk ratios for lung, colon, pancreas, and endocrine tumors, as well as melanomas[45]. Finally, when tumor incidence was examined in 608 cardiac transplant patients between 1980 and 1993 at the University of Pittsburgh, the prevalence of lung tumors was 25-fold higher than in the general population[40].

Thus, immunodeficiency and immunosuppressed individuals displayed an increased probability of developing a variety of cancers. This indicates that the immune system appears to be able to give protective immunity in preventing

human tumors.

Thus, after a century of controversy, substantial amounts of direct experimental data from mice, coupled with correlative data from humans, show that innate and adaptive immunity function together to protect the host against neoplastic disease and thereby converge on the original conviction of Burnet and Thomas: immunosurveillance exists.

1.2.4 Tumor immunoediting – refining tumor immunosurveillance

Based on the data review above, host immune responses should efficiently eliminate tumors. However, spontaneous tumor eradication was rare. More and more work realized that although the immune system constrains tumor growth, the tumor cell might escape this immune pressure. This concept evolved from the observation that tumors from immunocompetent hosts and immunodeficient hosts have different immunogenicities[48,75,94,95]. Specifically, tumors formed in the absence of an intact immune system could be more immunogenic when transplanted into wild type hosts than tumors that arise in immunocompetent hosts. This indicates that the immunogenicity of a tumor might be sculpted or edited by the microenviroment of the immune systems from which it was derived. In other words, the immune systems exert host-protecting as well as tumor promoting effects on developing tumors. Because of this dual opposing function of immunity, the term tumor immunosurveillance may no longer be appropriate to describe the process accurately. Thus, Dunn et al. proposed a broader and more comprehensive

hypothesis, “cancer immunoediting,” to replace the original “tumor immunosurveillance”[12,51].

In this hypothesis, cancer immunoediting emcompasses three phases: elimination (original concept of immunosurveillance), equilibrium (persistence), and escape (progression).

1.2.4.1 Elimination

Elimination represents the original concepts of cancer immunosurveillance. During this phase, innate immunity received the “danger” signal from the developing tumor after transformed cells have circumvented the intrinsic tumor suppressor mechanism. Macrophage, NK, NK T cells, and γδ T cells are recruited to the tumor site. NK cells and NK T cells recognize developing tumors via TCR interaction with either NKG2D ligands expressed on tumor cells, whereas γδ T cells interact with tumors cells using glycolipid-CD1 complexes. These events lead to IFN-γ production that is critical for the progression of the anti-tumor response. Specifically, IFN-γ at the tumor site induces the chemokine productions, which in turn attract more cells in the innate immune system to the tumor sites and further amplify the innate immune response to attack developing tumors. This positive feedback also could be seen in the macrophages which secrete low amounts of IL-12. IL-12 induces NK cells to secrete more IFN-γ, which can activate macrophage leading to a large amount of production of IL-12. Moreover, the production of IFN-γ can kill the tumor through antiproliferative, proapoptotic, and

antiangiostatic effects. In addition, NK cells can kill the tumor cells via perforin-dependent or TRAIL-dependent mechanisms. As a result of this process, tumor antigens released from the dead tumor cells can be recognized by DCs in the tumor sites. Antigen bearing DCs activated by cytokines and NK cells can migrate from the tumor sites to draining lymph nodes, where they activate the adaptive immunity by inducing specific $CD4^+$ T cells and $CD8^+$ T cells activation through the interaction of MHC-peptide complex and TCR. Activated tumor specific $CD8^+$ and $CD4^+$ T cells home to the tumor site, where they participate in the killing of tumor cells through direct or IFN-γ dependent mechanisms.

If the elimination phase is successful in deleting the developing tumor, it will represent a complete immunoediting process and won't proceed to the subsequent phase. If not, immunoediting will proceed to the dynamic equilibrium phase in which a continuous sculpting of tumor cells produces cells resistant to immune effector cells.

1.2.4.2 Equilibrium

In the equilibrium phase, tumor cells persist but are "equilibrated" by the immune system. This process leads to the immune selection of tumor cells with reduced immunogenicity. The evidence comes from studies in which spontaneous or chemically induced sarcomas in IFN-γ-receptor-deficient, nude, SCID mice are more highly immunogenic than tumors from immunocompetent mice[48,70,95-97]. These findings suggest that the process of host immune selection leads to

elimination of highly immunogenic tumor cells, whereas tumors with decreased immunogenicity and non-immunogenic tumor cells still grow.

Two other test results may support the existence of an equilibration phase. One comes from a mouse experiment showing that wild-type mice were tumor free after low-dose MCA administration. However, when these mice were depleted of $CD4^+$ and $CD8^+$ T cells at day 200 after administration, they rapidly developed sarcomas that have unusual growth characteristics when transplanted into naive recipients[98]. More evidence comes from clinical studies in which cancers could be transmitted from donor to transplant recipient. Specifically, two renal transplanted recipients grew metastatic melanomas one to two years after they received kidneys from the same donor[99]. Upon analysis, it was found that the donor had been treated for primary melanoma 16 years before her kidneys were donated but was considered tumor free at the time of her death. These observations suggest that the tumors that grow rapidly and progressively in an immune deficient or suppression environment had previously been maintained in the equilibrium phase by the host or the donor's competent immune system.

1.2.4.3 Escape

The final phase of immunoediting is termed escape in which tumor cells actively escape from attack of the innate and adaptive antitumor immune response through multiple immunoevasive strategies.

These include tumor-induced impairment of the antigen presentation machinery, activation of negative costimulatory signals in the tumor microenvironment (CTLA-4/B7, PD-1/PD-L1, Fas/ FasL), elaboration of immunosuppressive factors (IL-10, TGF-β, galectin-1, gangliosides, PGE_2), and overexpression of indoleamine 2,3 dioxygenase (IDO). In addition, cancer cells may promote the expansion and/or recruitment of regulatory cell populations that different regulatory cell populations contribute to this immunosuppressive network, including $CD4^+CD25^+$ regulatory T-cells (Tregs) and inducible T regulatory (Tr1) cells that impact negatively on the fate of effector T cells. Tumors can directly or indirectly impede the development of antitumor immune responses, either through the elaboration of immunosuppressive cytokines (such as TGF-β and IL-10) or via mechanisms involving T cells with immunosuppressive activities (i.e., regulatory T cells).

1.3 Tumor immunotherapy

Tumor immunotherapy is an anticancer approach in which the patient's immune system is stimulated to fight tumors. Over time, our understanding of the immune system and tumor immunology has increased. and his has enabled scientists to develop specific immunotherapies to enhance the immune response of a particular patient against unique targets.

The earliest reported efforts about cancer immunotherapy date back to 1893, when the New York surgeon William Coley noted the regression of sarcomas in patients with tumors with attack of erysipelas[100]. He felt that some of the beneficial effects

of erysipelas were due to the toxic products produced by the infections. In 1909, he reported on 24 patients with sarcoma, eight with carcinoma, and three with undefined histology but probably carcinoma or sarcoma: five sarcomas were cured and the others had a marked improvement. The results set the scene for similar clinical studies over the next four decades. Tumor regression and clinical improvement occasionally were seen; cures were very rare.

Now, the prevailing techniques of tumor immunotherapy can be divided into two broad groups called nonspecific and antigen-specific therapies. The latter can be attained either by vaccination or adoptive transfer. Vaccination means the administration of a particular antigen to induce a specific immune response. Adoptive transfer involves the physician transferring direcinto the patient the actual components of the immune system that already are capable of producing a specific immune response. Nonspecific immunotherapy refers to therapies that can stimulate the immune system by using a substance that activates or enhances immune cell function regardless of their antigen specificity. In the early days of immunotherapy, many nonspecific immunostimulants were tested as antitumor reagents in their own right, but today their use in this way has declined. The majority of these substances now is recognized for the supporting roles they play, such as enhancing cellular communication between immune cells, and therefore are being tested for use in combination with antigen-specific immune stimulation. I will review the adjuvant effects of nonspecific immunotherapeutic reagents in section 1.4. Next, I will focus on the specific immunotherapy.

1.3.1 Tumor cell-based immunotherapy

Vaccination for protection against infectious agents has been one of the most successful interventions in medicine[101]. Twenty-six infectious diseases are preventable through vaccination. However, cancer now is outstripping infectious diseases as a cause of death in developed countries, and there is an urgent need for effective ways to prevent it. In this section, a few examples of anticancer vaccination methods, including tumor-based, protein or peptide-based, DC-based, and gene-based vaccination, will be reviewed.

Before the identification of human cancer antigens, cancer vaccine approaches depended on immunization with whole cancer cells (both autologous and allogeneic preparations) or cancer cell extracts. Autologous tumor cell-based vaccine uses cells from the patient's own tumor that have been treated so that they no longer can replicate *in vivo*.

Tumor antigens that are not of a viral nature probably will be self-antigen, to which the patient would be expected to have higher levels of tolerance. Therefore, successful immunization relies on the ability of the vaccine to break tumor-specific tolerance. The basis of autologous tumor challenge is, therefore, to generate an effective immune response against what is naturally a less immunogenic cancer *in vivo*. Hence, the relevant autologous vaccine should be combined with an adjuvant. In one of the earliest trials, autologous irradiated tumor cells administered with BCG and pre-treatment low-dose cyclophosphamide to metastatic melanoma

patients showed a small but significant number of patients with responses to treatment[102]. Its clinical efficacy was improved by being conjugated to a hapten (dinitrophenyl, DNP)[103]. Later trials in clinics reported that 214 patients with clinical stage III melanoma (117 patients with stage IIIC and 97 with stage IIIB) treated with multiple intradermal injections of autologous, DNP-modified vaccine mixed with BCG had significantly longer durations of relapse-free survival and overall survival[104,105]. Based on these encouraging results, a phase I/II randomized, double-blind trial of this vaccine is ongoing[106].

Despite these successes, there also are clear technical disadvantages to this strategy. Patients must present with a resectable tumor that can be used to prepare the vaccine. Furthermore, it is not unusual for autologous tumor cells to be cultured for a period prior to use as a vaccine, often to increase the bulk of the cells. This has profound implications for patients whose disease may progress, as exemplified by a study from Dillman et al. in which a large proportion of the patients had clinical deterioration during the culture period and no longer met the inclusion criteria[107].

An alternative approach is to immunize with cells from allogeneic sources. This overcomes the requirement for tumor tissue from the patient and consequently the delay in preparation of vaccine, and provides unlimited material for vaccination.

Knight et al. showed that, in a murine melanoma model, allogeneic cell vaccination with the melanoma cell line K1735-M2 prolonged survival compared to non-immunized controls. This effect was dose sensitive as 5×10^5 cells gave 20

percent long-term protection but 5×10^4 cells gave rise to 100 percent mortality[108]. However, a lung carcinoma cell line was not able to cross-protect against melanoma[109]. This indicates that vaccination with an allogeneic tumor of the same type can induce rejection of a tumor while treatment with cell lines of a different cancer background does not. Besides, allogeneic whole tumor cell vaccines typically consist of cells from more that one tumor line in order to maximaize the antigen expression.

Specifically, a mixture of three cell lines plus a BCG named Canvaxin used to vaccinate metastatic melanoma patients showed encouraging results. Phase II trials of Canvaxin in stage IV melanoma demonstrated a five-year survival rate of 25 percent of 157 patients treated with vaccine, compared with only 6 percent of 1,521 historic controls treated with nonvaccine therapies[110]. Moreover, they also showed further immunological responsiveness in patients with DTH response[111] and humoral responses[112] correlating to clinical response. Another allogeneic vaccine, Onyvax-P, is composed of three cell lines (OnyCap23, LnCaP and P4E6) for the treatment of hormone-resistant prostate cancer[113], and test results demonstrated that 11 of the 26 patients vaccinated with Onyvax-P showed significant prolonged decreases in prostate-specific antigen (PSA) velocity. The median time to disease progression was 58 weeks, compared with historical control values of around 28 weeks. In addition, an analysis of immunological results suggested that a positive clinical outcome was correlated with a Th1 cytokine profile in combination with the reduction in PSAV.

Although there clearly are encouraging results from studies using tumor-cell vaccines, none has yet proved to be effective enough for routine use in the clinic. Tumor cells generally are poorly immunogenic, at least partly due to heterogeneous expression of costimulatory molecules and MHC[114]. A phase III trial recently was stopped as there was no evidence that survival in the treatment arm was diverging from that of the control arm, which included the BCG without the allogeneic cell component of the vaccine, itself being better than expected[115]. Therefore, in order to prime antitumor immune responses efficiently, tumor cells have been modified to express costimulatory molecules such as CD80 and CD86. Preclinical animal studies showed that that CD80-transfected tumor cells can elicit CTL responses[116] and protect against tumor challenge in curative models[117,118]. These data indicate that CD80 modification of tumor cells might have some clinical efficacy in treating patients. In the clinic, Raez et al. treated 19 patients with relapsed metastatic non-small-cell lung cancer with a whole-cell vaccine composed of an allogeneic human lung adenocarcinoma cell line transfected with CD80 and either HLA-A1 or -A2. The result showed a 32 percent clinical response rate with a median overall survival of 18 months compared with a previously reported median survival of less than one year for metastatic lung cancer patients[119]. Besides, cytokine modified tumor-cell vaccines have been used clinically. Tumor-cell vaccines modified to secrete IL-2, IFN-γ, and GM-CSF have shown clinical effectiveness. A number of cytokine-secreting tumor vaccines have been investigated in mouse models but have not yet reached the clinic, including IL15[120], IL-21[121], IL-23[122] and IL-27[123].

1.3.2 Protein or peptide based immunotherapy

The identification and molecular characterization of human TAAs has opened new approaches to the development of cancer vaccines. The first gene encoding a human TAA, the melanoma antigen-1 (MAGE-1) was reported in 1991[13]. Subsequently, many human TAAs have been identified and characterized[124]. The TAAs can be classified into six groups: class I HLA-restricted cancer/testis antigens such as MAGE-3, BAGE, GAGE, and NY-ESO-1; class I HLA-restricted differentiation antigens, e.g., melanocyte differentiation antigens, Melan-A/MART-1, tyrosinase, gp-100; class I HLA-restricted over expressed antigen, e.g., HER-2/neu; class I HLA-restricted mutational antigens, e.g., abnormal forms of P53; Viral Antigens, e.g., EBV and HPV; and Class II HLA-restricted antigen. When bound to histocompatibility complex molecules, TAAs or their peptide can be recognized by T cells and subsequently induces T and B cell activation. Therefore, animal and clinical studies were initiated to assess the therapeutic potential of active immunization or vaccination with TAAs or peptides in patients with cancers. Of all the examples mentioned above, NY-ESO-1 represents one of the most potent naturally occurring cancer antigens. With the exception of the testis, this protein is completely absent from normal tissues, hence its categorization as a CT antigen. NY-ESO-1 is found in about 30 percent of breast, prostate, and ovarian cancers as well as melanoma. These desirable features—that is, rarity in normal tissues, high immunogenecity, and significant presence in a relatively broad range of cancers—have made NY-ESO-1 a highly attractive target

for specific immunotherapy in certain cancer patients, especially those with metastatic melanoma[125,126].

Immunization with whole proteins has some advantages, as they potentially have multiple CTL epitopes and T helper cell epitopes not restricted to one HLA type. However, since most TAAs are self antigens, they have potential for induction of autoimmunity. Another disadvantage is that purified proteins elicit relatively poor immune responses on their own.

Rationally designed peptide vaccines may be able to overcome some of the limitations of whole protein vaccination. The main advantage of a peptide-based vaccine is that it provides a method for monitoring a specific immune response for a particular antigen. Other advantages are as follows: firstly, it bypasses the need for antigen-presenting cells to process a whole cell before presenting the antigen to the immune system; secondly, administration of a peptide antigen minimizes the potential for induction of autoimmunity; and thirdly, the preparation of peptides is relatively easy and cost-affordable[127]. Several studies have utilized single epitope peptides in combination with various types of vaccine adjuvants for the therapy of tumors[128-131]. However, each peptide-based vaccine is limited to the number of epitopes presented within a peptide vaccine. Lee et al. reported a case of gp100 peptide vaccination for treatment of a gp100-expressing melanoma[132]. After the vaccination, the patient was found to develop tumor progression that did not express the gp100-antigen. And, the tumor-infiltrating lymphocytes isolated from

these lesions were not reactive to gp100 but to MAGE-A12, which was not a component of the original vaccine. Besides, other important mechanisms of immune escape are equally problematic across peptide tumor vaccination strategy aimed at generating cellular immune response. Thus, multipeptide vaccines, together with MHC class II-restricted peptide[133], with a variety of different adjuvants[134-136], have been investigated in preclinical and clinical studies.

1.3.3 APC-based immunotherapy

In general, the success of vaccine strategies depends on the mode of antigen delivery, the choice of adjuvant, and the particular antigen being used. Both immunity and tolerance are controlled by a network of professional APCs, the most important of which are known as DCs [137,138]. Tissue-resident DCs that capture pathogen-encoded antigens are activated by stimuli generated in the course of a pathogen-induced inflammatory response. Activation of DCs occurs in two phases, maturation and licensing, and is an essential step that enables the antigen-loaded DCs to migrate to the draining lymph nodes. There, the DCs present the peptide to naive T cells, thereby inducing a cellular immune response that involves both $CD4^+$ T helper 1 (Th1) cells and cytolytic $CD8^+$ T cells[139,140]. In addition, DCs are important in launching humoral immunity, through their capacity to activate naive and memory B cells[141,142]. DCs also can activate NK cells[143] and NK T cells[144]. So, DCs can conduct all of the elements of the immune orchestra, and they therefore are a fundamental target and tool for vaccination.

In 1992, Inaba et al. found that mouse DCs can be cultured *ex vivo* from bone marrow precursors[145]. In a similar fashion, human DCs can be generated in culture from $CD34^+$ hematopoietic progenitors and, more commonly, from peripheral blood–derived monocytes[146,147]. Now, *ex vivo*-generated, antigen-loaded DCs have been used as vaccines to improve immunity[148]. Numerous studies in mice have shown that DCs loaded with tumor antigens can induce therapeutic and protective antitumor immunity[149]. In clinical studies, Vieweg and colleagues showed that patients with prostate cancer vaccinated with DCs transfected with mRNA encoding tumor antigens such as PSA or TERT, and patients with renal cancer vaccinated with DCs transfected with unfractionated tumor-derived mRNA, developed tumor antigen–specific $CD8^+$ T cell responses[150-153]. Banchereau et al. demonstrated that patients with metastatic melanoma who were vaccinated with DCs pulsed with several melanoma-derived peptides showed a better clinical outcome[154]. These works provided proof of the concept that using DCs as vaccines can work. Despite this, the efficacy of therapeutic vaccination against cancer has been questioned recently[155] because of the undeniably limited rate of objective tumor regressions that have been observed in clinical studies so far. A phase III clinical trial in patients with stage IV melanoma failed to demonstrate that DC vaccination provided increased benefit compared with standard DTIC chemotherapy[156]. The overall response was low in both patient groups (DTIC, 5.5 percent; DC vaccinated, 3.8 percent)[156]. However, the question is not whether DC vaccines work but how to orient further studies to refine the immunological and

clinical parameters of vaccination with DCs to improve its efficacy. It is conceivable that the suboptimal nature of the cytokine cocktail maturation protocol might have had an important role in the failure of the trial. Pathogen-mediated maturation of DCs is done mainly through the Toll-like receptors (TLRs) that are expressed on immature DCs and activated in response to distinct microbial compounds, PAMPs[157]. Cytokines, such as TNF-α, IL-1, and IL-6, also are capable of promoting DC maturation but cannot substitute for TLR stimulation[158]. The most widely used maturation protocol for human monocyte-derived DCs consist of four reagents, TNF-α, IL-1β, IL-6, and PGE2, also known as monocyte conditioned media mimic or cytokine cocktail, that were used in above mentioned clinical trial. The rationale for including PGE2 in the maturation protocol is to endow the *ex vivo*-generated DCs with the capacity to migrate[159], but PGE2, in the context of the tumor microenvironment, can mediate Th2 polarization and promote the differentiation of DCs secreting the immunosuppressive cytokine IL-10[160]. Therefore, the key negative impact of PGE2 on the function of *ex vivo*-generated DCs probably is the primary reason for the failure. In addition, it is important to appreciate the fact that a DC vaccination protocol is a complex, multistep process and that a myriad of seemingly trivial steps such as how the cells are frozen and thawed, how long the cells are matured, at what speed they are centrifuged, the quantity and timing of antigen they load, the mechanics of their administration, and the time intervals between boostings can have a critical impact on the outcome of

the treatment. Therefore, the complexity of the DC system requires rational manipulation of DCs to achieve protective or therapeutic immunity.

Improving the maturation protocol is a central challenge to improving the DC vaccine. Several rapid, two-day "fast-DC" protocols have been developed that generate DCs able to stimulate T cell responses *in vitro* as effectively as DCs generated by standard protocols, which usually require seven to nine days of culture[161,162]. In a recently published clinical trial, HER2/neu-positive breast cancer patients vaccinated with peptide-loaded DCs generated in a two-day culture of monocytes incubated with IFN-γ and LPS induced HER2/ neu-specific $CD4^+$ and $CD8^+$ T cell responses and measurable decreases in tumor volume[163]. Another alternative to optimizing the *ex vivo* DC maturation process is to inject antigen-loaded *ex vivo*-generated immature DCs into sites that have been pretreated with adjuvant to induce a local inflammatory reaction[164].

The forms of antigens presented by DCs also could be optimized. Traditionally, there are two forms of antigen loading to the DCs[165]. Firstly, antigens can be added exogenously, as peptides, whole proteins, tumor lysate, or apoptotic debris. Secondly, the DCs can be engineered to synthesize it endogenously by transfection with mRNA or cDNA encoding the antigen.

Exogenous provision of short peptides presented by the MHC class I and MHC class II molecules used to be the favorite form of antigen to load DCs[166]. Such peptides are synthesized by chemical means and are readily available for clinical

use. However, the logistical advantage of using peptides is offset by the need to determine the MHC haplotype of the patient, the paucity of known tumor-specific peptides, and the limited persistence of peptide-MHC complexes on DCs[165]. Whole antigen is more useful because it is likely to contain peptides that can be presented effectively by most MHC molecules[166]. However, use of protein-based antigens to load DCs is limited by access to clinical grade reagents. Another approach is to use overlapping long (20–25 aa) peptides covering most, but not necessarily all, of the coding sequence of the tumor antigen. This approach should provide both MHC class I and MHC class II epitopes, does not require knowledge of an individual's MHC haplotype, and seems to be highly effective[167].

The use of antigens encoded by nucleic acid, either cDNA or mRNA, also is attractive because their isolation and use in clinical settings is more straightforward than the use of exogenously provided peptides and proteins[165]. However, transfection of DCs with cDNA encoding antigen has not proven effective for loading DCs with the antigen[168]. By contrast, transfection of DCs with mRNA that encodes antigens has turned out to be an efficient method in both preclinical and phase I/II prostate cancer and renal cancer clinical trials[169]. One main drawback of transfection as an approach to expressing tumor-specific antigens in DCs is that the antigen is channeled primarily into the MHC class I presentation pathway, limiting the generation of effective $CD4^+$ T cell responses[170]. One approach to rechannel cytoplasmic antigens into the MHC class II presentation pathway is to fuse the antigens with a lysosomal targeting signal[171]. This approach has been shown to

enhance $CD4^+$ T cell stimulation *in vitro*[172] and augmented the induction of $CD4^+$ T cell responses in vaccinated patients[151].

In summary, DCs as APCs have been very well established and accepted in mice; multiple clinical trials have been carried out targeting different cancer using different methods of generating DCs, different antigens, and different antigen-loading techniques[173]. The *ex vivo* strategies should help to identify the parameters for *in vivo* targeting of DCs, which is the next step in the development of DC-based vaccination. In addition, DC is not the only professional APC to use in the setting of cancer vaccination with *ex vivo*-derived APC. A recent study has provided provocative evidence that monocytes loaded with antigens can do just about everything that DCs do — migrate to the lymph node and stimulate potent T cell immunity[174]. Even more intriguing are B cells. An increasing number of studies have shown that B cells loaded with antigens can stimulate robust T cell response *in vitro* and, more tellingly, *in vivo* in mice[175]. Human γδ T cells also have been shown to function as potent APCs *in vitro*[176]. A key advantage that B cell or γδ T cells–based APC has over DC and monocyte is that these two cell types can be expanded easily *in vitro*.

1.3.4 DNA vaccine

DNA vaccine based on simple vehicles for *in vivo* transfection and antigen production is a recently developed approach that has the potential to elicit strong

and long-lasting immunity while also having several practical advantages over other vaccines[177-180].

In the early 1990s, a number of animal model studies first indicated success in the delivery system and protein expression of vaccinating with DNA preparations[181,182]. Wolff et al. found that intramuscular injection of naked DNA led to the expression of the encoded gene by myofiber cells[181]. Subsequent study by Ulmer and colleagues demonstrated that intramuscular delivery of DNA encoding influenza protein nucleoprotein A antigen could elicit a $CD8^+$ T cell immune response protective against infection[183]. This study provided the rationale to develop DNA vaccines for therapy of diseases, including cancer.

With time, a number of murine systems have shown prophylactic and therapeutic successes in reducing the spread of cancer through DNA vaccines. And the major advantage of DNA vaccines is that both cellular (including $CD4^+$ and $CD8^+$ T cells) and humoral immune responses can be induced because the encoded antigen is processed through both endogenous and exogenous pathways, and peptide epitopes are presented by both MHC class I and class II complexes[184].

Amici et al. showed that DNA vaccine induced a Th1 skewed immune response in a transgenic FVB/neu mouse model[185]. Lindencrona et al. found that DNA vaccine encoding HER-2 and GM-CSF can induce Her-2 specific tumor immunity, the anti-tumor effect relied on by both $CD4^+$ and $CD8^+$ T cells. And IFN-γ were crucial cytokines during tumor rejection[186]. Kontani et al. showed that mice immunized

with a DNA vaccine encoding MUC1 polypeptide were able to reject a challenge with MUC1-transfected syngeneic tumor cells[187]. Vaccination against PSA using a DNA vaccine has been investigated as immunotherapy treatment of prostate cancer. Pavlenko et al. demonstrated that vaccination with plasmid vector carrying the PSA gene results in PSA-specific cellular responses and protection against tumor challenge[188]. Thus, a phase I trial of a PSA DNA vaccine was undertaken in patients with hormone-refractory prostate cancer. Among eight patients, a PSA-specific cellular immune response and a rise in anti-PSA IgG were detected in two patients after vaccination in the highest dose cohort.

In addition, DNA vaccine encoding virus antigens, which are the cause of transformation in a number of tumors, also has been explored in preclinical and clinical trials. For example, Chen et al. showed that DNA vaccines encoding HPV virus oncogene E7 or E7 fused to LAMP-generated potent antitumor immunity in the liver and lung metastases models[189]. Another study showed that animals that received DNA expressing the L1 gene of HPV type 16 intramuscularly, subcutaneously, and orally developed systemic anti-L1 IgG antibodies. Specific IgA antibodies also were found in vaginal washes from immunized mice. CTL activity mediated by $CD8^+$ cells also was achieved. Challenged with a melanoma cell line engineered to express the HPV16-L1 protein, mice showed a slower tumor growth rate and a longer survival time[190]. Other vaccines encoding E6 and E7 epitopes or whole proteins have been used successfully prophylactically and therapeutically in animal models[191,192]. In a phase I clinical trial, a therapeutic

HPV16 DNA vaccine, ZYC101, was tested against anal dysplasia. This DNA vaccine encoded four multiple HLA-A*0201 epitopes from the E7 protein. Ten of twelve subjects demonstrated increased immune responses to the relevant epitopes[193]. A more comprehensive DNA vaccine ZYC101a subsequently was in a phase 2/3 clinical trial for cervical dysplasia[194]. This vaccine is composed of complete HPV-encoding sequences contained within ZYC101, in addition to regions encoding segments of HPV-16 and HPV-18 E6 and E7 viral proteins. The results showed that ZYC101a was well tolerated in all patients and can promote the resolution of CIN 2/3 in women younger than age 25.

In summary, DNA vaccines hold considerable promise as a useful tool to prevent or treat tumorigenic growth and subsequent metastasis. However, several hurdles need to be overcome before DNA vaccines reach clinics widely. Firstly, it's difficult to choose the proper antigen for DNA immunization. The oncogenic process normally is the result of tissue outgrowth with self-antigen over-expression such as CEA and MUC1. Cells producing these molecules as a result of transfection with the vaccine DNA, such as myocytes, keratinocytes, or DC, would be expected to produce these molecules in the form expressed on normal cells rather than the form produced by tumor cells and thus promote autoimmunity rather than tumor immunity. Secondly, tumor environment might be conducive to evade the immune attack by various mechanisms to lead finally to tumor evasion. Thus, in the future, additional tactics must be employed in order to achieve an optimum immune response to initiate effective long-lived immunity.

1.3.5 Adoptive transfer of T cells

Adoptive T cell immunotherapy (ACT) is a treatment strategy for cancer in which T-cells derived from tumor-bearing hosts are activated and numerically expanded *ex vivo*, and then reinfused into the host with a goal of eliminating a tumor and preventing its recurrence[195-199]. Early attempts of ACT therapies using TILs and an immunoreplete patient met with some success. Pioneering investigation in the 1970s by Chester Southam and colleagues demonstrated that subcutaneous growth of human tumor autografts to patients bearing advanced cancers was inhibited by the cotransfer of autologous leukocytes in about half of the patients[200]. This finding suggested that T lymphocytes with a specific inhibitory effect on the implantation and growth of cancer cells were present in many patients and could be used as potential candidates for adoptive immunotherapy.

Later, adoptive T-cell therapy was established in murine models using *ex vivo*-expanded splenocytes as an effective antitumor strategy against syngeneic tumor. The antigen-specific $CD8^+$ T cells were identified as an essential immune effector in several studies[201-203]. In some cases, $CD4^+$ T cells also were found to play an important helper role[204,205] or $CD4^+$ T cells alone were sufficient to mediate an antitumor effect[206,207]. In the clinic, adoptive T cell therapy for cancer focuses mainly on therapy with CTLs, TILs, engineered T cells, and the use of adoptive T cell transfers to facilitate therapeutic cancer vaccines.

At present, there is a plethora of suitable CTL targets for many tumors[208]. In one

clinical study, CTLs derived from PBLs were used to treat patients with refractory, metastatic melanoma, and eight of the 20 patients had antitumor immune responses[209]. Furthermore, the infusion of autologous melanoma-associated antigens recognized by T cells specific (MART-1–specific) $CD8^+$ T cells into a patient with metastatic melanoma resulted in T cell infiltration into both the skin and tumor tissue[210]. These results were confirmed in an independent trial in which engraftment of the CTLs, which specifically bind tetramers loaded with MART-1 peptides, was detectable up to two weeks after T cell transfer in all patients, with a maximal frequency of 2 percent of the total $CD8^+$ T cells[211]. Despite this high level of engraftment in all patients, only three of 11 patients had clinical antitumor responses, and a selective loss of MART-1 expression in lymph node metastases in both of two evaluated patients was observed[211]. Therefore, perhaps the most worrisome issue revealed with CTL transfers is the emergence of antigen escape variants[212,213].

Another adoptive transfer therapy strategy is to transfer TILs isolated from fresh patient biopsy specimens. The adoptive transfer of these cells showed promise in preclinical models[214], but clinical experiences almost uniformly were disappointing[215-217]. However, two recent studies suggest that prior host conditioning with chemotherapy increases the response to adoptive immunotherapy with TILs[218,219]. When 13 patients with progressive metastatic melanoma were given cyclophosphamide and fludarabine, a drug regimen that is immunosuppressive but does not have anti-melanoma efficacy, six of the patients

had objective clinical responses to treatment and four others demonstrated mixed responses, with significant shrinkage of one or more metastatic deposits[218]. This approximately 50 percent objective response rate was confirmed in a subsequent report from the same group[219]. Moreover, an alternative vaccine adoptive transfer approach may circumvent many of the limitations posed by adoptive transfer using TILs. Chang and coworkers carried out a phase I clinical trial in patients with either advanced melanoma or renal cell carcinoma[220]. Patients were vaccinated with irradiated autologous tumor cells together with adjuvant BCG. Seven days later, draining LNs were removed for activation with anti-CD3 mAb, followed by expansion in IL-2. Activated LN cells were administered intravenously with the concomitant administration of IL-2. Among the 11 patients with melanoma, one had a partial tumor regression, and among the 12 patients with renal cell carcinoma, there were two complete and two partial tumor regressions[220], suggesting that there might be some clinical benefit to this adoptive transfer approach to boost the clinical efficacy of therapeutic cancer vaccines.

Genetic modification of T cells to engineer improved antitumor effects is an attractive strategy in many settings[221]. Introduction of a chimeric antigen receptor composed of an antibody-based external receptor and the intracellular domains for T-cell signaling provides an efficient means of redirecting T-cell responses to surface tumor antigens. These constructs can function to retarget T cells *in vitro* in an MHC-unrestricted manner to attack the tumor while retaining MHC-restricted specificity for the endogenous TCR. Three pilot clinical trials recently have been

reported. The results showed that the approach was safe, but poor expression and persistence of the transgene encoding the T body receptor[222] or poor persistence of the T cells was observed *in vivo*[223]. Methods to enhance survival of adoptively transferred T cells include the introduction of costimulatory molecules[224] or chimeric cytokine receptors composed of an extracellular domain (e.g., GM-CSF receptor) and the intracellular signaling molecule for the IL-2 receptor to allow antigen-driven, helper-independent proliferation[225]. Besides, strategies to improve effector function may be achieved by countering inhibitory proteins expressed by T cells, such as CTLA-4, src homology phosphatase-1, suppressor of cytokine signaling, or cbl-b[226]. Thus, the means to transduce T cells efficiently and to maintain transgene function in T cells for adoptive transfer represent surmountable challenges, and several promising clinical studies already are under way to evaluate the use of genetically modified T cells in cancer.

In summary, vaccine and adoptive T-cell therapy provides a rigorous means of dissecting the requirements for successful immunotherapy and is an increasingly feasible treatment modality for patients who have cancer. With recent scientific and technologic advances, lessons learned from murine models gradually are being translated into clinical trials. Although the majority of studies have been performed in patients who have advanced disease, immunotherapy is particularly well suited for prevention and for treatment of patients who have low tumor burden. The convergence of early disease detection and antigen-specific immunotherapy may lead to safe and effective strategies for long-term tumor immunoprotection. For the

treatment of established tumors, the development of strategies to undermine tumor immune escape mechanisms and the integration of adoptively transferred antigen-specific T cells in combination with chemotherapy, anti-angiogenesis agents, or other targeted molecules will enhance the prospect of achieving and maintaining clinical responses in patients who have otherwise refractory disease.

1.4 Adjuvants – strategies for optimizing vaccine for tumor immunotherapy

Adjuvants (from the Latin, adjuvant = to help) are substances or formulations that have been used to improve vaccine efficacy from the early 1920s[227,228]. It has been proposed that adjuvants may act in one or more of five discrete ways[229]. A summary of these is given below[230].

(i) Immunomodulation describes the ability of an adjuvant to modify the cytokine network. A high degree of immunomodulation can result in a more rapid response, a quantitatively greater response (or less antigen to achieve an equivalent response), and the selection of a Th1 or Th2 response.

(ii) Presentation describes the ability of an adjuvant to maintain the essential native configuration of an immunogen for presentation to B cells. A high level of presentation should result in an increased proportion of neutralizing antibody, that is, antibody that recognizes native epitopes. It is possible that the duration of the immune response also may be increased, as a result of an increased availability of

native antigen to dendritic cells.

(iii) Cytotoxic T-lymphocyte (CTL) induction describes the ability of an adjuvant to deliver antigen in sufficient quantity to the cell cytosol of an antigen-presenting cell to permit class 1 processing and subsequent induction of a cluster of differentiation of CTL responses.

(iv) Targeting defines the ability of an adjuvant to deliver antigen more efficiently, and even selectively, to immune effector cells. There are many ways in which this can be achieved, including use of optimal particle size to achieve draining and trapping in lymph nodes, and targeting specific receptors, e.g., the mannose receptor on macrophages.

(v) Depot generation describes the ability of an adjuvant to retain antigen at the dose site. Short-term depots usually release antigen over one to two weeks; long-term depots may retain antigen for months. Depots increase the efficiency of delivery of antigen to an APC by minimizing losses due to liver uptake and detoxification.

Adjuvants can be classified according to their source, mechanism of action, or physicochemical properties. Alison[231] classified adjuvants into three groups: (i) active immunostimulants, being substances that increase the immune response to the antigen; (ii) carriers, being immunogenic proteins that provide T-cell help; and (iii) vehicle adjuvants, being oil emulsions or liposomes that serve as a matrices for

antigens as well as stimulating the immune response. An alternative adjuvant classification divides adjuvants according to administration route, namely mucosal or parenteral. A third classification divides adjuvants into particulate or nonparticulate adjuvants[229]. A fourth, more recently proposed, system of classification divides adjuvants into the following groups: gel-based adjuvants, tensoactive agents, bacterial products, oil emulsions, particulated adjuvants, fusion proteins, or lipopeptides[232].

At present, there are only four adjuvants worldwide that are approved for clinical use: aluminum-based salt (alum), Imiquimod (Aldara), Incomplete Freunds (IFA) and a squalene oil–water emulsion (MF59). Although many other adjuvants have been proposed over the years and proved to be effective in animal models, these have failed to be successful in humans largely because of toxicity, stability, bioavailability, and cost.

For the development of a vaccine for cancer immunotherapy, the prerequisites for an ideal cancer adjuvant differ from conventional adjuvants for many reasons. First, the tumor Ags usually are self-derived and therefore are poorly immunogenic. Second, the patients who will receive the vaccines are immuno-compromised because of, for example, impaired mechanisms of antigen presentation, non-responsiveness of activated T cells, and enhanced inhibition of self-reactivity by regulatory T cells. Third, tumors develop escape mechanisms to avoid the immune system. Thus, adjuvants for cancer vaccines, especially therapeutical

vaccines, need to be more potent than for conventional infectious vaccines. In summary, the ideal cancer adjuvant should rescue and increase the immune response against tumors in immuno-compromised patients, with acceptable profiles of toxicity and safety. Next, I will review some main adjuvants used currently in ongoing anticancer clinical trials.

1.4.1 Aluminum compounds

Aluminium adjuvants have been used in practical vaccination for more than half a century to induce early, high-titer, long-lasting protective immunity. In general, aluminium adjuvants are regarded as safe when used in accordance with current vaccination schedules[233]. Thus, they are the most widely used adjuvants in human vaccines.

The adjuvant activity of aluminium compounds (aluminium phosphate or hydroxide) first was described by Glenny and colleagues in 1926. Glenny observed that injecting the diphtheria toxoid as an alum precipitate led to a significant increase in the immune response against the toxoid[234,235]. Vaccine preparations based on this approach are called alum-precipitated vaccines. However, one report from Holt found that such preparations could be highly heterogeneous, depending on which anions (such as bicarbonate, sulfate, or phosphate) were present at the time of precipitation[236].

In 1931, Maschmann found that aluminium-hydroxide hydrated gels have the

ability to adsorb protein antigens from an aqueous solution, and these gels can be preformed in a well-defined and standardized way[237]. Vaccine preparations based on this approach are called aluminium-adsorbed vaccines. In 1946, Ericsson devised a method by which diphtheria toxoid was coprecipitated into a matrix of aluminium phosphate[238]. Holt, shortly after, demonstrated that preformed aluminium phosphate, prepared from equimolar amounts of aluminium chloride and trisodium phosphate, acted as an adsorbant and was adjuvant active wisth diphtheria [239]. At present, preformed hydroxide and aluminium phosphate gels has almost completely replaced the alum precipitation in vaccine preparations. Occasionally, the word “alum” is used to describe both aluminium hydroxide and aluminium phosphate gels.

The immunostimulating effect of the aluminium adjuvants is highly complex and the mechanism is not very well understood. It now is generally accepted that aluminium adjuvants are Th2 stimulators because they mainly induce Th2-type immune response for many antigens, and are particularly effective in stimulating IgE and IgG1 production[240]. And they are not capable to induce cytotoxic T cells and are poor at enhancing delayed hypersensitivity reactions[241]. Thus, alum is not likely to protect against diseases for which Th1 immunity and MHC class I restricted CTL are essential for protection, e.g., cancer or tuberculosis. However, it is interesting to note that a complex between Al(OH)3 and IL-12 (Al(OH)3/IL-12) induced a Th1 response rather than a Th2 reponse when used as an adjuvant[242], and the Th1-promoting effect of the Al(OH)3/IL-12 complex was augmented greatly by

the coadministration of exogenous IL-18[243]. In a recent clinical trial, 60 patients with high-risk resected melanoma were randomized to receive antigen (melanoma peptides gp100, MART-1, and tyrosinase) with either IL-12/alum or IL-12/GM-CSF[244]. The result showed that alum with IL-12 induced sustained immune responses and the generation of functional IFN-γ–secreting CD8 T cells as compared to IL-12/GM-CSF[244]. Therefore, a combination of alum with other adjuvants might be a new promising strategy for future cancer immunotherapy.

1.4.2 Mycobacterium bovis Bacillus Calmette-Guérin

A variety of bacterial cells and their fractions have been used as adjuvants for the host stimulation against cancer in both preclinical and clinical situations. The mycobacterium bovis bacillus Calmette-Guérin (BCG) strain is a tuberculosis vaccine strain that is almost nonpathogenic, yet retains the immunogenic properties of tuberculosis[245]. Several reports have suggested that phagocytosis of BCG mycobacteria is a potent inducer of maturation of DCs. Infection of DC with BCG facilitated secretion of inflammatory cytokines, including TNF-α, IL-1β, and IL-12, and up-regulation of CD40, CD80, CD83, CD86, and MHC class I molecules. Immature dendritic cells (iDCs) exhibit potent antigen-presenting ability through uptake of BCG[246,247]. Thus, BCG mycobacteria may serve as an adjuvant in cancer therapy.

In a clinical cancer study, patients were immunized with autologous colon-tumor/BCG vaccine or autologous colon tumor alone. PBL from 64 percent

of patients immunized with the autologous colon-tumor/BCG vaccine responded to the CTAA 28A32-32K antigen, which is a colon-associated tumor antigen, whereas only one responded to the CTAA28A32-32K antigen[248]. In another multicenter, randomized controlled phase III clinical trial, stages II and III colon cancer patients were randomized to receive either OncoVAX therapy (autologous tumor cells with BCG vaccine) or no therapy after surgical resection of the primary tumor. Analysis of prognostic benefit in the pivotal phase III trial, with a 5.8 year median follow-up, showed that a beneficial effect of OncoVAX is statistically significant for all endpoints, including recurrence-free interval, overall survival, and recurrence-free survival in Stage II colon cancer patients. For melanoma immunotherapy, Berd et al. treated 64 patients with metastatic melanoma using a melanoma vaccine (irradiated tumor cells mixed with BCG) preceded by low-dose cyclophosphamide, and monitored immunologic effects and antitumor activity. Of 40 assessable patients with measurable metastases, five had responses, four complete and one partial, with a median duration of 10 months[249].

Besides, intravesical BCG is an effective therapeutic option for patients with superficial bladder cancer. A number of randomized trials have been done to evaluate the benefit of adjuvant intravesical BCG in intermediate and high risk bladder cancer patients after transurethral resection (TUR). Several systematic reviews[555,556] of these data indicated that BCG adjuvant following TUR significantly reduced the risk of recurrence in patients with superficial bladder cancer compared to TUR alone.

These studies support the idea that BCG may act as an effective adjuvant for the active immunotherapy of cancers. To further explore effective components of BCG that are responsible for antitumor immunity or the mechanisms by which BCG can potentiate the host immune system, it is recognized that the host immune responses are enhanced by complete Freund's adjuvant (CFA) containing dead mycobacterial cell wall. Azuma et al. reported that the active immunoadjuvant component in mycobacteria is cell-wall skeleton (CWS) and examined in detail the biochemical and immunologic properties of these CWS fractions[250,251]. The cell wall skeleton of BCG-CWS, consisting of a peptidoglycan covalently linked to arabinogalactan and mycolic acids, was identified as an agonist for TLR2 and TLR4[252]. Now, BCG-CWS has been used as an effective adjuvant for patients with various cancers, showing possible antitumor effects without any major adverse effects[253,254].

1.4.3 Toll-like receptor ligands

1.4.3.1 Lipopolysaccharides and its derivate MPL

Lipopolysaccharide (LPS), a major constituent of the Gram-negative bacterial outer membrane, can trigger a variety of inflammatory reactions, including the release of proinflammatory cytokines and other soluble factors. The LPS molecule is composed of a highly conserved lipid A, a core oligosaccharide, and O-antigen[255]. The lipid A moiety is responsible for the proinflammatory properties of LPS, and therefore is a target for the development of medical therapies for the treatment of cancer. Recognition of LPS occurs largely by the mammalian LPS receptor — the

TLR4–MD2–CD14 complex — which is present on many cell types, including macrophages and dendritic cells[256]. *In vivo*, LPS has been shown to induce the maturation and survival of dendritic cells[257] and trigger the release of many proinflammatory cytokines, in particular TNF-α, IL-1β, and IL-6.

In 1943, Shear et al. first demonstrated that LPS had an antitumor effect against mouse tumor[258]. Later, Parr et al. showed that Lipid A had a similar antitumor effect as whole endotoxin[259]. Since then, various types of Lipid A have been tested either alone or as adjuvant in therapeutic anticancer vaccine in clinical trials. Specifically, Mitchell demonstrated clinical responses in melanoma patients given subcutaneous injection of a melanoma vaccine consisting of allogeneic melanoma cell lysates plus DETOX adjuvant (consisting of MPL and purified mycobacterial cell-wall skeleton). Of the 106 evaluable patients, 20 had objective clinical regression of tumor masses, five with complete remission[260]. In another clinical trial, 38 patients with resected stage III melanoma were immunized with a polyvalent melanoma antigen vaccine mixed with DETOX. A non-randomized control group of 35 patients was treated similarly with vaccine plus alum. Although the antibody response rate was significantly higher in the DETOX group (79 percent) compared with that in the alum group (21 percent), median disease-free (DF) survival for the DETOX group (17.8 months) was shorter than that in in the alum group (32.1 months).

1.4.3.2 TLR 9 agonist-unmethylated CpG dinucleotides

Oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs directly stimulate B cells and DCs, thereby promoting the Th1 and pro-inflammatory cytokines and the maturation of professional antigen-presenting cells. Yamamoto et al. were the first to report that synthetic ODNs with sequences patterned after those found in bacterial DNA could activate NK cells to secrete IFN-γ[261]. Later, Krieg et al. found that specific sequence motifs present in bacterial DNA consisting of an unmethylated CpG dinucleotide flanked by two 5' purines (optimally GpA) and two 3' pyrimidines were responsible for triggering this "innate" immune response[262,263]. They further established that TLR9 in mice is responsible for the recognition of CpG motifs by immune cells[264], a finding subsequently confirmed for humans[265,266]. TLR9 is narrowly expressed in human B cell and plasmacytoid DCs and broadly expressed in B cells, monocytes, and probably all DC subsets of mice[264-267]. It detects the unmethylated CpG dinucleotides that are prevalent in bacterial and viral genomic DNAs but are in vertebrate genomes. So far, at least three families of immune stimulatory CpG ODN with distinct structural and biological characteristics have been described[268] — A-class CpG ODN (also known as D type), B-class CpG ODN (also known as K type), and C-class CpG ODN. Most studies of CpG activity utilize B-class CpG, which are strong B cell stimulators but induce relatively little pDC secretion of IFN-α.

CpG enhances tumor-associated antigen-specific humoral and cellular responses to

a wide variety of antigens, including peptide or protein antigens, dendritic cell vaccines, and autologous cellular vaccines in both prophylactic and therapeutic vaccines in numerous animal models[269-273]. The mechanisms that contribute to the strong adjuvant activity of CpG ODN potentially include synergy between TLR9 and the B-cell receptor, which preferentially stimulates antigen-specific B cells[262]; inhibition of B-cell apoptosis[274]; enhanced immunoglobulin G (IgG) class switch DNA recombination[275,276]; and dendritic cell maturation and differentiation, resulting in enhanced activation of Th1 cells and strong CTL generation. Conjugation of a CpG ODN directly to an antigen can enhance antigen uptake and reduce antigen requirements[277]. Comparisons of different adjuvants in mouse models have demonstrated CpG ODN to be unsurpassed at inducing Th1-type responses[278,279]. The Th1 bias induced by TLR9 stimulation is maintained even in the presence of vaccine adjuvants such as alum or IFA that normally promote a TH2 bias[280,281].

In humans, most types of immune cells do not express TLR9, and so are not activated directly by CpG DNA. All of the cellular immune effects of CpG ODN in humans are thought to result directly and indirectly from activating TLR9-expressing plasmacytoid DC and B cells. TLR9-stimulated B cells and pDCs show increased expression of costimulatory molecules (such as CD80 and CD86), resistance to apoptosis, upregulation of the chemokine receptor CCR7, and secretion of Th1-promoting chemokines and cytokines, such as monocyte inflammatory protein-1 (MIP-1), IFN-γ-inducible 10-kDa protein (IP-10) and other

IFN-inducible genes[282]. pDC activated through TLR9 secrete IFN-α, which drives the migration and clustering of pDC in the marginal zone and outer T-cell areas of the lymph node, where they are better able to stimulate adaptive immune responses[283]. Co-activation of naive, germinal center or memory B cells through the B-cell-antigen receptor and TLR9 induces their differentiation into plasma cells[284]; for memory B cells, activation through TLR9 alone is sufficient to drive differentiation to plasma cells[285]. Moreover, the efficiency of CpG-induced plasma cell differentiation could be enhanced by interactions with pDC, together with B-cell receptor crosslinking[286].

In clinical trials, CpG ODN has been used as adjuvant for hepatitis B vaccination either in combination with alum[287] or alone[288]. In a randomized, double-blind controlled Phase I/II dose-escalation study, healthy individuals received three intramuscular injections of an alum-absorbed HBV vaccine either in saline or mixed with a B-class ODN, CPG 7909[287]. HBsAg-specific antibody responses appeared earlier and had higher titers at all time points from two weeks after the initial prime up to 48 weeks in CPG 7909 recipients compared with those individuals who received vaccine alone. Moreover, most of the subjects received CPG 7909 as adjuvant developed protective levels of anti-HBs IgG within just two weeks of the priming vaccine dose, compared with none of the subjects receiving the commercial vaccine alone[287]. In another small clinical trial, eight melanoma patients received four monthly vaccinations of low-dose CpG 7909 mixed with MART-1 analog peptide and IFA. All patients exhibited rapid and strong

antigen-specific T cell responses: the frequency of Melan-A-specific T cells was more than 3 percent of circulating $CD8^+$ T cells, significantly higher than the frequency seen in eight control patients treated similarly but without CpG. The enhanced T cell populations consisted primarily of effector memory cells, which in part secreted IFN-γ and expressed granzyme B and perforin *ex vivo*[289].

Taken together, the results from animal studies and human clinical trials show that stimulation of TLR9-expressing cells is sufficient to induce strong and sustained humoral and cellular memory immune responses, offering several advantages over conventional vaccines. The adjuvant effect of CpG may have three components: a CpG-induced enhancement of APC function; a CpG-dependent induction of a cytokine/chemokine microenvironment supportive of antigen-specific immunity; and an improvement in antigen uptake mediated by DNA-binding receptors on APCs.

1.4.4 Heat shock protein

Heat shock proteins (HSPs) are ubiquitous soluble intracellular and highly conserved molecules that are present, and can be induced, in all eukaryotic and prokaryotic species, including plants. They were discovered in 1962 when Ritossa noted that temperature shock had induced odd puffing patterns and an unusual profile of gene expression in the polytene chromosomes of salivary glands in Drosophila melanogaster larva[290]. In 1974, the first products of these genes were identified and termed heat shock proteins[291]. Later, it became known that in

addition to raised temperature, exposure to oxidative stress, nutritional deficiencies, ultraviolet irradiation, chemicals, ethanol, viral infection, and ischaemia-reperfusion injury also can induce the expressions of these proteins[292-297]. Heat shock proteins are categorized into several families that are named on the basis of their approximate molecular weight (e.g., the 60 kDa Hsp60 family). They can exist in an unbound state or bound to specific client proteins. HSPs function as molecular chaperones in numerous processes, such as protein folding, assembly and transport, peptide trafficking, and antigen processing under physiologic and stress conditions.

The immunological functions of HSPs began to emerge in the 1980s when Srivastava et al. found that homogeneous preparations of certain HSPs that were isolated from cancer cells elicited immunity to cancers, whereas corresponding preparations from normal tissues did not[298]. The protein that induced tumor protection has been shown to be the heat shock protein gp96. Later, the heat shock proteins calreticulin, Hsp70, Hsp90, and grp170 also were shown to elicit similar effects by similar mechanisms[299-304]. Now, HSPs have been demonstrated to activate $CD8^+$ and $CD4^+$ lymphocytes; induce innate immune response including NK cell activation and cytokine secretion; and induce maturation of dendritic cells[305-307].

Autologous heat shock protein-peptide complexes produced from each patient's tumor is a logical personalized strategy that may obviate the need to identify the

unique antigens contained in the individual vaccine. Clinical trials have been performed to define the safety and characterize immunologic responses of autologous HSPs in immunotherapy for cancer. Autologous HSP-based immunotherapy studies in humans started in 1995 in Berlin and 1997 in New York[308]. Now, the gp96 HSP-peptide complexes (HSPPC-96) have been evaluated in early trials of pancreatic cancer[309], gastrointestinal tract cancers[310], lymphoma[311], chronic myelogenous leukemia, renal cell carcinoma[312] and melanoma[313]. For example, in one clinical trial, two of 28 melanoma patients vaccinated with HSPPC-96 had a completed response and three had stable disease at the end of follow-up. ELISPOT assay with PBMCs of 23 subjects showed a significantly increased number of postvaccination melanoma-specific T-cell spots in 11 patients, with clinical responders displaying a high frequency of increased T-cell activity[313]. Similar immune response and related clinical significance also was found in vaccine treated patients with colorectal cancer[310] and with pancreatic adenocarcinoma[309]. Recently, the results of a randomized, open-label, multicenter Phase III trial in patients with stage IV melanoma were announced[314]. In this trial, HSPPC-96 was given once weekly for the first four weeks and every other week after that. The control patients received the physician's choice, typically including IL-2 and/or dacarbazine/temozolomide, and/or tumor resection. Approximately 300 patients were accrued and randomized 2:1 in favor of HSPPC-96 treatment. The results showed that the survival plots of vaccine-treated and control patients were quite similar. However, when the data were analyzed for the sub-stages of stage IV

melanoma, the patients in the M1α category in the vaccine treatment group survived an estimated median of 626 days as compared with 383 days in the control patients. In another clinical trial done by Wood[557], patients who had nephrectomy were randomly assigned to receive either HSPPC-96 or observation alone. The results showed that patients with earlier stage (patients with T1, T2 and T3a diseases) of renal carcinoma drew clinical benefit from HSPPC-96 treatment with better prognosis whereas those with later stages did not.

Another strategy is to transduce tumor cell lines with HSP. This approach could be particularly promising in tumors in which dominant antigens are poorly characterized or a single HSP-antigen complex is inadequate to confer protection. Wang et al. showed that CT26 colon carcinoma cells transduced with cDNA of Hsp110 had reduced tumor growth as compared to the wild-type CT26 tumor in immunocompetent mice[315]. Immunization of mice with inactivated CT26-HSP110 cells significantly inhibited the growth of wild-type CT26 tumor[315]. Huang et al. demonstrated that vaccination with an engineered melanoma cell line expressing HSP 70 led to augmentation of NK and CTL activities and protection against tumor challenge in mice[316].

In summary, HSPs are potent inducers of immunity and have been harnessed as vaccine adjuvants targeted to cancers. HSP-based vaccine strategies — including vaccines derived from patient tumor material and from transduced tumor cells lines — are promising for immunotherapy. Advanced clinical studies using autologous

tumor-derived HSPs are underway.

1.4.5 Cytokines

1.4.5.1 IL-2 and its family members

IL-2 is a 15,000-KDa a-helical cytokine produced predominantly by activated $CD4^+$ and $CD8^+$ T cells. It is produced rapidly and transiently upon engaging the TCR and costimulatory molecules such as CD28 on naive T cells. Activated DCs, NK cells, and NK T cells also produce IL-2. IL-2 stimulates the proliferation of activated T cells and facilitates the induction of CTLs. Moreover, it induces the proliferation of B cells that have been stimulated with IgM-specific antibody or CD40 ligand. IL-2 also stimulates the generation, proliferation, and activation of NK cells[148].

In 1985, IL-2 combined with autologous lymphokine-activated killer cells first was used to treat patients with metastatic cancer[317]. After that, a series of clinical trials that combined various cancer vaccines with IL-2 have been published. In an early randomized clinical trial[558], IL-2 was administered alone or with LAK cell in 181 patients, 97 had renal cell cancer and 54 had melanoma. There were 10 complete responses among the 85 assessable patients who received IL-2 plus LAK cells, compared with four among the 79 who received IL-2 alone. Complete response continues in seven patients at 50-66 months. Improved survival was seen for patients received IL-2 plus LAK cells compared with those who received IL-2

alone ((36-month survival: 31% versus17%).

In another clinical trial done by Rosenberg, IL-2 could enhance the anti-tumor activity of the gp100 peptide vaccine[318]. Eight of 19 patients who received the peptide plus high dose IL-2 were seen with objective remissions, whereas no objective cancer regressions were seen in any of the 11 patients who received the peptide in incomplete Freund's adjuvant. In a recent phase II clinical trial[559], A recombinant modified vaccinia virus Ankara (MVA) encoding human 5T4 (MVA-5T4) combined with high dose IL-2 were given by intramuscular injection to 25 of patients with metastatic renal carcinoma. The results showed that the combination treatment induced higher levels of 5T4-specific humoral and cellular immunity as compared to the MVA-5T4 alone[560]. Twelve patients had stable disease, which was associated with increased effector T cells.

Because high doses of IL-2 have toxicity[317,319], subsequent trials tried to use low doses of IL-2 but got frustrating results[134,320-322]. Thus, there are no convincing data to support combining various vaccines with lower doses of IL-2 except for one report from Rosenberg's study[318].

Much data now support an essential role for IL-2 for Treg production[323,324]. These ideas are much different from the early paradigm in which IL-2 is central for immune responses by promoting T cell growth and effector differentiation. For cancer patients, increasing Treg cells is likely to be detrimental by preventing induction of tumor-specific T cell responses and may explain the limited efficacy

of IL-2 in antitumor therapy. Now, two new family members, IL-15 and IL-21, have been indentified. They share some immune-stimulating activities with IL-2 but are not primarily involved in immune-regulation, and have been proposed as alternative candidates for cancer immunotherapy[325,326].

1.4.5.2 IL-12 and its family members

IL-12 is a heterodimeric protein composed of two covalently linked p35 and p40 subunits and first was recovered from the supernatant of phorbol-ester-induced EBV-transformed B cell lines[327,328]. Its biological functions are mediated by the IL-12 receptor composed of two chains (ß1 and ß2)[329]. IL-12Rβ1 binds IL-12 p40, and it is associated with Tyk2, whereas IL-12Rβ2 recognizes either the heterodimer or the p35 chain and is associated with Jak2. Signaling through the IL-12 receptor complex induces phosphorylation, dimerization, and nuclear translocation of several STAT transcription factors (1, 3, 4, 5), but the predominant response and most of the biological responses to IL-12 are mediated by STAT4[330]. IL-12 is produced mostly by activated hematopoietic phagocytic cells (monocytes, macrophages, neutrophils) and dendritic cells. It is a potent inducer of IFN-γ production from T, NK, and other cell types, and it has been shown to be a potent inducer of differentiation of Th1 cells[331].

The antitumor and adjuvant activities of IL-12 have been shown extensively in murine models, including melanomas, mammary carcinomas, colon carcinoma, renal carcinoma, and sarcomas[332]. In clinic, it has been investigated in patients with

advanced solid tumors and hematologic malignancies, as either monotherapy or in combination with antigen-associated proteins as adjuvant. For example, Lee et al. immunized 48 patients with stage III or IV melanoma with two tumor peptides derived from gp100 and tyrosinase, with or without s.c. administration of IL-12. IL-12 augmented peptide-specific delayed-type hypersensitivity reactivity to the gp100 antigen in 34 of 40 patients[333]. Moreover, the treatment boosted the gp100-specific and tyrosinase-specific peripheral immune response, as measured by IFN-γ release in 37 of 42 patients[333]. In another trial done by Cebon et al.[334], IL-12 was administered s.c. or i.v. in 21 of stage III or IV melanoma patients expressing Melan-A in their tumors. Melan-A peptides were administered intradermally. One patient achieved complete response and one had stable disease in the i.v. arm. One patient achieved partial response, and five had stable disease in the s.c. arm[334]. Peterson et al. reported a trial in which 20 advanced melanoma patients were immunized with PMBC loaded with Melan-A/Mart-1 peptide plus IL-12. The results showed that two complete responses, five minor or mixed responses, and four stabilizations of disease were achieved. The overall median survival was 12.25 months and seven patients remained alive at the time of data analysis, with all patients followed for more than 12 months[335].

Systemic administration of IL-12 in cancer patients is limited by toxicity. IL-12 gene therapy might be an alternative strategy. Based on the promising results obtained in preclinical IL-12 gene therapy studies[336], clinical trials have been designed with the aim of achieving production of the cytokine at the tumor site,

while maintaining low serum concentrations to reduce systemic toxicity. In one study conducted by Sun et al., patients with melanoma received s.c. injections at weekly intervals of autologous tumor cells transduced with IL-12[337]. Two patients developed delayed-type hypersensitivity reaction against their autologous melanoma cells and one had a minor clinical response[337].

In addition, two new family members, IL-23 and IL-27, have been discovered. Similar to IL-12, IL-23 and IL-27 are produced predominantly by macrophages and dendritic cells and affect IFN-γ production by T and NK cells[338]. All three cytokines (IL-12, IL-23, and IL-27) seem to play roles in the priming and reactivation of polarized T cell responses. In murine models, IL-23 as adjuvant has been used to treat tumors effectively[339]. Thus, further clinical studies could be developed by exploiting the antitumor effects of new IL-12 family members and combining IL-12 with its new members.

1.4.5.3 GM-CSF

GM-CSF has been shown to stimulate monocytes and neutrophils, reducing the risk for febrile neutropenia in patients with cancer. It also augments the numbers and activity of DCs, enhancing cellular immunity[340]. A number of clinical trials have utilized GM-CSF as an adjuvant for cancer vaccines.

GM-CSF has been administered with irradiated autologous or allogeneic melanoma cells and transduced into these types of cells[111,341] or combined with tumor peptides

to enhance immune responses[342,343]. For example, in a study done by Weber et al.[343], 48 patients with resected stage IIA or IIB melanoma were immunized with gp100 and tyrosine in IFA, with or without GM-CSF. Immune responses were seen in the majority of patients post-vaccination. There was a trend for GM-CSF to increase modestly the levels of immune activation as measured by all these assays. In another phase II clinical trial, 25 patients with melanoma were immunized with three melanoma HLA-A2-binding peptides[344]. Patients were randomized into three groups: (i) peptides with Montanide ISA-51; (ii) peptides + Montanide + GM-CSF 10 μg; or (iii) peptides + Montanide + GM-CSF 50 μg. Nine patients showed a successful immune response, as measured by delayed-type hypersensitivity testing to gp100 peptides. There did not seem to be any evidence of an enhanced immune response by combining the vaccine with either of the low-dose GM-CSF regimens used. The authors concluded that higher doses of GM-CSF may be needed. This conclusion may be supported by a randomized phase I clinical trial[345]. Twenty staged IIB, III or IV melanoma patients were immunized with VACCIMEL (The vaccine VACCIMEL consisted of three irradiated allogeneic melanoma cell lines with BCG as an adjuvant). Besides, patients received local injections of placebo, GM-CSF 150, 300, 400, or 600 μg split into four doses. The result showed GM-CSF induced statistically significant increased DTH reactions, with the maximal effect occurring in the 400 μg high dose group.

GM-CSF also has been fused with tumor-associated antigen and used to pulse antigen-presenting cells as a vaccine. A variety of tumor types, both hematological

cancers and solid tumors, have been shown in phase I/II studies to respond to adoptive therapy using antigen-primed DCs, including myeloma[346], lymphoma[347], prostate cancer[348], melanoma[154], and colon cancer[349]. Research by Small and colleagues, for example, evaluated immunotherapy of HRPC (hormone-refractory prostate cancer) with antigen-loaded DCs[348]. The DCs had been loaded *ex vivo* with a recombinant fusion protein composed of prostate-specific acid phosphatase (PAP) linked chemically to GM-CSF. Patients who had evidence of an immune response to PAP had a longer progression-free survival interval than patients with no evidence of immune response, suggesting that induction of immunity was associated with clinical response[348].

1.4.6 Costimulatory molecules

For effective T cell immune response, recognition of antigen (signal 1) — interaction of CD3/TCR complex with peptide-MHC complex expressed on APCs — is not efficient. An additional signal from a costimulatory receptor (signal 2) is required for T cell activation. The costimulatory receptor can be divided into two main groups: the immunoglobulin superfamily, such as B7/CD28 (CD28 is a receptor for B7-1 and B7-2), and TNF/TNFR superfamily.

1.4.6.1 B7/CD28 Family

A CD28 signal is considered to be the primary costimulatory receptor that controls initial clonal expansion and provides early signals to augment expression of

anti-apoptotic members of the Bcl-2 family to prevent cell death[350]. It also regulates early cytokine production in particular IL-2, and IL-2R signals may provide additional early anti-apoptotic signals[351]. B7-1(CD80) and B7-2(CD86) have been used in various cancer therapies by fusing them with Ig[352-354], overexpressing them on tumor cells[355-358]. When translating these findings into clinic for treatment with 12 metastatic melanoma patients, 50 percent of the injected lesions remained stable or regressed after vaccination, an objective clinical response was observed in one patient, and disease stabilization in two others[359]. Besides, Orabona C et al, found that CD28-Ig can induce DCs to secrete IL-6 and IFN-γ, thus eradicated a growing tumor *in vivo*[360].

The obstacle of using CD28 ligand as an adjuvant is that B7 also bind to another receptor CTLA-4 which has been shown to inhibit T cell response and regulate peripheral T cell tolerance[361-364]. A monoclonal antibody that blocks CTLA-4 binding has been developed and has been studied extensively as a means to overcome immune checkpoints and break tolerance[365]. A CTLA-4 blockade has entered clinical trials for patients with a variety of tumors, including prostate, ovarian, breast, colon, renal carcinomas, and melanoma[365,366].

More recently, several new B7/CD28 family members have been identified that integrate into stimulatory or inhibitory activity for T-cells. The five new B7 family members, ICOS ligand, PD-L1, PD-L2, B7-H3, and B7-H4, mainly are expressed on APCs as well as on cells within lymphoid organs, providing new means for

regulating T cell activation and tolerance[363,367]. The new CD28 family members, ICOS, PD-1, and BTLA, are inducibly expressed on T cells and are important for regulating previously activated T cells[363]. Investigation of applications of these new costimulatory molecules in cancer therapy is underway[368,369].

1.4.6.2 TNF/TNFR Family

Several members of the TNF/TNFR family function after initial T cell activation to sustain T cell division and promote T cell survival[370]. CD40/CD40L (CD154), OX-40L/OX-40 (CD134), 4-1BBL/ 4-1BB (CD137), LIGHT/HVEM, CD30L/CD30, CD70/CD27, GITR L/GITR, all can have costimulatory effects on T cells and thus can be potential targets for tumor immunotherapy.

CD40 is expressed on a wide range of cells including APCs — such as DCs, macrophages and B cells — as well as on cells with high proliferative potential, including tumor cells[371]. Its ligand CD154 is up-regulated on T cells after CD28 signaling[371]. Evidence for the role of the CD40–CD154 signaling in enhancing antitumor immunity came initially from studies that demonstrated that conferring CD154 expression to tumors is sufficient to promote regression of some transplantable murine tumors[372,373]. Thereafter, a soluble form of CD154 as well as an anti-CD40 antibody was proved to have antitumor effect in animal models[374,375]. A phase I clinical trial delivered recombinant human CD40L protein systemically to patients with advanced non-Hodgkin's lymphoma or solid tumors[376]. Of the 32 patients treated, one subject achieved a complete response, one a partial response,

and 12 achieved stable disease. And clinical trials with anti-CD40 monoclonal antibodies are ongoing[377].

OX-40 is expressed primarily on activated $CD4^+$ T cells with some $CD8^+$ T cells after TCR engagement. OX40 can costimulate some IL-2 production and blocking OX40 signaling suppresses the accumulation of effector T-cells[370,378,379]. Furthermore, OX40 costimulation appears to be most important in T-cell memory responses[370,379]. Another important function of OX-40 is to sustain the expression of anti-apoptotic Bcl-2 family members, hence promoting cell survival[380]. The potent biological effects observed following OX40-mediated signaling imply that OX40 might be an excellent target for cancer therapy. Stimulating OX40 on T-cells with an agonist monoclonal antibody results in therapeutic activity[381]. Consistently, another study has shown that engagement of OX40 during tumor-specific adoptive immunotherapy improves the ability of T cells to eradicate lung and brain metastases[382]. More recently, a phase I clinical trial of an agonistic anti-OX40 monoclonal antibody has been initiated for patients with advanced cancers, such as melanoma, lung, breast, prostate, colon, and lymphoma, who have failed standard cancer treatments[383].

4-1BB (CD137) is expressed on the surface of activated T cells[384], as well as on NK cells[385], monocytes[386], and DC[387]; its ligand 4-1BBL is expressed on B cells and macrophages. *In vitro* exposure to anti-CD137 mAbs can stimulate the proliferation of $CD8^+$ T cells with CTL activity and production of Th1 cytokines

IFN-γ and TNF-α[388]. And 4-1BB signaling also can protect T cells against apoptosis[389-391]. Moreover, engagement of CD137 can induce proliferation of monocytes[392] and facilitate their differentiation to DC. Initially, it was found that administration of anti-CD137 mAbs can break the tolerance and induce a tumor-destructive immune response in mouse models[393,394]. However, the clinical use of such MAbs may be problematic since they depress antibody formation and abrogate T cell-dependent humoral antibody responses[395,396]. The alternative approach is to transfect tumor cells to express the CD137 ligand (CD137L) and increase their immunogenicity[397], but antitumor response by vaccinating with tumor cells transfected to express CD137 ligand is less effective than by injecting anti-CD137 mAb[398,399]. A more effective way to engage CD137 toward tumor destruction is to transfect tumor cells to express a cell-bound form of anti-CD137 single-chain Fv fragments (scFv). For example, cells from sarcoma Ag104 became immunogenic when transfected to express anti-CD137 scFv3 but not when transfected to express CD137 ligand[385]. Ye et al. transfected cells from the M2 clone of the K1735 mouse melanoma to express anti-CD137 scFv and showed therapeutic efficacy against small M2 tumors growing s.c. or in the lung[400]. Furthermore, a recent study showed that mammary carcinoma cells (MMC) transfected to stably express surface scFv completely eradicate the tumor growth in the neu-transgenic mouse breast cancer model[401]. Clinical trial with the CD137 mAb is expected in the near future.

Taken together, costimulatory signals play a critical role in inducing and maintaining cell-mediated immune response and thus might be promising targets for helping to increase the safety and efficacy of tumor immunotherapy. However, some of these molecular pathways are very well characterized and require further investigation.

In summary**,** adjuvants are crucial components of immunotherapy. However, the field of pharmacological adjuvants still is at a very early stage of development. Many components from mushrooms have been shown to be potential immunomodulatory activities.

1.5 The history and immunobiology of fungal immunomodulatory proteins

For centuries, mushrooms have been shown to contain abundant biologically active compounds for the promotion of good health and vitality, enhancement of the body's adaptive capabilities, and treatment of various diseases worldwide, especially in Asian countries[402-405]. To the ancient Romans, they were "the foods of the gods" and to the early Egyptians they were "a gift from the God Osiris," whereas the Chinese considered them as "the elixir of life." The practice of using medicinal mushrooms in Chinese traditional medicine dates back into antiquity and has been recorded in ancient Chinese manuscripts[406]. More than 14,000 species of mushroom have been found and at least 270 species are known to possess various therapeutic properties[402,407]. In recent years, the ability of mushrooms to modulate

the immune system and inhibit tumor growth has been studied and investigated[403,408-410]. After early studies were conducted with whole mushrooms, some of the most effective components, such as polysaccharides, polysaccharopeptides, and polysaccharide-proteins, subsequently have been isolated and identified[411]. The major immunomodulating activity of these bioactive compounds includes their mitogenicity, potential stimulation of hematopoietic stem cells, and activation capacity of immune cells like NK cells, DCs, and T cells[411-416].

Historically, most medicinal mushroom species were relatively scarce and were collected from the forests where they grew on dead or living trees and forest litter. Nowadays, almost all important medicinal mushrooms have been subjected to large-scale artificial cultivation by solid substrate or low moisture fermentation, thus removing the historical scarcity factor and allowing large commercial operations to develop. Overall, the world production of cultivated edible and/or medicinal mushrooms was recorded as 4909 × 103 tons in 1994, increasing to 6158 × 103 in 1997, with an estimated value in excess of US $14 billion[417]. Output yield of the leading 10 species cultivated made up about 92 percent of total world production and of these, six species —Agaricus bisporus, the white button mushroom (31.8 percent); Lentinula edodes (25.4 percent); Pleurotus spp. (14.2 percent); Auricularia auricular (7.9 percent); Flammulina velutipes (4.6 percent); and Volvariella volvaceaea (7.9 percent) — made up 87 percent of the total production[418].

Of these species, *Flammulina velutipes* (also called golden needle mushroom) possess immunomodulatory, anti-tumor, anti-viral, anti-fungal, and cholesterol-lowering activities[419-422]. Its major fruiting body protein, Fip-Fve, has been shown to play a significant role in the mushroom's immunomoduatory effect [423]. The Fip-fve is classed as a new distinct family of fungal immunomodulatory proteins (Fips), which include LinZhi-8[424,425], Fip-vvo[426], Fip-gts[416], Fip-vvl[427], purified from *Ganoderma lucidium*, *Volvariella volvacea*, *Ganoderma tsugae*, and *Volvariella volvacea*, respectively. Fips exhibit a high homology in their amino acid sequences[426], which comprise ~110 amino acid residues with a molecular mass of ~13 kDa, and are significantly similar to the VH region of the immunoglobulin in both amino acid sequence and predicted secondary structure[428]. Fips are mitogens *in vitro* for human peripheral mononuclear cells (hPBMCs) and are mediated by active monocytes and T-lymphocytes to induce the production of cytokines, IL-2, IL-12, IFN-γ, and TNF-α[414,424,429].

Native Fve protein is an acetylated protein consisting of 114 amino acid residues with an estimated molecular weight of 12.7 kDa[423]. Its 3D structure determined by x-ray crystallography revealed that it is a non-covalently linked homodimer, and has a novel structure, wherein each subunit of the homodimer consists of an N-terminal alpha-helix followed by a fibronectin III (FN III)-type fold (appendix 4). Sugar-binding studies suggested that Fve could be a lectin with specificity for complex cell surface carbohydrates[430]. Fve has potent mitogenic stimulatory effects on both mouse splenocytes and hPBMCs and enhances IL-2, IFN-γ, and TNF-α

production[415,423]. The comparison of structures and their functions of different mitogens was shown in appendix 6. Besides the mitogenic effect, Hsu et al. reported that Fve can inhibit IL-5-mediated survival of eosinophils through the modulation of cytokine receptor expression and apoptotic signal protein production. Moreover, mice fed with Fve protein induced systemic production of IFN-γ, and coadministration of Fve with antigen could drive a strong Th1 skewed immune response[431]. Taken together, Fve has a characteristic ability to induce production of high levels IFN-γ by immune cells *in vitro* and *in vivo*, presumably through its mitogenic stimulatory effects on the immune response cells.

1.6 Objectives and significance of the study

To clarify further the immunomodulatory activity of Fve, the interactions of Fve with immune cells, T cells, DCs, and NK cells were explored. Also, the potential application of Fve in the development of tumor vaccine was investigated. The adjuvant effects of Fve in the clinical application and for immunotherapy of other diseases such as asthma were not examined in this study.

The specific aims of the research were:

1. To elucidate the characteritics of Fve in the immune system.
    - The interaction between Fve and T cells was investigated. The surface markers staining, proliferation, and cytokine production of T cells was assayed *in vitro*.

- The interaction between Fve and antigen presenting cells DCs in innate immunity was investigated. The surface markers of DCs were examined after stimulation by Fve *in vitro* as well as *in vivo* using FACS analysis. The cytokine production by antigen-specific T cells was analyzed to evaluate the functional maturation of DCs in transgenic mice models.
- The transwell experiments were assayed to elucidate the possible roles of T cells in the maturation of DCs by Fve.
- The interaction between Fve and NK cells in innate immunity was investigated. The cytokine released by NK cells after stimulation by Fve were analyzed by ELISA

2. To investigate the adjuvant effect of Fve in cervical cancer immunotherapy.

- HPV type-16 E7 protein was used as the antigen model to evaluate the E7-specific humoral and cellular immune response *in vitro*. E7-specific antibodies and cytokine production from T cells was the read-out.
- TC-1 tumor cell, which expresses E7 protein, was used as the animal tumor model to evaluate the ability of protection and therapeutics *in vivo*. The tumor size and survival rate were monitored in different groups.

Results from the tumor model study may provide an alternative model to test the immunomodulatory functions of Fve *in vivo*. More importantly, clarification of the relationship between Fve and different immune cells could contribute to a better understanding of the molecular mechanism of immunomodulatory proteins in the

immune system. This should provide useful information on how to optimize the vaccine to prevent and treat HPV-related and other cancers in clinics in the future.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Animals

The BALB/c, BALB/cJ and C57BL/6 mice were originally obtained from The Jackson Laboratory (Bar Harbor, Me, USA) and were bred in the Animal Holding Unit of National University of Singapore (NUS).

Breeding pairs of OT-I, OT-II and DO11.10 mice having transgenic Vα2Vβ5 TCRs specific for $OVA_{257–264}$ epitope in the context of H-$2K^b$, $OVA_{323–339}$ epitope in the context of I-A^b and $OVA_{323–339}$ epitope in the context of I-A^d respectively, were originally acquired from Jackson Laboratory (Bar Harbor, Me, USA) and were maintained and bred in the pathogen-free environment in the Animal Holding Unit of NUS. All animal procedures were performed according to approved protocols and in accordance with the Institutional Animal Care & Use Committee (IACUC) of NUS.

Female mice (6-8 weeks old) were used to study function of Fve and to evaluate the potential clinical application of Fve in treatment of cancer.

2.1.2 Edible mushroom

Flammulina velutipes (Golden needle mushroom) was purchased from Taiwan, Republic of China (Fig. 2.1).

2.1.3 Bacterial strains

Escherichia coli strain TG-1 [Genotype: *sup*E *hsd*Δ5 *thi* Δ (*lac-proAB*) F' (*tra*D36 *proAB*$^{+}$ *lac*I^{q} *lacZ*ΔM15)] was used for the expression of recombinant protein.

2.1.4 Bacteria culture media

Chemical Recipe	Company
Bacto-tryptone	Difco Laboratories (Detroit, MI, USA)
Bacto-yeast extract	Difco Laboratories (Detroit, MI, USA)
Bacto-agar	Difco Laboratories (Detroit, MI, USA)
Glycerol	Merck (Whitehouse station, NJ, USA)
NaCl	Sigma (St Louis, MO, USA)
KH_2PO_4 and K_2HPO_4	Sigma (St Louis, MO, USA)
Ampicillin	Calbiochem (San Diego, CA, USA)
IPTG	Calbiochem (San Diego, CA, USA)

Luria-Bertani (LB) medium composes of 10 g of bacto-tryptone, 5 g of bacto-yeast extract, and 10 g of NaCl in 1000 ml deionized H_2O.

2.1.5 Cell lines

TC-1 tumor cell line was kindly provided by Dr TC Wu, Johns Hopkins University, USA and was used as HPV type-16 tumor model experiments. The maintenance of

TC-1 cells has been described previously[432]. HPV-16 E6, E7, and ras oncogene were used to transform primary C57BL/6 mice lung epithelial cells. The cells were grown in RPMI 1640, supplemented with 10% (v/v) FBS, 50 units/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.4 mg/ml G418 at 37 °C with 5% CO2. On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed three times with PBS, and the designated concentrations of cell number for tumor inoculation was re-suspended in 200 μl of PBS.

Anti-CD4 (clone, GK1.5), anti-CD8 (clone, 2.43) and anti-IFN-γ (clone, R4-6A2) antibodies were purified from the supernatant of hybridoma cells (ATCC) by passage through a protein-G column (Amersham).

2.1.6 Cell culture media and supplements

Chemical Recipe	Company
Cell culture medium RPMI 1640	HyClone (Logan, UT, USA)
L-glutamine	HyClone (Logan, UT, USA)
sodium pyruvate	HyClone (Logan, UT, USA)
sodium bicarbonate	HyClone (Logan, UT, USA)
HEPES	HyClone (Logan, UT, USA)
Hank's balanced salt solution (HBSS)	HyClone (Logan, UT, USA)
fetal calf serum (FCS)	HyClone (Logan, UT, USA)
fetal bovine serum (FBS)	StemCell Technologies (Vancouver, BC, Canada)
2-mercaptoethanol	Life Technologies (Grand Island, NY, USA)
penicillin and streptomycin solution	HyClone (Logan, UT, USA)
Geneticin® selective antibiotic (G418) solution	Life Technologies (GibcoBRL) (Grand Island, NY, USA)
Trypsin solution	Life Technologies (Grand Island, NY, USA)
[^{3}H]-thymidine	Amersham Pharmacia (Piscataway, NJ, USA)

2.1.7 Plasmids and reagents for gene cloning

The pGEX is a glutathione S-transferase (GST) fusion protein expression system used extensively as a high yield expression and easy purification system. GST is a 26 kDa tag to any recombinant protein, that it can be removed as an endopeptidase (thrombin, Factor Xa protease or enterokinase) cleavage site sequence which is incorporated between the tag and the protein. Elution of the purified protein is accomplished under mild, nondenaturing conditions. This prokaryotic expression plasmid was purchased from Pharmacia (Uppsala, Sweden).

Pfu DNA polymerase, T4 DNA ligase, shrimp alkaline phosphatase, restriction enzymes such as Bam *HI*, Eco *RI* were purchased from Promega (Madison, WI, USA). Wizard® Plus SV minipreps DNA Purification System was purchased from Promega (Madison, WI, USA). DYEnamic™ ET Terminator Cycle Sequencing Kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.1.8 Reagents for protein purification and identification

Protein purification resins such as Cynogen Bromide-activated Sepharose 4B, Sephadex G-25, Butyl Sepharose 4 Fast Flow, Q Sepharose Fast Flow, Hi-Trap Q-Sepharose, Hi-Trap SP-Sepharose, and the Fast Performance Liquid Chromatography (FPLC) system for protein chromatography were purchased from Pharmacia Amersham Biosciences AB (Uppsala, Sweden). Glutathione agarose, DNase, and lysozyme were purchased from Sigma (St. Louis, MO, USA). Protein

concentrator Amicon® Centricon Plus-20 (PL-10, PL-30) was purchased from Millipore (Bedford, MA, USA).

Bio-Rad Protein Assay kit was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Coomassie Plus Protein Assay Reagent and SuperSignal® West Pico Chemiluminescent were purchased from Pierce (Rockford, IL, USA). Coomassie blue R was purchased from Sigma (St Louis, MO, USA). BioMax film was purchased from EASTMAN KODAK Company (Rochester, NY, USA).

2.1.9 Antibodies and microbeads

All antibodies were purchased from BD PharMingen (San Diego, CA, USA) unless specified otherwise.

For the detection of antigen-specific IgG1 in mouse sera, the rat anti-mouse Igκ light chain (clone 187.1) and biotin-conjugated rat anti-mouse IgG1 mAb (clone LO-MG1-2, Serotec Ltd., Oxford, England) were used. The purified mouse IgG1 (clone 107.3) was used as the standard.

For cytokine ELISA, mAbs of rat anti-mouse IFN-γ (clone R4-6A2), biotin-conjugated rat anti-mouse IFN-γ (clone XMG1.2), rat anti-mouse IL-2 (clone JES6-IA20), biotin-conjugated rat anti-mouse IL-2 (clone JES6-5H4), rat anti-mouse IL-4 (clone BVD6-24G2), biotin-conjugated rat anti-mouse IL-4 (clone BVD4-1D11), rat anti-mouse IL-6 (clone MP5-20F3), biotin-conjugated rat anti-mouse IL-6 (clone MP5-32C11), rat anti-mouse TGF-β (clone, A75-2.1),

biotin-conjugated rat anti-mouse TGF-β (clone A75-3.1) were used. Rat anti-mouse TNF-α (clone AF-410-NA), biotinylated rat anti-mouse TNF-α (clone BAF410), rat anti-mouse IL-10 (clone JES052A5) and biotin-conjugated rat anti-mouse IL-10 were purchased from R&D Systems (Minneapolis, MN, USA).

Rat anti-mouse CD3ε (clone 145-2C11) and CD28 (clone 37.51) mAbs and recombinant mouse IL-2 were used for stimulation of T cells *in vitro*. Percp-conjugated rat anti-mouse CD3 (clone 17A2), FITC-conjugated rat anti-mouse CD8β (clone 53-5.8), biotin-conjugated rat anti-mouse CD4 (clone GK1.5), APC-conjugated rat-anti-mouse CD4 (clone RM4-5), PE-conjugated anti-mouse OX-40 (clone OX86, eBioscience San Diego, CA, USA), PE-conjugated anti-mouse 4-1BB (clone 17B5, eBioscience San Diego, CA, USA), biotin-conjugated hamster anti-mouse CD69 (clone H1.2F3), streptavidin-PerCP, were used for the surface marker staining of T cells. APC-conjugated rat-anti-mouse IFN-γ (clone XMG-2.1) was used for the intracellular cytokine staining of T cells. Rat anti-mouse CD16/CD32 (clone D34-485), biotin-conjugated rat anti-mouse CD8α (clone 53-6.7), APC-conjugated rat anti-mouse CD4 (clone RM4-5), PE-conjugated rat anti-mouse CD11c (clone HL3), FITC-conjugated anti-mouse I-A^b (clone AF6-120.1), FITC-conjugated anti-I-A^d (clone AF6-120.1) FITC-conjugated anti-CD86 (clone GL1), PE-conjugated rat anti-mouse CD40 (clone 1C10), FITC-conjugated rat anti-mouse CD80 (clone 16-10A1), FITC-conjugated hamster anti-mouse CD54 (clone 3E2) and streptavidin-conjugated PerCP were used for surface marker staining of splenic

DCs.

Immune cell separation microBeads, which recognize CD4, CD8, CD19, CD11c, or NK1.1 were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.1.10 Reagents for immunoassay

Anti-mouse Ig κ light chain, mouse IgG1, IgG2a, recombinant IL-2, IL-6, IL-4, IL-10, IFN-γ, TNF-α, TGF-β were used as ELISA standards, and monoclonal antibodies for mouse cytokine ELISA were purchased from PharMingen (San Diego, CA, USA). Biotin-conjugated anti-mouse total immunoglobulins, ExtraAvidine Alkaline Phosphatase (AP), ExtraAvidin-Peroxidase conjugate, para-nitrophenyl phosphate (pNPP) substrate for AP, and 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate for HRP were purchased from Sigma (St. Louis, MO, USA). Biotin conjugated anti-mouse, anti-rat IgG1 and IgG2a were purchased from Serotec (Oxford, England, UK).

2.2 Methods

2.2.1 Preparation of native Fve protein, recombinant E7 protein, and recombinant GST protein.

2.2.1.1 Purification of Fve protein from *Flammulina velutipes*

Two kilograms of *Flammulina velutipes* (golden needle mushroom) was purchased from Taiwan. The fresh fruit bodies of mushroom were homogenized with 2 L of 5

% acetic acid (v/v) in the presence of 0.1 % (v/v) 2-mercaptoetheanol. The homogenate was centrifuged for 20 min and soluble proteins in the supernatant were precipitated by addition of ammonium sulphate to 95 % saturation. After stirring for an overnight, the precipitate was collected by centrifuge for 20 min again. The pellet was then dissolved and dialyzed against 4.5 L of 10 mM Tris-HCl (pH 8.0) at 4 ˚C for 4 days with 6-9 changes of dialysis solution. The dialyzed solution was firstly applied to Q column, which had been pre-equilibrated with 10 mM Tris-HCl (pH 8.0). The flows through fractions were then further purified by SP column, which was pre-equilibrated with 10 mM sodium acetate (pH 5.0). The column was washed with 10 mM equilibration buffer and then eluted with a linear gradient of 0-1 M NaCl in 10 mM sodium acetate (pH 5.0). The fractions were then further purified on a Q column (pH 8.0) and SP column (pH 5.0). Purified Fve was dialyzed with 2 L of 10 mM PBS (pH 7.4) and stores at -80˚C.

2.2.1.2 Construction of plasmid DNA pGEX-E7

The cDNA of HPV-16 E7 (A gift kindly given by Dr SW Chan, Institute of Molecular and Cellular Biology, ASTAR, Singapore) was subcloned into pGEX-4T1 expression vector. DNA fragment of E7 was amplified by polymerase chain reaction (PCR) using a set of primers: E7-F 5’-TTGTTGGATCCCATGGAGATACACCTACATTG-3’ and E7-R 5’-TTACTGAATTCTTATGGTTTCTGAGAACAGATG-3’. The primers were synthesized by QIAGEN Operon (Alameda, CA, USA). The PCR was performed

under standard conditions (94 ^{o}C for 1 min, 55 ^{o}C for 1 min and 72 ^{o}C for 2 min for 25 cycles) using *pfu* DNA polymerase. The amplified products were separated on a 1% of agarose gel. The band corresponding to the expected PCR product size was cut out and the DNA were purified by Wizard® Lamba Preps DNA purification system. The purified DNA was digested with Bam *HI* and Eco *RI*, and the resulting fragment was then cloned into the Bam *HI* and Eco *RI* sites of pGEX- 4T1 vector. Then the nucleotide sequencing was analyzed by the ABI 377 DNA Sequencer (Applied Biosystems, USA). The pGEX-E7 recombinant plasmid was transformed into *Escherichia coli* TG-1 for protein expression.

2.2.1.3 Preparation of recombinant E7 protein

E7 was expressed as GST-fusions protein from pGEX-4T1. Small scale of pGEX-E7 transformed TG-1 bacteria was seeded in LB medium. The overnight culture was transferred to 1L of LB medium containing ampicillin (100 μg/ml) in 1 in 40 proportion and grown at 37 $^{\circ}$C with 250 rpm vigorous shaking until the OD_{600} reach to 0.6-0.8 (approximately 2-3 hrs). The recombinant protein was induced by IPTG at a final concentration of 0.1 mM and further incubation of 4-6 hrs at 30 $^{\circ}$C in a shaker incubator at 200 rpm. Cells were harvested by centrifugation at 6000 rpm for 10 minutes and the pellet was used for protein extraction.

The pellet of E7 transformed bacteria was re-suspended in 250 ml ice-cold lysis buffer (TBS pH 7.5, 1 mM PMSF (Sigma, MO, USA), 20 μg/ml DNase *I* and 1 % Tween 20). The cell suspension was then sonicated at 4 $^{\circ}$C for 50 seconds, 18

cycles with 30 seconds intervals. Total cell lysate was centrifuged at 16 000 rpm for 25 min at 4 °C. The supernatant was collected for further affinity purification on glutathione agarose beads. Initially, glutathione agarose beads (Sigma, MO, USA) was dispensed into a chromatography column and then washed with TBS (pH 7.5). Supernatant from the total cell lysate was then loaded onto the column and subsequently washed with TBS. GST-E7 was eluted with elution buffer (Glutathione 0.15 g, Tris-base in a total volume of 50 ml dH_2O) and then analyzed by SDS-PAGE. Pure fractions of GST-E7 protein were pooled together and cleaved with thrombin. Purified E7 was dialyzed against PBS (pH 7.4) and used for further studies.

2.2.1.4 Preparation of recombinant GST protein

Glutathione S-transferase is the GST enzyme of Schistosoma japonicum worms which terms as Sj26. The expression and purification was described previously[433]. Briefly, vector pGEX4T-1 was transformed into E.*coli* strain TG-1 for expression of GST. The protein product was induced with 0.1 mM IPTG at 30 °C in a shaker incubator at 200 rpm for 1 hr. Recombinant GST protein was obtained by passing the cell lysate through glutathione agarose beads.

2.2.1.5 SDS-PAGE and immunoblot analysis

Protein samples were routinely loaded on 7.5 % SDS-PAGE tricine resolving and stacking gel for electrophoresis. Samples were mixed with 2 × sample buffer (100

mM DTT, 4 % SDS, 0.01 % Coomassie blue, 24 % glycerol, pH 6.8) to a concentration of 1 × and boiled for 15 minutes to denature the proteins before being loaded into the wells. SDS-PAGE molecular weight standards, broad range protein markers (Bio-Rad, CA, USA) were used. Electrophoresis was carried out with anode (0.2 M Tris, pH 8.9) and cathode buffers (0.1 M Tris, 0.1 % SDS, pH 8.25) on a vertical electrophoresis apparatus (Hoefer, Pharmacia) at 50-100V. After electrophoresis has completed, the proteins in the gel were stained with Coomassie plus Reagent. For western blotting, the proteins were transferred from the gel to a bio-blot nitrocellulose membrane (Amersham, Piscataway, NJ) and probed with specific antibody. The membrane was pre-equilibrated with PBST solution containing 2 % BSA for 1 hr and then reacted with anti-E7 mAbs (Oncogene, Boston, MA) for overnight at 4 ^{o}C. After three washes with PBST, the membrane was incubated with anti-mouse IgG-HRP for 1 hr at room temperature. The immunoreactive protein bands were detected using the ECL detection reagents (Amersham).

2.2.1.6 Determination of proteins concentration

The concentrations of native Fve, and recombinant E7 protein, recombinant GST protein were analyzed by 7.5 % Tricine SDS-PAGE (Schagger and Von Jagow, 1987) were determined by the Coomassie plus protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as standards, and quantified by Bio-Rad Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Richmond,

CA, USA).

2.2.1.7 Endotoxin removal

Removal of LPS and LPS-associated molecules from Fve protein and GST protein using Polymyxin B-Agarose (Sigma-Aldrich) was performed according to manufacturer's instruction. 10 mL of Polymyxin B-agarose were poured into disposable polypropylene columns (Pierce, Rockford, IL, USA) and washed three times in 20 mL of PBS. The Polymyxin B-agarose was then poured into a 50 mL polypropylene tube. Fve protein or GST protein at 1 mg/mL were added to Polymyxin B-agarose at a ratio of 5 mg protein to 10 mL Polymyxin B-agarose. The mixture was then preceded shaking for overnight at 4 ˚C. Next, the Polymyxin B-agarose was re-packed into the disposable polypropylene columns, washed with PBS until the absorbance at 280nm of the eluents dropped to baseline. The eluents containing the protein of interest, which were excluded from the Polymyxin B-agarose, were collected, concentrated by Amicon cell, and assayed for endotoxin levels using the Limulus Amebocyte Lysate (LAL) QCL-1000 (Cambrex Bio Science, USA).

2.2.2 Preparation of immune cells

2.2.2.1 Preparation of single splenocyte suspension

Spleens were removed from mice and placed in Petri dishes in the presence of 10 ml of HBSS. Single cell suspension was made by disrupting the tissues using two

frosted-slides. Red blood cells (RBCs) were lyses by adding 1.5 ml of RBC lysis buffer (10 mM Tris, 0.83% NH4Cl, PH8.2-8.4) for 90 seconds. The cells were then washed three times with HBSS and cell number was determined using hematocytometer. The cells were re-suspended in complete RPMI-1640 medium for further use.

2.2.2.2 Preparation of accessory cells/antigen presenting cells

Mitomycin C-treated splenocytes, splenic DCs, B cells from naïve mice and BM-DCs from 5-days culture were used as accessory cells (ACs) or antigen presenting cells. Mitomycin C (Roche Diagnostics GmbH, Mannherim, Germany) was dissolved in PBS at a concentration of 0.5 mg/ml. Cells were washed twice with HBSS and suspended in PBS to a concentration of 50 μg/ml and the tube was wrap with aluminum foil. The tube was incubated in 37 ^{o}C water bath for 20 min. After that, cells were washed 3 times with large volume of HBSS and suspended in RPMI-1640 medium.

2.2.2.3 Preparation of bone marrow-derived DCs

The method for generating bone marrow-derived dendritic cells (BM-DCs) with GM-CSF was adapted from previous publication[434]. In brief, bone marrow cells were harvested from femurs and tibias of normal C57BL/6 mice and washed with PBS. 4-6 × 10^6 of cells were re-suspended in complete RPMI-1640 medium containing recombinant mouse GM-CSF (20 ng/ml, BD PharMingen) and cultured

in 100-mm diameter petri dishes. On day 3 half of the medium was replaced with fresh medium supplemented with GM-CSF (10 ng/ml). On day 5 or 6 of cultured bone marrow cells were harvested for dendritic cell purification.

2.2.2.4 Preparation of splenic DCs

The splenic DCs were purified as describe before[435] with modifications. Spleens (from eight mice) were minced with scissors and digested in 10 ml HBSS (with Ca^{2+} and Mg^{2+}) containing collagenase (400U/mL, Roche Molecular Biochemicals) for 30 min at 37 °C. Next, EDTA (1 ml, 0.1 M) was added at room temperature for 5 min to disassociate the DCs from any complexes with T cells. The digested tissue samples were filtered through a 40 μm nylon mesh to remove undigested fibrous materials. All subsequent steps were performed at room temperature using a HBSS (without Ca^{2+} and Mg^{2+}). Cells in the filtrate was recovered by centrifugation, the pellet was re-suspended in a 1.068 g/cm3 OptiPrep medium and centrifuged at 600g for 15 min. The low-density fraction was collected (2-4% of the total) and re-suspended in the running buffer (PBS with 0.5% BSA and 2 mM EDTA).

2.2.2.5 Lymphocytes purification using AutoMACS separator

Magnetic microbeads of autoMACS separator (Miltenyi Biotec GmbH Bergisch Gladback, Germany) was employed to purify splenic $CD90^+$ ($Thy1.2^+$) T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD11c^+$ splenic DCs, $CD11c^+$ BM-DCs, $CD19^+$ B cells, and $NK1.1^+$ NK cells according to the manufacturer's protocol. Briefly, every 10^7

single cells were re-suspended in 90 μL of staining buffer (0.5% bovine serum albumin (BSA, Sigma-Aldrich), 2 mM EDTA (1st BASE**,** Singapore) in PBS, pH 7.4) and 10 μL of corresponding MicroBeads were added. After incubation for 20 min at 4 °C and extensive washing with staining buffer, the cells were subjected to magnetic separation using AutoMACS. The purities of $CD90^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD11c^+$ splenic DCs, $CD11c^+$ BM-DCs, $CD19^+$ B cells, and $NK1.1^+$ NK cells are above 97%, 95%，90%，97%，98%， 95% and 90%，respectivley.

2.2.3 Cell aggregation assay

$CD4^+$ T cells, $CD8^+$ T cells, $NK1.1^+$ NK cells from spleen in C57BL/6 mice were purified by AutoMACS. $CD11c^+$ BM-DCs were purified from GM-CSF cultured BM-cells at day 5. Purified $CD11c^+$ BM-DCs, $CD4^+$ T cells, $CD8^+$ T cells and $NK1.1^+$ NK cells were stimulated with 20 μg/ml of Fve for 24 hrs, respectively. The morphologies of the cells were examined by light microscopy at 24 hrs. Non-stimulated cells used as negative controls.

2.2.4 Cell proliferation assay

For splenocytes proliferation, spleen cells collected from C57BL/6 mice were re-suspended in the RPMI complete medium, and incubated in the presence of different doses of Fve in triplicate wells of a 96-well plate (5×10^5 cells/well). For T cell proliferation assay, $CD90^+$ T cellls were purified from splenocyte of CD57BL/6 or BALB/c mice by AutoMACS. Mitomycin C-treated splenocytes (3 ×

10^5 cells/well) or mitomycin C-treated B cells (3×10^5 cells/well) were co-cultured with purified $CD90^+$ T cells (1×10^5 cells/well) with GST (20 μg/ml), or Fve (20 μg/ml) for 72 hrs. For another set of experiment, mitomycin C-treated purified BM-DCs (2×10^4 cells/well) were co-cultured with purified $CD90^+$ T cells (1×10^5 cells/well) from C57BL/6 mice with PBS or Fve (20 μg/ml) for 72 hrs. The ^{3}H-thymidine was added 18 hrs before cell harvest and thymidine incorporation was measured by liquid scintillation counting after 72 hrs.

2.2.5 *In vitro* cytokine production by Fve-stimulated T cells

For cytokine profiling experiment, 2×10^6 of $CD90^+$ T cells purified from the C57BL/6 mice spleens were seeded into a 6-well U-bottom plate together with 2×10^5 of Mitomycin C-treated BM-DCs. Cells were treated with or without Fve (20 μg/ml). The supernatants were collected after 24 hrs and the cytokine productions (IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α) by T cells were measured by ELISA.

For cytokine kinetics experiment, $CD90^+$ T cells (2×10^6 cells /ml) from the C57BL/6 mice spleens were incubated either alone (T cells) or with BM-DCs at a 10:1 ratio (T cell/DC) in the presence or absence of 20 μg/ml of Fve for 24, 48, 72 hrs. The transwell groups were also set up correspondingly. The supernatants were collected and cytokine production (IFN-γ, IL-2) by T cells was measured by ELISA.

2.2.6 Flow cytometry analysis

2.2.6.1 Cell surface marker staining and flow cytometry analysis of the Fve-stimulated T subsets

For T subsets population analysis, splenocytes from C57BL/6 were stimulated with or without 20 μg/ml *in vitro*. The cells were collected at 24, 48, 72 hrs time point. Then the cells were stained with rat anti-mouse CD3-PercP, CD8-FITC, CD4-APC monoclonal antibody or IgG isotype control. Flow cytometry was performed using FACSCalibur (Becton Dickinson immunocytometry systems, San Jose, CA).

For T cell activation marker staining, $CD90^+$ T cells purified from the splenocytes of C57BL/6 mice were incubated for 24 hrs either alone (T cells) or with BM-DCs at a 10:1 ratio (T cell:DC) in the presence or absence of 20 μg/ml of Fve. The cells were collected and suspended in the staining buffers. The mAbs (rat anti-mouse CD3-PercP, CD8-FITC, CD4-APC, OX-40-PE, 4-1BB-PE, hamster anti-mouse CD69-PE) were used to stain the cells and flow cytometry analysis was performed using FACSCalibur.

2.2.6.2 Surface marker staining and flow cytometry analysis of the Fve-stimulated dendritic cells.

For BM-DCs surface marker staining, day 5 GM-CSF-cultured Bone marrow cells were stimulated with different concentrations of Fve protein (10, 20, 100 μg/ml), or LPS (1 μg/ml) *in vitro* for 16-18 hrs. The cells were stained with rat anti-mouse

CD11c-APC, I-A^b-FITC, CD80-FITC, CD86-PE, or hamster anti-mouse CD54-FITC mAbs. For splenic DCs surface marker staining, C57BL/6 and BALB/c mice were intraveneously injected with PBS, Fve or LPS 12 hrs prior to cell harvest. DCs were enriched from spleen for staining of MHC class II and CD86. Rat anti-mouse CD11c-APC, I-A^b-FITC, or I-A^d-FITC, CD86-PE mAbs was used. Flow cytometry analysis was performed using FACSCalibur.

2.2.6.3 Surface marker staining and flow cytometry analysis of the Fve-stimulated DCs with/without the help of T cells

Transwell experiment was performed in six well plates with tissue culture inserts of 0.4 μM pore size (Corning, NY). Purified BM-DCs (1×10^6 cells/well) were seeded at the bottom well and incubated alone, or with $CD90^+$ T cells from the splenocytes of C57BL/6 mice at a T cell/ DC ratio of 10:1, or with $CD90^+$ T cells (T cell:DC = 10:1) separated in trans-well (DC/T) in the presence or absence of Fve protein (20 μg/ml) for 24 hrs. For another experiment, $CD90^+$ T cells were purified from the splenocytes of C57BL/6 mice and stimulated with/without Fve protein (20 μg/ml) for 24 hrs. Then the activated T cells or freshly purified T cells were cocultured with purified BM-DCs (T cell/DC ratio= 10:1) for 24 hrs. The surface markers were stained with Rat anti-mouse CD11c-APC, I-A^b-FITC, CD86-PE, CD40-FITC or CD80-FITC mAbs and analyzed by flow cytometry.

2.2.7 Analysis of DC-directed $CD4^+$ and $CD8^+$ T cells activities

There are two set of experiments for this purpose. For the first set of experiment, BALB/c mice (n = 8 mice/group) were intraveneously injected with PBS or Fve. The DCs from the pool of eight spleens from each group of mice were isolated 12 hrs later. Purified DCs were pulsed with 0.1 μM of $OVA_{323-339}$ peptide for 2 hrs at 37 °C and washed three times. Then splenic DCs (5×10^3 cells/well) were co-cultured with $CD4^+$ T cells (2×10^4 cells/well) from DO 11.10 mice for 72 hrs. The ^3H-thymidine was added 18 hrs before cell harvest and thymidine incorporation was measured by liquid scintillation counting after 72 hrs. Supernatants were collected and cytokine production was measured by ELISA.

For the second set of experiment, C57BL/6 naive mice were intravenously injected with PBS, 100 μg of OVA, 20 μg of Fve, or 100 μg of OVA plus 20 μg of Fve 24 hrs prior to $CD11c^+$ DC harvest. Purified $CD11c^+$ DCs were pulsed with 1 μM of $OVA_{323-339}$ peptide or 1 μM of $OVA_{257-264}$ peptide respectively (AnaSpec, Inc., San Jose, CA) for 2 hrs at 37 °C and washed extensively thereafter. Naïve $CD4^+$ cells were purified from OT-II transgenic mice. Naive $CD8^+$ cells were purified from OT-I transgenic mice. DC (5×10^3 cells) were incubated with 5×10^4 of $CD4^+$ T cells or 5×10^4 of $CD8^+$ T cells respectively in U-bottom 96-well plates in 200 μl of RPMI medium in triplicates for 72 hrs. Supernatants were collected and cytokine production was measured by ELISA.

2.2.8 Cytokine analysis of Fve-stimulated NK cells *in vitro*

Different cell numbers of purified $NK1.1^+$ NK cells from BALB/c mice were stimulated with/without 10 μg/ml Fve *in vitro*. After 24 hrs, supernatant were collected for cytokine assay using ELISA.

2.2.9 Immunization protocol in the E7 model experiments

For E7-specific B cell immune response, C57BL/6 and BALC/cJ mice were subcutaneously immunized with PBS, 20 μg of E7 or 20 μg of E7 plus 20 μg of Fve at day 0, 14, 28. Sera were collected weekly and used for antibodies analysis by ELISA.

For E7-specific T cell immune response, C57BL/6 mice were subcutaneously immunized with PBS, 20 μg of E7, or 20 μg of E7 plus 20 μg of Fve at day 0 and 14. Splenocytes were collected at day 28 for cell culture. The culture processes were described in section 2.2.10-13.

2.2.10 Short-term T cell culture *in vitro*

The method for single cell suspension was described in section 2.2.2.1. Cells were cultured with RPMI-1640 medium with 10% heat-inactivated BCS (Stem cell technologies, BC, Canada), 2 mM 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (Hyclone Laboratories, Logan, UT), and 5.5×10^{-2} mM 2-mercaptothanol (life technology, Grand Island, NY). To

determine the cytokine production in the freshly isolated splenocytes, 100 μl of splenocytes (4×10^5 cells/well) were added into the 96-well U-bottom plate followed by 100 μl of E7 (1, 10, 20 μg/ml) diluted in culture medium. For E7-specific T cell immune response, the splenocytes were cultured in 6-well (2×10^7 cells/well) or 24-well (4×10^6 cells/well) plates in the presence of E7 protein (10 μg/ml) for 9 days. Recombinant mouse IL-2 (Pharmingen) was added to the cultured cells on day 3 and day 6 to a final concentration of 10 U/ml.

2.2.11 Separation of dead cells from short-term cultured splenocytes by Ficoll-Pague centrifugation

Nine days after the primary cell culture with antigen, the cells were collected and suspended in 5 ml of HBSS. The cell suspension was transferred to 15 ml tube. Threr milliliters of Ficoll-Paque (Amersham Bioscience Corp. Piscataway, NJ) was added carefully to the bottom of the tube without disturbing the interface of two layers. The cells were then centrifuged for 20 min at 800g. After that, live cells were collected from the interface, washed 3 times with HBSS and suspended in the culture medium. To determine the cytokine production from the short-termed cultured T cells, 50 μl of T cells (2×10^6 cells/ml) collected after the Ficoll-Paque centrifugation and 50 ml of antigen presenting cells (6×10^6/ml) were added into the 96-well U bottom plate followed by 100 μl of E7 protein (1, 10, 20 μg/ml).

2.2.12 Intracytoplasmic cytokine staining

For intracytoplasmic cytokine staining, 96-well plate was coated with 200 μl/well of anti-CD3 mAb (5 μg/ml) diluted in PBS and incubated at 37^{o}C for 2 hrs. The well was then washed twice with 1 ml PBS. 2 × l0^{5} of cells/well of short-term cultured cells were added to each well in the presence of 2 μg/ml of rat anti-mouse CD28 mAb for 12 hrs. Three μM of Monensine (Sigma, St. Louis, MO) was added 6 hrs before harvesting the cells from the culture. Cells were then washed twice in staining buffer and stained with rat anti-mouse CD8-FITC or rat anti-mouse CD4-PerCP monoclonal antibody. After fixation with 1 % of formaldehyde solution (BDH laboratory supplies, England) containing 0.1 % of Saponin (Sigma) for 30 min at room temperature, the cells were washed twice with permeabilization buffer (staining buffer with 0.1 % Saponin) and stained with anti-IFN-γ, IL-10, IL-4, TNF-α for another 30 min at room temperature. The cells were then washed twice with permeabilization buffer and resuspended in 1% formaldehyde solution. Flow cytometry was performed using FACSCalibur.

2.2.13 Stimulation of T cells by anti-CD3 and anti-CD28 mAbs

Thirty microliter of rat anti-mouse CD3 mAb at a concentration of 10 μg/ml was added to 96-well U-bottom plate. The plate was incubated at 37 ^{o}C for two hrs. After that, each well was washed twice with 100 μl of PBS. One hundred microliters of cell suspension (1 × 10^{6} cells /ml) and 100 ml of rat anti-mouse CD28 mAb (2 μg/ml) were added to each well.

2.2.14 Immunoassay

2.2.14.1 Mouse cytokines ELISA

The 96-well high- binding plate (Costar) was coated with 25 μl of purified rat anti-mouse IFN-γ (2 μg/ml), TNF-α (0.8 μg/ml), TGF-β (2 μg/ml), IL-4 (1 μg/ml), IL-10 (2 μg/ml), IL-2 (1 μg/ml), IL-6 (1 μg/ml) diluted in coating buffter (0.1 M NaHCO3, PH 8.2) and incubated at 4^{o}C overnight. After washing the plate with washing buffer (0.05% Tween 20, 1% BSA in TBS), the wells were then blocked with blocking buffer (PBS containing 0.05% Tween-20 and 1% BSA) at room temperature for 1 hr. 25 μl of cultured supernatants were added to wells and the plate was incubated at 4^{o}C overnight. 25 μl of recombinant mouse IFN-γ, TNF-α, TGF-β, IL-4, IL-10, IL-2, IL-6 were added for quantification purpose. In the third day, plate was washed and 50 ml of biotinylated anti-mouse IFN-γ (2 μg/ml), TNF-α (0.3 μg/ml), TGF-β (2 μg/ml), IL-4 (1.5 μg/ml), IL-10 (0.4 μg/ml), IL-2 (1 μg/ml), IL-6 (1 μg/ml) mAbs were added to each well and incubated at room temperature for 1 hr. After washing, 25 μl of ExtraAvidin® - alkaline phosphatase (1:2000) diluted in blocking buffer were added and incubated at at room temperature for another 1 hr. Signal was developed by adding pNPP substrate and read with a microplate reader at 405 nm (Tecan, Ges.m.b.H, Austria).

2.2.14.2 Mouse antibodies ELISA

Mice were subcutaneously immunized with PBS, 20 μg of E7 or 20 μg of E7 plus

20 μg of Fve at day 0, 14, 28. Sera were collected weekly and used for antibodies analysis by ELISA. For IgG1 analysis, 96-well plate was coated with (5 μg of E7 protein per well) and incubated at 4 °C overnight. The wells were then blocked with blocking buffer. Diluted sera were added and incubated at 4 °C overnight. After washing, the plate was incubated with biotin-conjugated anti-mouse IgG1 at room temperature for 1 hr, followed by addition of ExtrAvidin®-alkaline phosphatase for another 1 hr. Signal was developed by adding pNPP substrate and read with a microplate reader at 405 nm (Tecan, Ges.m.b.H, Austria). For IgG2c detection, ELISA Kit (Bethyl Laboratories, Montgomery, TX, USA) was used according to the manufacturer's protocol.

2.2.15 Murine tumor model protocol

2.2.15.1 *In vivo* tumor protection assay

Mice were subcutaneously immunized with PBS, 20 μg of E7, 20 μg of Fve, or 20 μg of E7 plus 20 μg of Fve at day 0, 14, 28. 5×10^4 of TC-1 cells were inoculated into the right flank of mouse at day 30. Tumor size was measured every two days in two perpendicular dimensions and expressed as length × width (mm^2). The survival was monitored.

2.2.15.2 *In vivo* tumor therapeutic assay

Mice were subcutaneously inoculated with 5×10^4 of TC-1 cells in the right flank at day 0. Mice were then subcutaneously immunized with PBS, 20 μg of E7, 20 μg of

Fve, or 20 μg of E7 plus 20 μg of Fve at day 3, 10, 17. Tumor size was measured every two days and survival was monitored.

2.2.15.3 *In vivo* tumor metastatic assay

For the metastasis protection assay, mice were subcutaneously immunized with PBS, 20 μg of E7, 20 μg of Fve, or 20 μg of E7 plus 20 μg of Fve at day 0, 14, 28. 5×10^4 of TC-1 cells were intravenously injected into the tail vein of mice 2 days after the last immunization. For tumor metastasis therapeutic assay, mice were intravenously injected with 2×10^4 of TC-1 cells by tail vein at day 0. Mice were then subcutaneously immunized with PBS, 20 μg of E7, 20 μg of Fve, or 20 μg of E7 plus 20 μg of Fve at day 3, 10, 17. The survival was monitored.

2.2.15.4 *In Vivo* depletion of $CD4^+$, $CD8^+$ T Cells and IFN-γ

Mice were subcutaneously immunized with PBS, 20 μg of E7, 20 μg of Fve or 20 μg of E7 plus Fve at day 0, 14, 28. To deplete $CD4^+$, $CD8^+$ T cells and IFN-γ, mice were intraperitoneally injected with 800 μg of anti-CD4, 500 μg of anti-CD8 and 500 μg of anti-IFN-γ mAbs at days -4, -1, 6, 13, 20, 27, 34, 41, 48. 5×10^4 of TC-1 cells was inoculated into the right flank at day 30. Tumor size was measured every two days and survival was monitored. The depletion of $CD4^+$, $CD8^+$ T cells was assessed at day 6 after the first immunization and the depletion in the spleen was higher than 95% as determined by flow cytometry analysis.

2.2.15.5 T-cell adoptive transfer

Mice were immunized with PBS, 20 μg of E7 or 20 μg of Fve, or 20 μg E7 plus 20 μg Fve at days 0, 14 and 28. Splenocytes were collected at day 30 and $CD90^+$ T cells were isolated using CD90 microbeads (Miltenyi Biotec) for cell transfer experiment. The purity of T cells was above 98% determined by flow cytometry analysis. 8×10^6 of freshly purified $CD90^+$ T cells were adoptively transferred into recipient mice at day -1, 3, 6, 9. Recipient mice were inoculated subcutaneously in the right flank with 5×10^4 of TC-1 cells to initiate tumors at day 0. Tumor size was measured every two days.

2.2.16 Statistical analysis

Statistical significance was determined using SPSS, Version 11.0.1 (SPSS Inc., Chicago, IL, USA). For all tests, $p < 0.05$ was considered to indicate statistical significance. Results for tumor sizes are given as mean ± SEM and were analyzed by one-way ANOVA. Antibody cytokine production between groups was analyzed by Student's *t* test. In tumor protection and therapeutic and adoptive transfer experiments, the tumor free and survival analysis were carried out using the Kaplan-Meier and the log-rank test. $P < 0.05$ was considered as statistically significant.

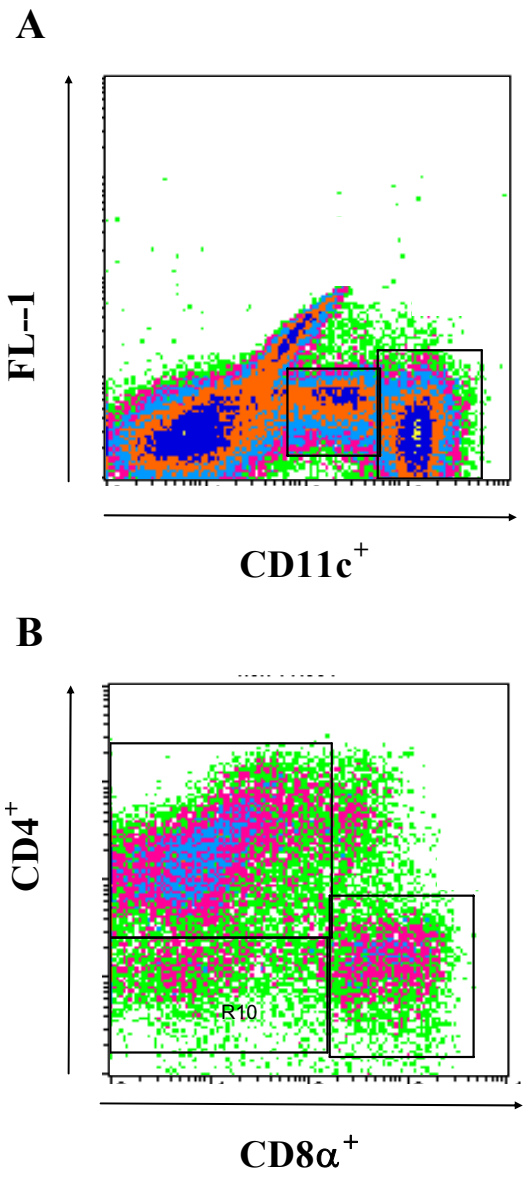


Figure 2.1 Immunofluorescent staining of freshly isolated splenic DCs for CD4 and CD8α. DC were extracted and enriched from the spleens of C57BL/6 mice and were stained with anti-CD11c (PE conjugate), anti-CD4 (APC conjugate), and anti-CD8α (Percp conjugate). Splenic DCs were separated into $CD11c^{int}$ and $CD11c^{hi}$ populations (A) and $CD11c^{hi}$ DCs could be further divided into $CD4^{+}$, $CD8\alpha^{+}$ and $CD4^{-}CD8\alpha^{-}$ sub-populations (B). The cells were gated for $CD11c^{+}$ cells with the high forward scatter of DC. The CD4 and CD8α fluorescence distribution was then plotted for the gated DCs (B).

Chapter 3

Immunological characterization of Fve-stimulated immune cells

3.1 Introduction

Native Fip-Fve (Fve) is an acetylated protein consisting of 114 amino acid residues with an estimated molecular weight of 12.7 kDa[423]. Its 3D- structure determined by x-ray crystallography revealed that it is a non-covalently linked homodimer, wherein each subunit of the homodimer consists of an N-terminal alpha-helix followed by a fibronectin III-type fold (appendix 4). Sugar–binding studies suggested that Fve could be a lectin with specificity for complex cell surface carbohydrates[430]. Fve has potent mitogenic stimulatory effects on both mouse splenocytes and hPBMCs, and enhances IL-2, IFN-γ, and TNF-α production[415,423]. Hsu et al. reported that Fve could inhibit IL-5-mediated survival of eosinophils through the modulation of cytokine receptor expression and apoptotic signal protein production. Moreover, mice fed with Fve protein-induced systemic production of IFN-γ and coadministration Fve with antigen could drive a strong Th1 skewed immune response[431].

Take together, published studies to date support the notion that Fve protein could activate total splenocytes mitogenically and have some immunomodulatory effects

on the immune system, but there is no systematic study and detailed immunological characterization being carried out to elucidate the effects of Fve on isolated immune cells such as T subsets, DCs, and NK cells. Therefore, the first part of this study aimed to characterize further the mitogenic effect of Fve on splenic T–subsets. The surface marker expression, proliferation, and cytokine production of T cells was assayed *in vitro*.

The interaction between Fve and innate immunity also is worth exploring. DCs are highly specialized antigen-presenting cells that play a pivotal role in regulating the T cell responses. Characterizing the maturational state of the Fve-stimulated DCs is important because several studies indicate that immature DCs are involved in maintaining tolerance[436-439] whereas fully mature DCs display several distinct features: (1) they gain the capacity to activate naïve T cells owing to their high expression levels of MHC antigen-presenting molecules, adhesion molecules, and costimulatory molecules such as CD86, CD40, CD80; (2) they are very efficient at presenting the antigens to T cells leading to T cell activation, differentiation, and polarization. In this study, murine DCs from bone marrow and spleen were characterized after Fve stimulation *in vitro* and *in vivo*. Besides, their maturation state *in vivo* correlated with their ability to process and present Ag to specific $CD4^+$ and $CD8^+$ T cells was also explored.

Another important and distinct cell type in the innate arm of the immune system is the NK cell. NK cells and T cells share a common biopotential progenitor; NK

cells share a common killing mechanism with CTLs and, like $CD4^+$ Th and CTL cells, NK cells secrete IFN-γ but not IL-2. The effector functions of NK cells can be regulated by the integrated processing of signals transduced by stimulatory or inhibitory cell surface ligands/receptors[440]. Thus, it would be interesting and important to explore the nature of interaction between Fve and NK cells and the possible immunological outcomes of such interactions.

In summary, the main objective of this chapter is to explore and elucidate the immunomodulatory effects of Fve on T cells, DCs, and NK cells by *in vitro* and *in vivo* experimental approaches such as [^{3}H]-thymidine uptake for cell proliferations, ELISA for cytokine profiles, and FACS analysis for surface marker profiles.

3.2 Results

3.2.1. *In vitro* immunological characterization of Fve-stimulated splenocytes and splenic T cells

3.2.1.1 Extraction and purification of Fve from cultivated *Flammulina velutipes*

Fve protein is the major component of the crude extract from the mushroom fruit bodies of *Flammulina velutipes* (appendix 2). A native form of Fve protein was extracted from the fruit bodes of the cultivated golden needle mushroom (appendix 2). Fve protein was purified, using ion exchange chromatography as described (section 2.2.2.1). The yield of Fve protein was 40 mg from 1 kg wet-weight of fresh mushroom. The purified Fve protein was analyzed on a 7.5% SDS-PAGE and the molecular weight of the protein is about 12.7 kDa. (Fig. 3.1). The purified Fve protein was used in all the experiments described in this thesis.

3.2.1.2 Dose-dependent proliferation of Fve-stimulated splenocytes

To examine the mitogenic effect of Fve on mouse splenocytes, splenocytes from C57BL/6 mice were cultured with Fve protein at final concentrations from 0 to 20 μg/ml for 72 hrs, and then the cell proliferation was analyzed by [^{3}H]-thymidine incorporation (section 2.2.4). As shown in Figure 3.2A, Fve stimulated splenocytes' proliferation in a dose-dependent manner.

To exclude the possibility of endotoxin LPS contamination in the Fve protein, cell proliferation experiments were performed using Fve protein before and after polymyxin B agarose treatment. As shown in Figure 3.2B, the Fve-induced splenocytes proliferation remained unchanged before and after the removal of LPS, suggesting that the proliferation of splenocytes by Fve was not induced by LPS contamination.

3.2.1.3 The percentages of $CD4^+$ T cells and $CD8^+$ T cells in Fve-stimulated splenocytes

To characterize the Fve-induced T cell proliferation, splenocytes were stimulated by 20 μg/ml of Fve for 24, 48, and 72 hrs, and the surface markers CD3, CD4, and CD8 were analyzed by flow cytometry (section 2.2.6.1). The flow cytometric data were summarized in Table 3. As shown in Table 3, the percentages of Fve-stimulated $CD4^+$ T cells in the cultured splenocytes were 28%, 35%, and 39% at 24, 48 and 72 hrs respectively, higher than those of non-stimulated $CD4^+$ T cells (24%, 24%, 9%) and of GST-stimulated $CD4^+$ T cells (27%, 23%, 9%). Similarly, and more evidently, the percentages of Fve-stimulated $CD8^+$ T cells in the cultured splenocytes were 17%, 20%, and 43% at 24, 48, and 72 hrs respectively, much higher than those of non-stimulated $CD8^+$ T cells (13%, 9%, 5%) and of GST-stimulated $CD8^+$ T cells (10%, 7%, 7%). These data indicate that non-stimulated and GST-stimulated $CD4^+$ and $CD8^+$ T cells might go to apoptosis over time, whereas Fve-stimulated $CD4^+$ and $CD8^+$ T cells could proliferate and

survival longer.

When analyzing the ratio of $CD4^+$ T cells and $CD8^+$ T cells (ratio = CD4/CD8, Table 3), the ratios of CD4/CD8 were almost unchanged at 24 hrs and 72 hrs in a non-stimulated group (from 1.9 to 2.0) but decreased significantly from 1.6 to 0.9 after Fve stimulation, indicating that Fve may have more effects on $CD8^+$ T cells than on $CD4^+$ T cells.

3.2.1.4 Aggregations of purified $CD4^+$ T cells and $CD8^+$ T cells in response to Fve

Since Fve induced aggregation or adhesion of $hPBMC^{424}$ and purified T cells from hPBMC (unpublished data from our lab), it's interesting to examine whether a similar phenomenon could happen in the mouse. For this purpose, purified $CD4^+$ T cells and $CD8^+$ T cells from mouse spleen were co-cultured with 20 μg/ml of Fve protein for 24 hrs (section 2.2.3). Microscopic visualization of stimulated cells revealed that $CD4^+$ T cells (Fig. 3.3A) and $CD8^+$ T cells (Fig. 3.3B) form large aggregates, whereas unstimulated cells remained in a single-cell suspension. The cell aggregated within 30 min after Fve stimulation and continued to expand in size for the duration of the culture period.

3.2.1.5 Up-regulation of CD69, OX-40 and 4-1BB expression on Fve-stimulated T cells

Purified $CD90^+$ T cells were stimulated with/without Fve in the presence or

absence of BM-DCs as accessory cells (ACs) for 24 hrs, and cell surface expression of CD69, OX-40, and 4-1BB were analyzed by flow cytometry. As shown in Figure 3.4, CD69, OX-40 and 4-1BB were up-regulated on $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells after Fve stimulation in the absence of accessory cells as compared to those on the non-stimulated T cells. The up-regulations of these markers increased greatly in the presence of accessory cells. These results indicate Fve could activate T cells independent of accessory cells, although the magnitude of the surface marker expression levels was greatly enhanced in the presence of BM-DCs as accessory cells. Moreover, further cytometric analysis of T subsets revealed that OX-40 was highly expressed on $CD4^+$ T cells as compared to those on $CD8^+$ T cells.

3.2.1.6 Accessory cell-dependent T cell proliferation in response to Fve stimulation

To explore further the mitogenic effect of Fve on T cells, purified $CD90^+$ T cells were stimulated with/without 20 μg/ml of GST protein, or 20 μg/ml of Fve for 72 hrs with/without accessory cells, and the cell proliferation was assayed by ^{3}H-thymidine incorporation (section 2.2.6.1). As shown in Figure 3.5, in the absence of accessory cells, T cells slightly proliferated in response to Fve as compared to those in non-stimulated and GST-controls, but the level of proliferation was very low. In contrast, the level of Fve-stimulated T cell proliferation was greatly enhanced in the presence of accessory cells, 10 times

higher than that in Fve-stimulated T cells without accessory cells. This indicates that Fve induces T cell proliferation is an accessory cell-dependent manner. Moreover, BM-DCs (Fig. 3.6) and B cells (Fig. 3.7) both can act as accessory cells to induce T cell proliferation.

To determine whether Fve-induced proliferation of $CD4^+$ and $CD8^+$ T cells was accessory cell dependent or not, splenic $CD4^+$ and $CD8^+$ T cells were isolated by AutoMACS (section 2.2.2.5) and co-cultured with 20 μg/ml of Fve for 72 hrs, and the cell proliferation was examined by [^{3}H]-thymidine incorporation. GST protein-stimulated T cells were used as control. As shown in Fig 3.8, Fve-induced proliferation of both T cell subsets in an accessory cell-dependent manner. Moreover, the proliferation levels of $CD4^+$ and $CD8^+$ T cells were similar at 48 hrs time point. However, the level of proliferation of $CD8^+$ T cells was higher at 72 hrs as compared to that of $CD4^+$ T cells.

3.2.1.7 The cytokine profile of Fve-stimulated T cells

To explore the cytokine profile of Fve-stimulated T cells, T cells from the splenocytes of C57BL/6 mice were cultured with 20 μg/ml of Fve protein for 72 hrs and the culture supernatants were harvested for cytokine assay by ELISA (section 2.2.5). As shown in Figure 3.9, Fve induced $CD3^+$ T cells to produce IFN-γ, IL-2, IL-6 in the presence, but not in the absence, of the accessory cells. Production of IL-4, TNF-α, and IL-10 by the Fve-stimulated T cells was at basal level. Moreover, the kinetics study of IFN-γ and IL-2 production revealed that a high

level of T cell-derived IL-2 was detected at 24 hrs, and then gradually decreased and finally reduced to basal level by 72 hrs after Fve stimulation (Fig. 3.10B). Since IL-2 is the growth factor of T cells, the reduction of IL-2 with time probably was inversely correlated to the increased Fve-induced T cell proliferation as described earlier (Fig. 3.5). On the other hand, significant levels of IFN-γ were detectable at 24 hrs time point and then reached maximum levels at 48 hrs after Fve stimulation, followed by a slight decline at 72 hrs (Fig. 3.10A).

To investigate whether the IFN-γ and IL-2 production by T cells required direct cell-cell contact between BM-DCs and T cells, similar experiments were set up using the transwell culture system. BM-DCs were placed in the lower chambers in the presence of Fve, while T cells were placed on the upper chambers separated by a porous membrane. The production of IL-2 and IFN-γ was reduced significantly in transwell culture at 24 hrs as compared to the normal cell culture conditions (i.e., in the "DC + T cell + Fve" group with cell-cell contact). Interestingly, under the transwell culture conditions, high level production of IFN-γ was observed only at 48 hrs instead of 24 hrs (Fig 3.10A) , and the production of IL-2 was significantly attenuated (Figure 3.10B), suggesting that the direct DC-T cell contact is important for the T cells to be fully activated.

3.2.2 Phenotypic and functional analysis of Fve-stimulated DCs

3.2.2.1 Fve failed to up-regulate MHC class II, costimulatory molecules on BM-DCs *in vitro*

It is well known that dendritic cell maturation status is a key parameter for DC-T cell cognate interaction, which determines the outcome of T cell activation and differentiation. The DC maturation process involves a series of changes including reduction of antigen uptake, up-regulation of surface MHC class II, and co-stimulatory molecules[441]. In this experiment, the phenotypic changes associated with DC maturation were monitored by surface marker staining followed by flow cytometry analysis. Briefly, purified BM-DCs were stimulated with Fve at concentrations ranging from 10 to 100 μg/ml, or LPS at 1 μg/ml as a positive control for 16–18hrs, and surface marker expression on BM-DCs was analyzed by flow cytometry after immunofluorecent staining (section 2.2.6.2). As shown in Figure 3.11, BM-DCs stimulated with LPS showed up-regulation of MHC class II, CD86, CD80, and CD40 as compared to the non-stimulated control. However, up-regulation of these surface markers was not observed on DC stimulated with various concentrations of Fve, except that there was a slight up-regulation of MHC class II on DC stimulated with 100 μg/ml of Fve. The results suggest that Fve is incapable of inducing DC's phenotypic maturation under *in vitro* culture conditions.

3.2.2.2 Fve induced splenic dendritic cells phenotypic maturation *in vivo,* licensed them for Th1 priming

In view of the observation that Fve failed to induce phenotypic maturation of BM-DCs under the conventional *in vitro* culture conditions, the next question to be addressed was whether Fve could induce DC maturation *in vivo*. To this end, splenic $CD11c^+$ DCs were enriched from spleens of BALB/c mice that were injected intravenously with Fve 12 hrs prior to harvest, as described in method and materials (section 2.2.6.2, Fig. 2.1A&B) and the surface expression of MHC class II and CD86 was analyzed. PBS-injected mice were served as controls. As shown in Figure 3.12A, the up-regulation of MHC class II and CD86 molecules can be seen on both $CD11c^{hi}$ and $CD11c^{int}$ subsets isolated from Fve-treated mice, as compared to that from PBS treated-mice. Furthermore, up-regulation of MHC class II and CD86 was seen in all three subtypes of $CD11c^{hi}$ DCs (Fig. 3.12B), especially in the $CD8\alpha^+$ DCs. This indicates that Fve can stimulate phenotypic maturation of splenic DCs *in vivo*.

Functional characterization of the DCs isolated from Fve-injected mice subsequently was carried out using $CD4^+$ T cells purified from OVA-specific TCR-transgenic DO11.10 mice . As shown in Figure 3.13A, the OVA-specific $CD4^+$ T cells co-cultured with DCs derived from the Fve-injected mice had a higher cell proliferation as compared to that of PBS control. To further assess the polarization effect of these splenic DCs on $CD4^+$ T cells, culture supernatants were

collected for cytokine profiling by ELISA. As shown in Figure 3.13B, $CD4^+$ T cells co-cultured with Fve-treated DCs showed increased production of the Th1-skewed cytokines IFN-γ and IL-2 and proinflammatory cytokine IL-6, whereas the signature Th2 cytokine IL-4, was not up-regulated. The data suggest that Fve-treated splenic DCs may preferentially drive Th1 differentiation.

3.2.2.3 Fve preferentially enhanced antigen-specific $CD8^+$ T cell activation to produce high levels of IFN-γ and IL-2

To further confirm the *in vivo* effects of Fve on mouse DCs, similar experiments were carried out using C57BL/6 mice. Splenic $CD11c^+$ DCs were enriched from spleens of C57BL/6 mice intravenously injected with Fve 12 hrs prior to harvest (section 2.2.2.4) and the surface expression of MHC class II and CD86 was analyzed by flow cytometry. PBS and LPS-injected mice served as controls. Consistent with the above results (Fig. 3.12) in BALB/c, the up-regulation of MHC class II and CD86 molecules can be seen on both $CD11c^{hi}$ and $CD11c^{int}$ subsets isolated from Fve and LPS-treated mice, as compared to that from PBS treated mice (Fig. 3.14C). Besides, up-regulation of MHC class II and CD86 was seen in all three subtypes of $CD11c^{hi}$ DCs (Fig. 3.14D). This further indicates that Fve stimulate phenotypic maturation of splenic DCs *in vivo*.

To explore further whether Fve-stimulated DCs can prime antigen-specific $CD4^+$ and $CD8^+$ T cell activation, *in vitro* functional assays for these Fve-stimulated DCs were performed using OVA-specific $CD8^+$ and $CD4^+$ T cells from OT-I and OT-II

mice, respectively (section 2.2.7). Analysis of culture supernatants of OVA-specific $CD8^+$ T cells co-cultured with OVA-laden DCs isolated from OVA plus Fve co-injected mice showed that production of IFN-γ and IL-2, IL-6 was greatly increased as compared to that from all the other experimental control groups (Fig. 3.15A). Such a remarkable enhancement of IFN-γ and IL-2 production by OVA-specific $CD4^+$ T cells was not observed in similar parallel functional assays performed using $CD4^+$ T cells from OT-II mice (Fig. 3.15B). Besides, there was no increased induction of IL-4, the signature cytokine for Th2 responses, by both $CD4^+$ and $CD8^+$ OVA-specific T cells. These data suggest that Fve can efficiently enhance the OVA-specific $CD8^+$ T cell immune response, probably by modifying the ability of DCs to present antigen.

3.2.2.4 Fve-activated T cells helped phenotypic maturation of BM-DCs in a cell- cell contact dependent manner

The data from section 3.2.3.1 revealed that DCs failed to mature in response to Fve stimulation *in vitro*. In contrast to *in vitro* data, results from *in vivo* experiments showed that Fve induced phenotypic and functional maturation of splenic DCs. The difference in the results from *in vivo* and *in vitro* experiments indicates that there might be some other cell types or factors involved in the DC maturation *in vivo*. It has been reported that naïve T cells activated by superantigen could help DC maturation *in vivo*[442]. Besides, as shown by data presented in Figures 3.5, 3.6, 3.7, and 3.8, Fve can stimulate T cell activation. This prompted me to explore further

whether Fve-induced activated T cells can help DC maturation or not.

To this end, purified $CD90^+$ T cells from C57BL/6 mice were cultured with purified $CD11C^+$ BM-DCs in the presence of 20 μg/ml of Fve for 24 hrs. Expression profile of the surface markers on BM-DCs was analyzed by flow cytometry. As shown in Figure 3.16, the expression of MHC class II, CD86, but not CD40 and CD80, was significantly up-regulated on DCs co-cultured with the combination of Fve and T cells as compared to those in the "DC alone," "DC + Fve," and "DC + T cell" controls. Transwell experiments (section 2.2.6.3) subsequently were set up to determine whether cell-cell contact between DCs and T cells is necessary for DC maturation. As shown by Figure 3.16, there was no up-regulation of MHC class II and CD86 on DCs under the transwell culture conditions.

Another set of experiments was performed to address further whether pre-activation of T cells by Fve had a similar role for DC maturation as seen in the above experiments. Purified $CD90^+$ T cells were pre-activated by Fve protein for 24 hrs, followed by extensive washings to remove Fve protein before co-cultured with DCs for another 24 hrs (designated as "DC + T (Fve)" in Fig. 3.17). The surface markers of DCs were analyzed by flow cytometry after immunofluorecent staining. The cytometric analysis results revealed that DCs co-cultured with the Fve-preactivated T cells showed up-regulation of the MHC class II and CD86, with an expression profile similar to that of the "DC + T + Fve" positive control.

Taken together, these data suggest that Fve-activated T cells could help DC maturation in a cell-cell contact dependent manner.

3.2.3 Cytokine profile of Fve-stimulated NK cells

NK cell represents an important and distinct cell type in the innate arm of the immune system. A preliminary study was conducted to explore the effects of Fve on NK cells purified from BALB/c mice by *in vitro* co-culture experiments (section 2.2.8). Titrated numbers of NK cells were co-cultured with 10 μg/ml of Fve protein for 24 hrs and supernatants were collected for cytokine assays by ELISA. Microscopic examination showed that significant cell aggregation occurred at 24 hrs (data not shown). Cytokine profiling data revealed that Fve-stimulated NK cells produced significant levels of IL-6 without increased production of IFN-γ, IL-2, IL-4, and IL-10. The production of IL-6 without IFN-γ cytokine production profile is a rather unexpected observation that deserves further investigation.

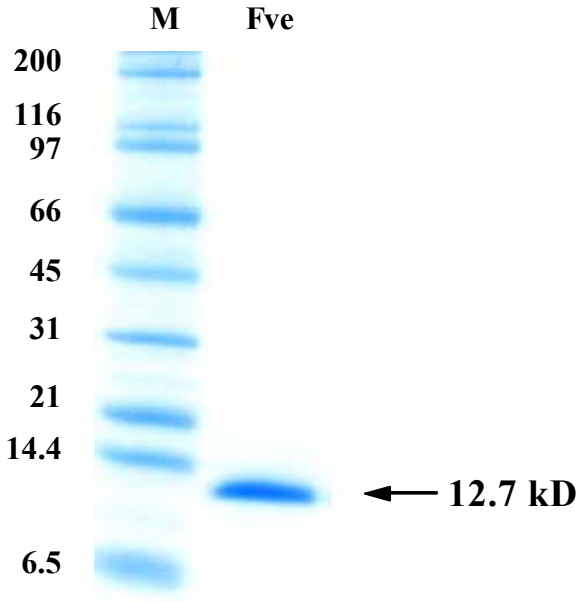


Figure 3.1 SDS-PAGE analysis of purified native Fve protein. Fve protein purified from *Flammulina velutipes* by ion exchange chromatography was analyzed by 7.5% of Tricine SDS-PAGE. Fve shows a single band with molecular mass of 12.7 kD. Lane 1: molecular mass markers; lane 2: purified Fve protein from *Flammulina velutipes* (golden needle mushroom).

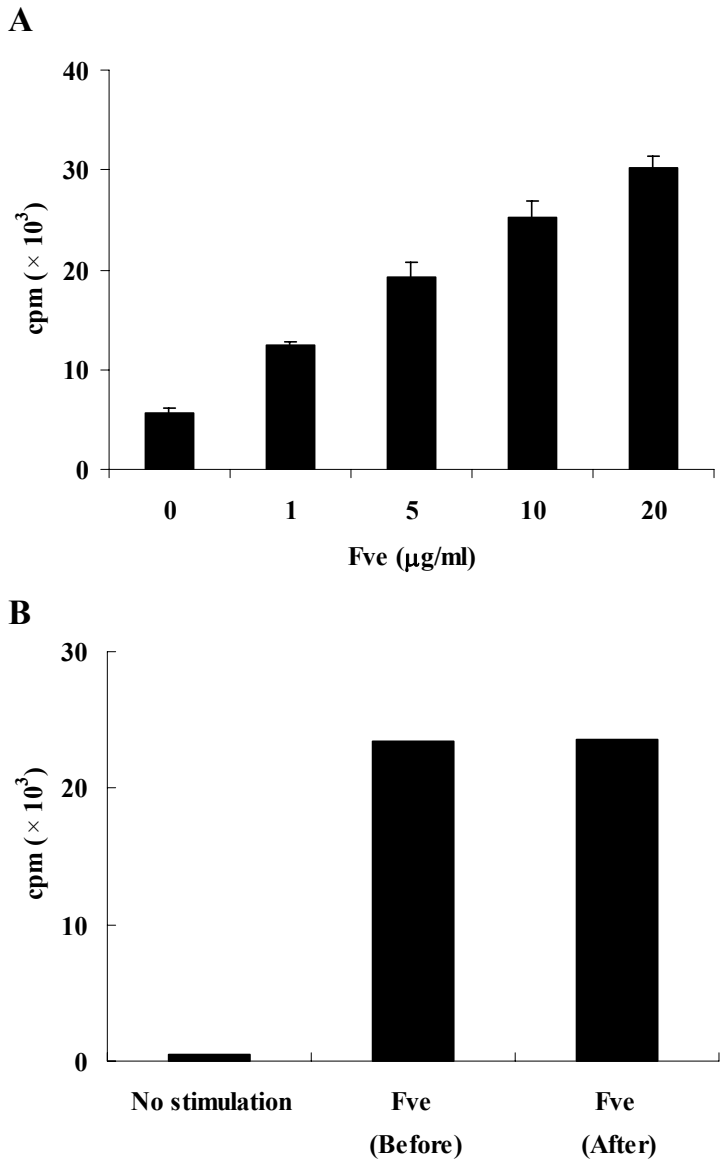


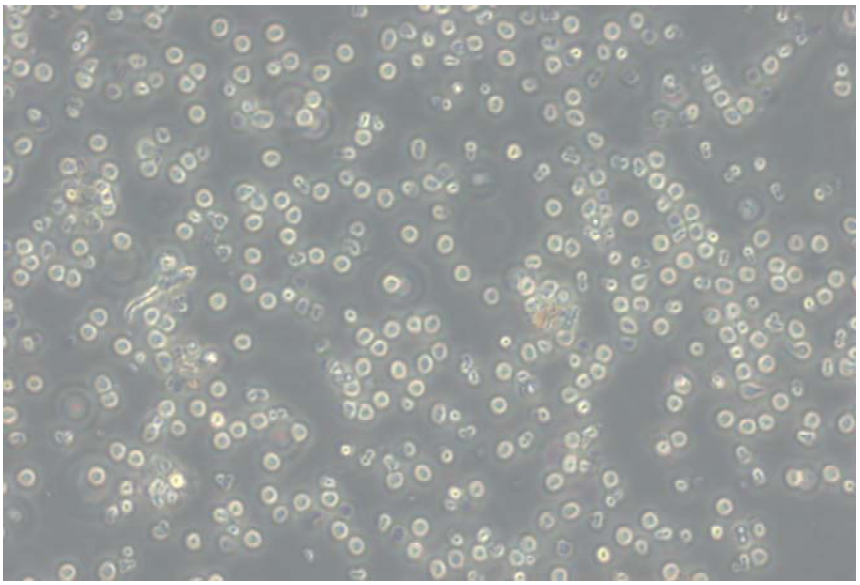
Figure 3.2 Fve stimulated mouse splencyte proliferation. Splenocytes collected from C57BL/6 naïve mice were resuspended in the RPMI complete medium, and incubated in the presence of different doses of Fve in triplicate wells of a 96-well plate (5×10^5 cells/well) (A). To exclude the possibility of endotoxin's effect on splenocytes proliferation, splenocytes (5×10^5 cells/well) from C57BL/6 naïve mice were incubated with 20 μg/ml of Fve (before and after polymyxin B agarose treatment) (B). The cultures were pulsed for 18 hrs with [^{3}H]-labeled thymidine. The cells were harvested and thymidine incorporation was measured by liquid scintillation counting after 72 hrs

Table 3 The percentage of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in total splenoctyte after stimulated by Fve *in vitro* and the ratio of CD4/CD8 T cells.

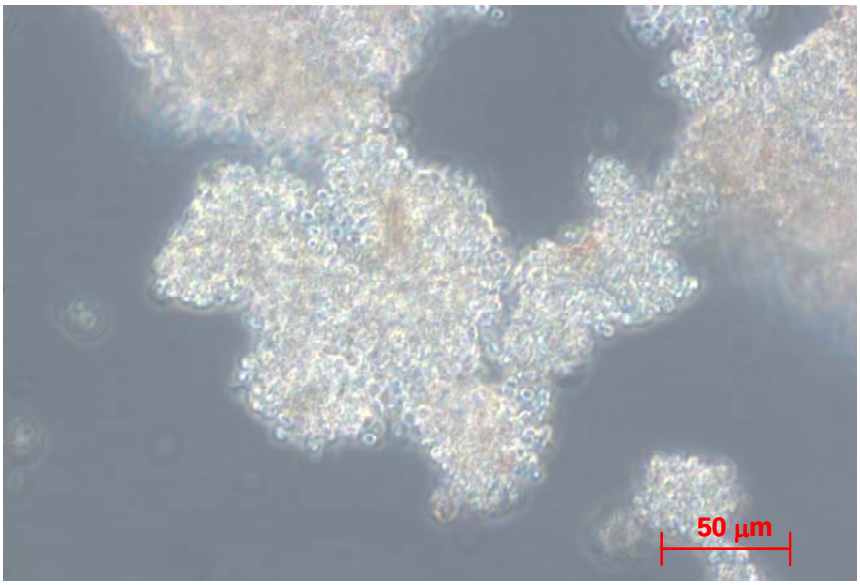
Groups	$CD3^+CD4^+$ T cells (%)			$CD3^+CD8^+$ T cells (%)			CD4/CD8 ratio		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Non-stimulation	24	24	9	13	9	5	1.9	2.7	2.0
GST (20 μg/ml)	27	23	13	10	7	7	2.8	3.4	1.9
Fve (20 μg/ml)	28	35	39	17	20	43	1.6	1.7	0.9

A. Purified $CD4^+$ T cells

Non-stimulation



Fve stimulation



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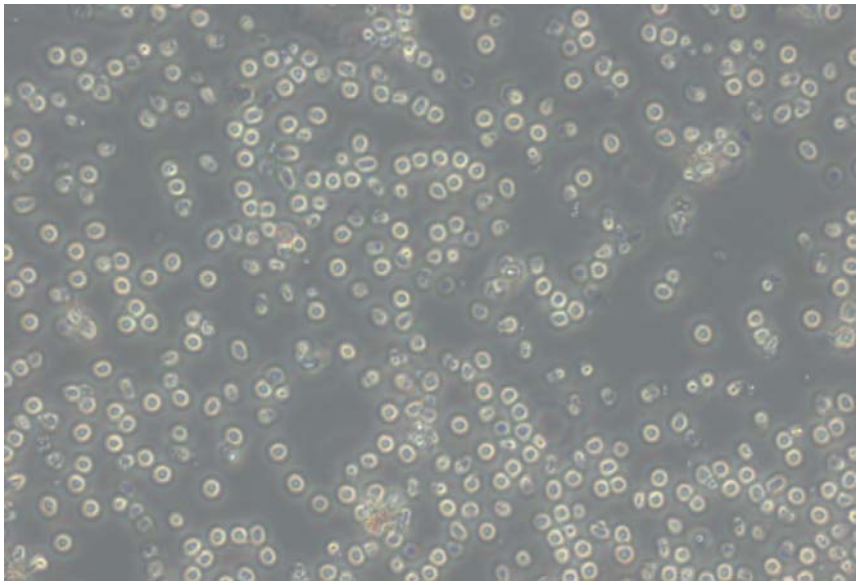
B. Purified $CD8^+$ T cells

Non-stimulation



Fve stimulation

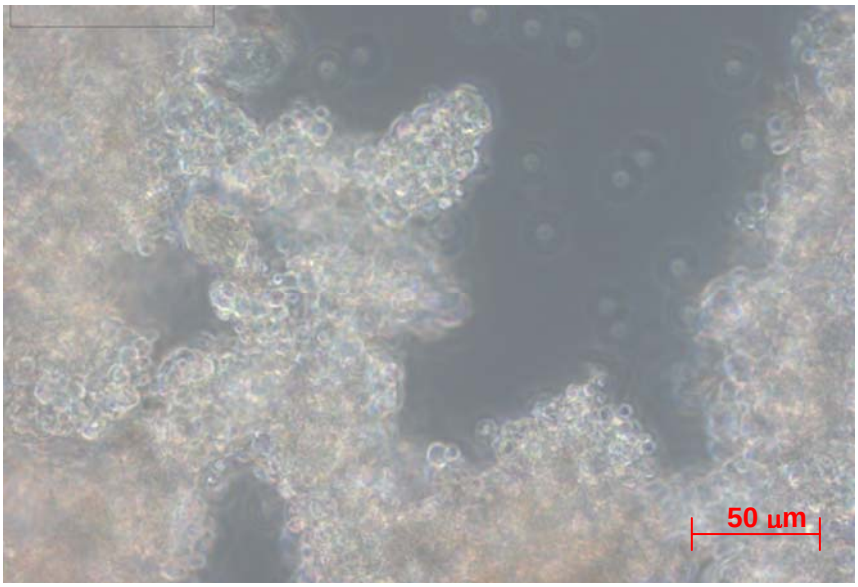
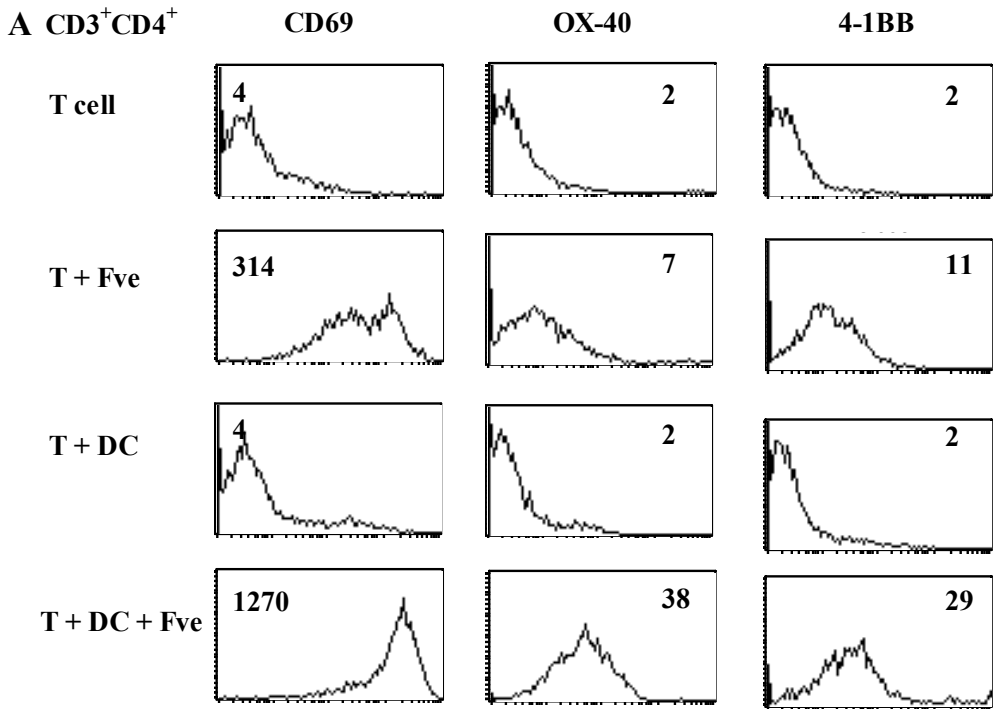


Figure 3.3 The aggregations of purified mouse CD4$^+$ T cells and CD8$^+$ T cells after Fve stimulation. CD4$^+$ T cells and CD8$^+$ T cells from spleen of C57BL/6 mice were purified by AutoMACS. Purified CD4$^+$ T cells and CD8$^+$ T cells were stimulated with 20 µg/ml of Fve for 24 hrs. The morphology of the CD4$^+$ T cells (A) and CD8$^+$ T cells (B) was examined by light microscopy at 24 hrs. Non-stimulated CD4$^+$ T cells and CD8$^+$ T cells used as negative controls.



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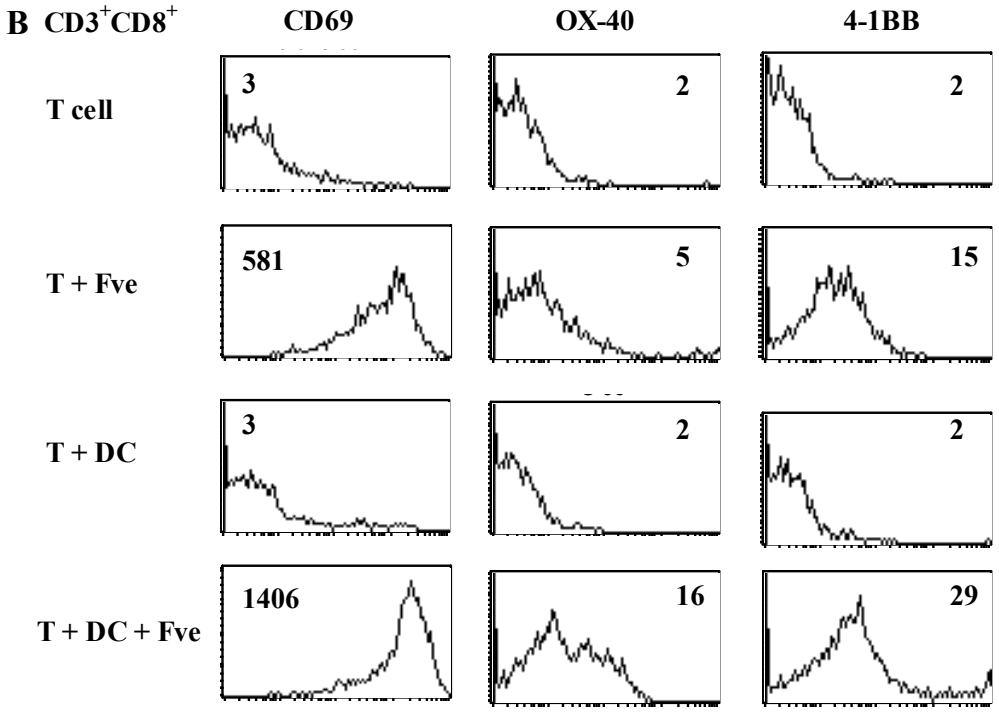


Figure 3.4 Fve stimulated CD69, OX-40, 4-1BB up-regulation on T cells. $CD90^+$ T cells purified from the splenocytes of C57BL/6 mice were incubated for 24 hrs either alone or with BM-DCs at a 10:1 ratio (T cell/DC) in the presence or absence of 20 μg/ml of Fve. The cells were stained with rat anti-mouse CD3−percp, CD4−APC, and anti-CD8β−FITC, CD69−PE, OX-40−PE or 4-1BB−PE. The cells were gated for $CD3^+CD4^+$ (A), $CD3^+CD8^+$ cells (B) for activation analysis by flow cytometry. The numbers in the histograms represent the mean fluorescence intensities (MFIs) of the surface markers on T cells.

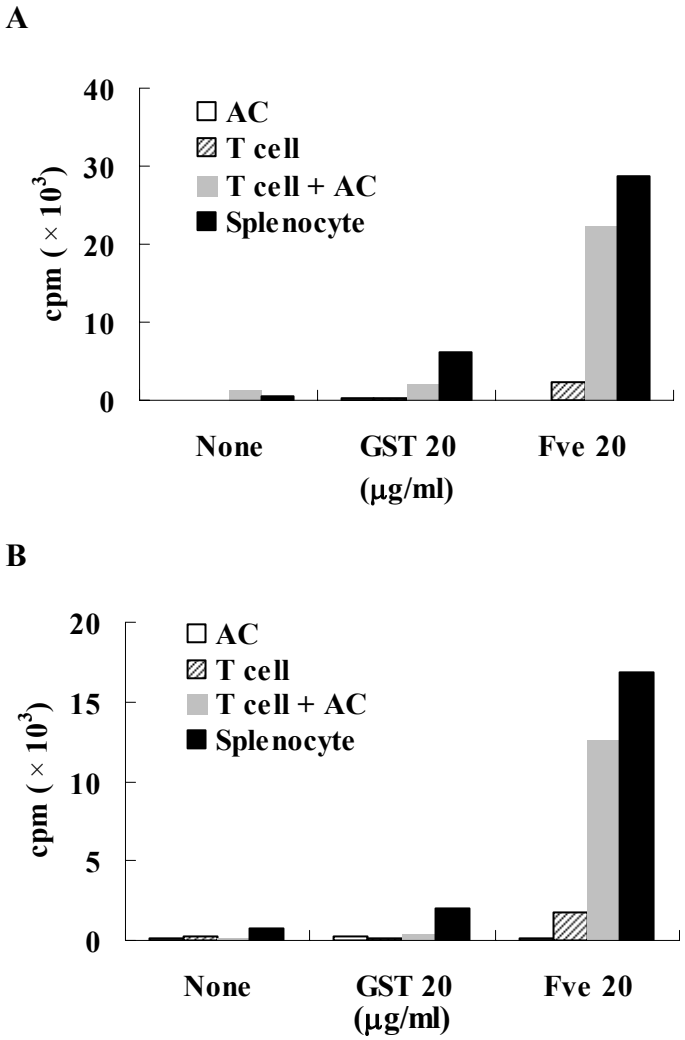


Figure 3.5 Fve stimulated mouse spleen T cells proliferation in an accessory cell-dependent manner. For accessory cell-dependent proliferation assay, $CD90^+$ T cells from splenocytes of C57BL/6 (A) or BALB/c (B) mice were purified from spleen cells by AutoMACS. Mitomycin C-treated splenocytes (3×10^5 cells/well) as accessory cells were co-cultured with purified $CD90^+$ T cells (1×10^5 cells/well) with GST (20 μg/ml), or Fve (20 μg/ml) for 72 hrs. The cultures were pulsed with [^{3}H]-thymidine for the last 18 hrs of the co-culture. The cells were harvested and thymidine incorporation was measured by liquid scintillation counting at 72 hrs. *AC (antigen presenting cell, accessory cell), refers to mitomycin C-treated splenocyte.

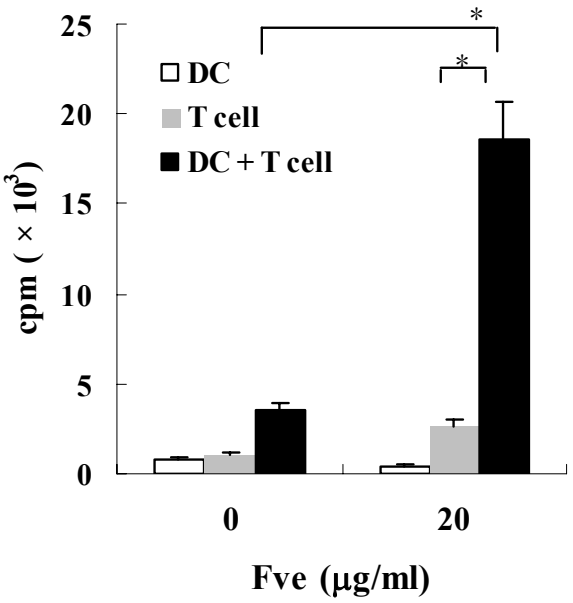


Figure 3.6 DCs can be accessory cells in the T cell proliferation stimulated by Fve. Purified $CD90^+$ T cells (1×10^5 cells/well) from C57BL/6 mice were co-cultured with mitomycin C-treated BM-DCs (2×10^4 cells/well) in the presence or absence of Fve (20 μg/ml) in triplicates using the 96-well plate. The DCs and $CD90^+$ T cells alone were included for comparison. The cultures were pulsed with [^{3}H]-thymidine for the last 18 hrs of the co-culture. The cells were harvested and thymidine incorporation was measured by liquid scintillation counting at 72 hrs. *: $p < 0.05$.

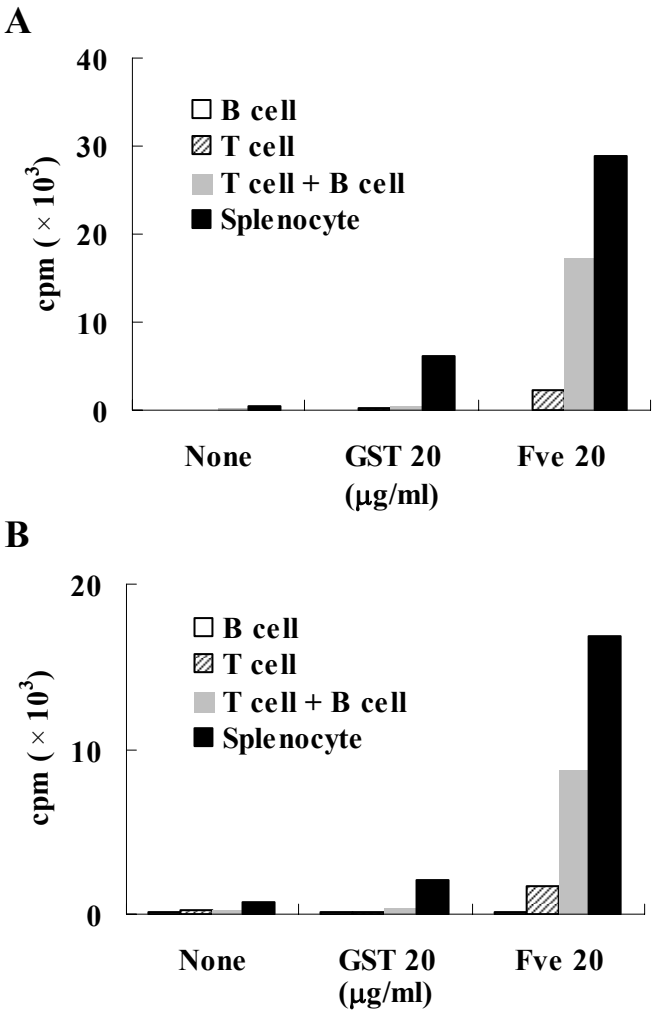


Figure 3.7 B cells as accessory cells in the T cell proliferation stimulated by Fve. For accessory cell-dependent proliferation assay, mitomycin C-treated B cells (3×10^5 cells/well) were co-cultured with $CD90^+$ T cells (1×10^5 cells/well) from C57BL/6 (A) or BALB/c mice (B) with GST (20 μg/ml), or Fve (20 μg/ml). The cultures were pulsed with [^{3}H]-thymidine for the last 18 hrs of the co-culture. The cells were harvested and thymidine incorporation was measured by liquid scintillation counting at 72 hrs. B cell refers to mitomycin C-treated B cell. The experiments were performed three times and similar results can be observed. Data are representative from one of these experiments are presented.

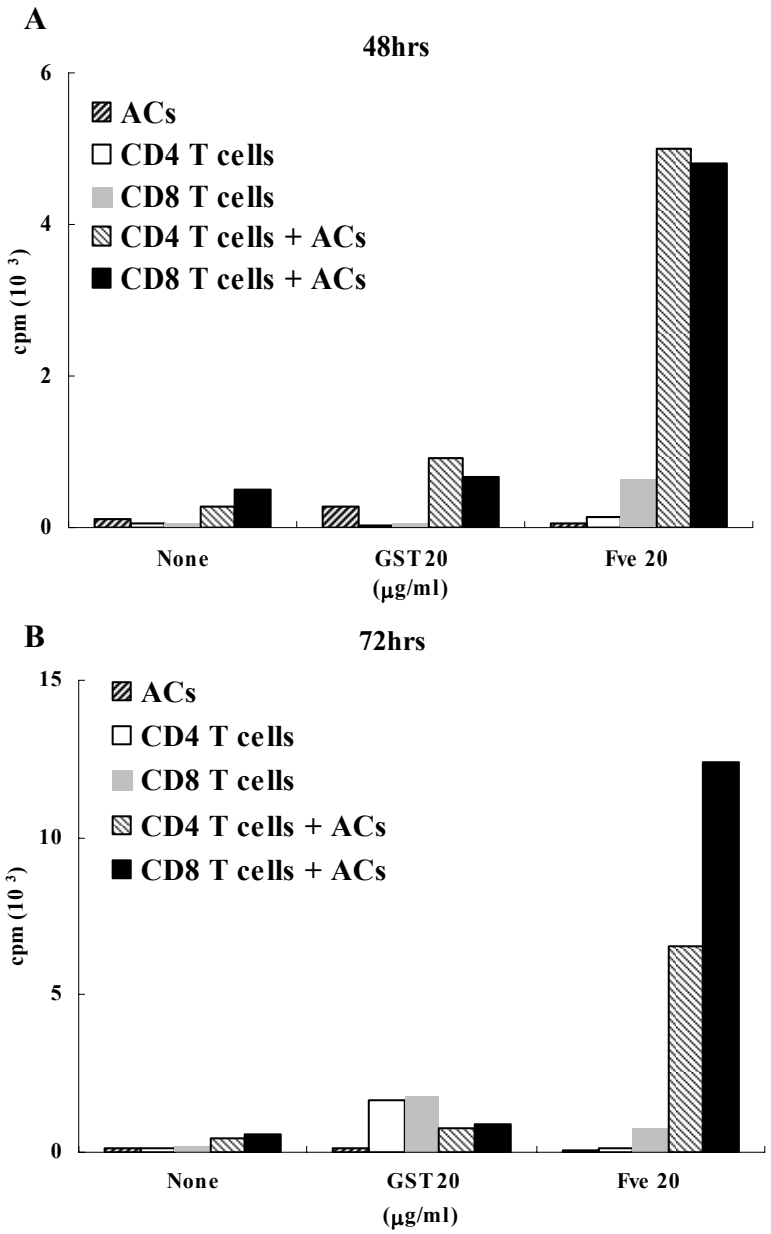
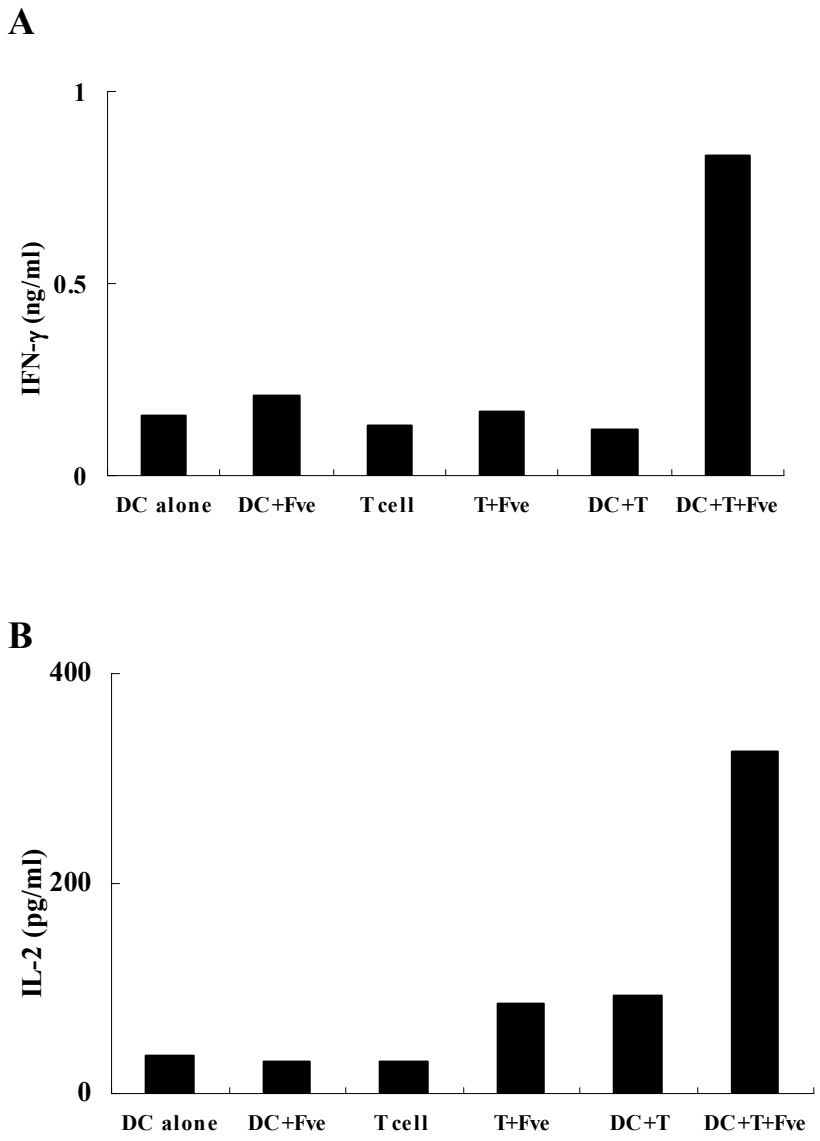


Figure 3.8 Accessory cell-dependent proliferation of Fve-stimulated T cell subsets. Mitomycin C-treated splenocytes (3×10^5 cells/well) as APCs were co-cultured with purified $CD4^+$ or $CD8^+$ T cells (1×10^5 cells/well) from C57BL/6 mice with GST (20 μg/ml), or Fve (10 μg/ml) for 48 hrs (A) or 72 hrs (B). The cells then were harvested and thymidine incorporation was measured. *ACs (accessory cella), refers to mitomycin C-treated splenocyte. The experiments were performed twice and similar results can be observed. Data are representative from one of these experiments are presented.



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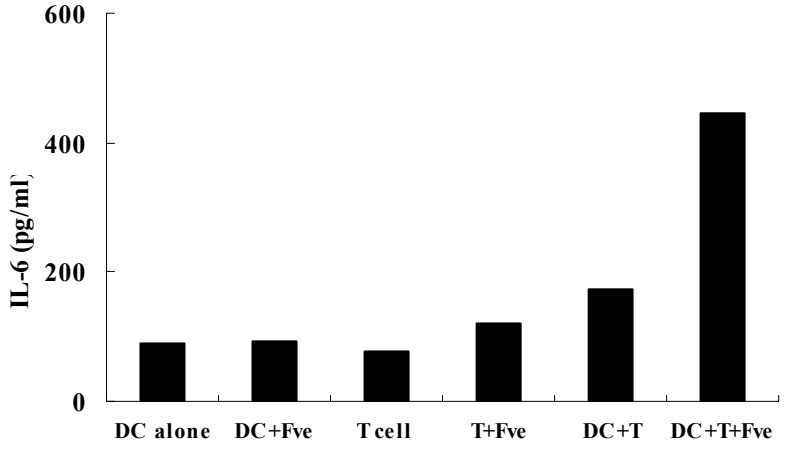

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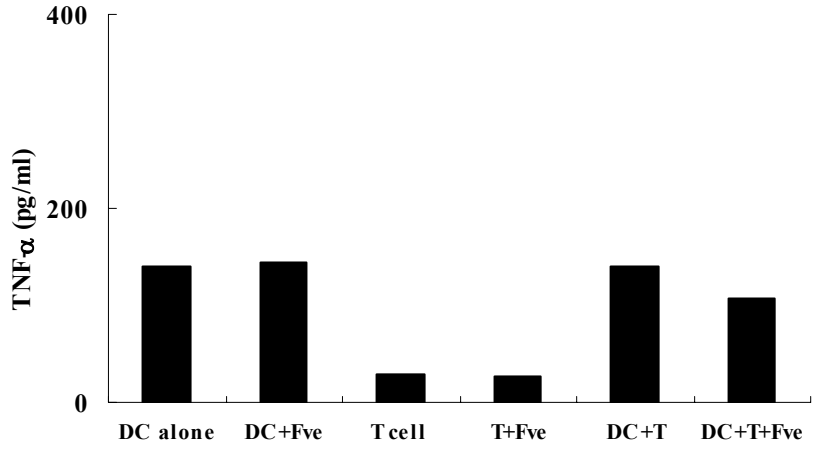

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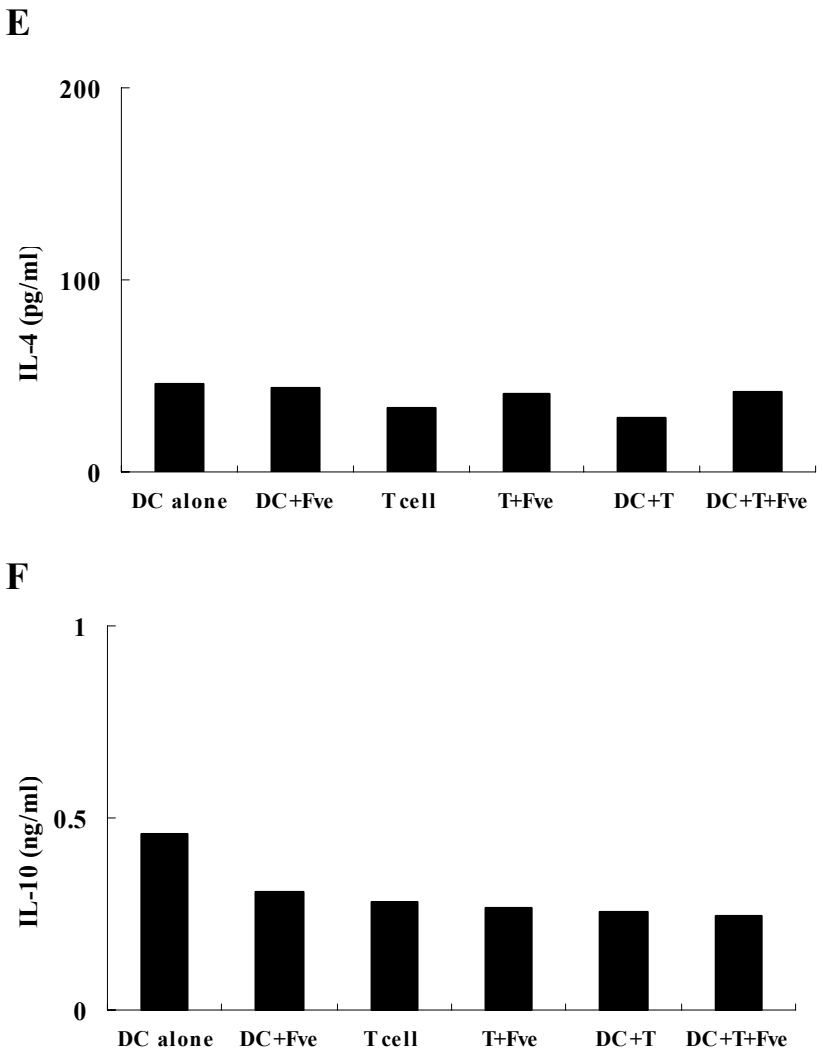


Figure 3.9 Profile of cytokine production by T cells stimulated with Fve *in vitro*. $CD90^+$ T cells (2×10^6 cells/ml) from the spleen of C57BL/6 mice were incubated either alone or with BM-DCs at a 10:1 ratio (T cell/DC) in the presence or absence of 20 μg/ml of Fve for 24 hrs. The supernatants were collected and cytokine production by T cells was measured by ELISA. The experiments were performed three times and similar results can be observed. Data are representative from one of these experiments are presented.

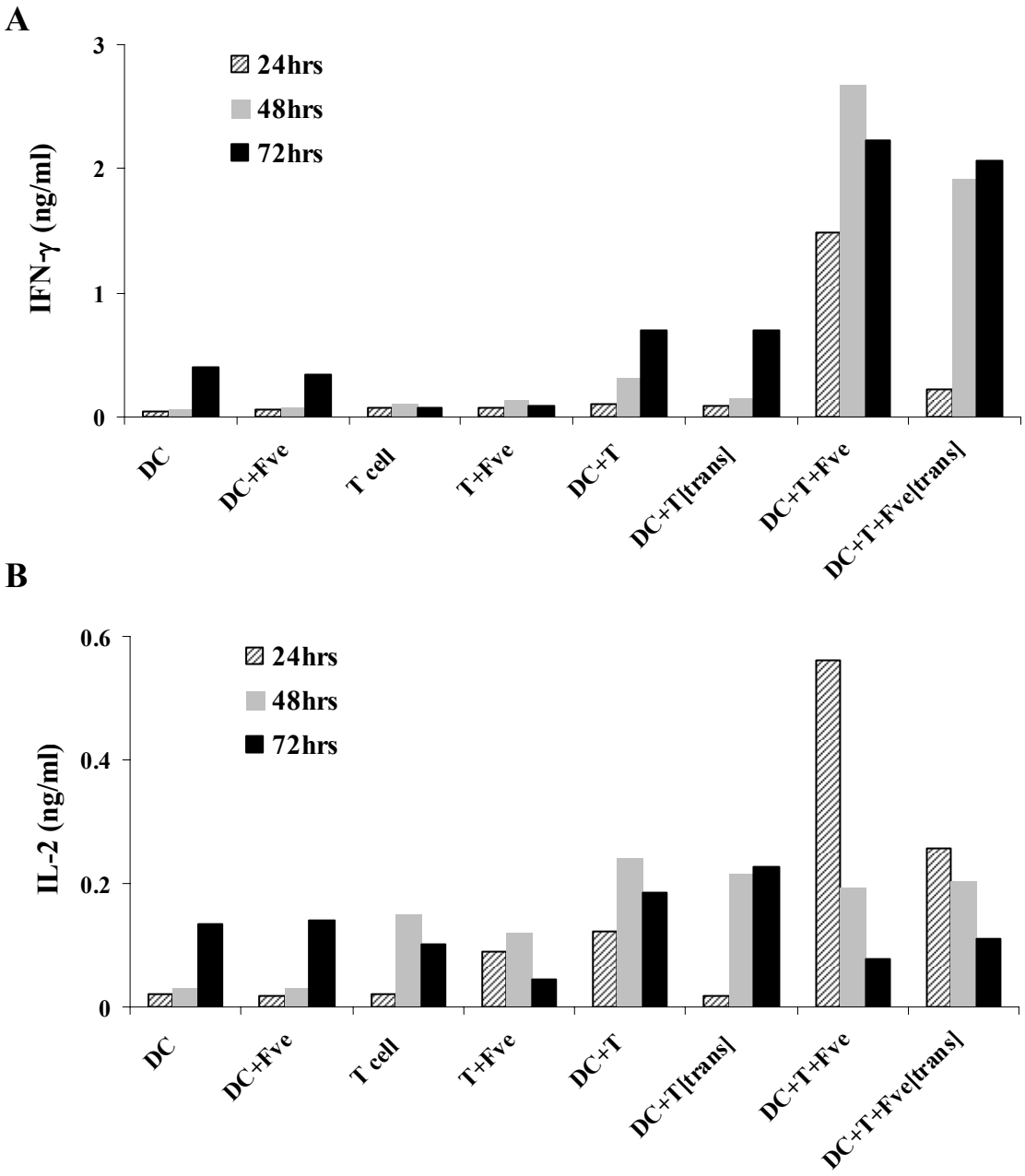


Figure 3.10 Fve stimulated murine T cells to produce IFN-γ and IL-2 in different kinetics. $CD90^+$ T cells (2×10^6 cells /ml) from the spleens of C57BL/6 mice were incubated either alone or with BM-DCs at a 10:1 ratio (T cell/DC) in the presence or absence of 20 μg/ml of Fve for 24, 48 or 72 hrs. Transwell groups also were set up correspondingly. The supernatants were collected and cytokine production (IFN-γ, IL-2) by T cells was measured by ELISA.

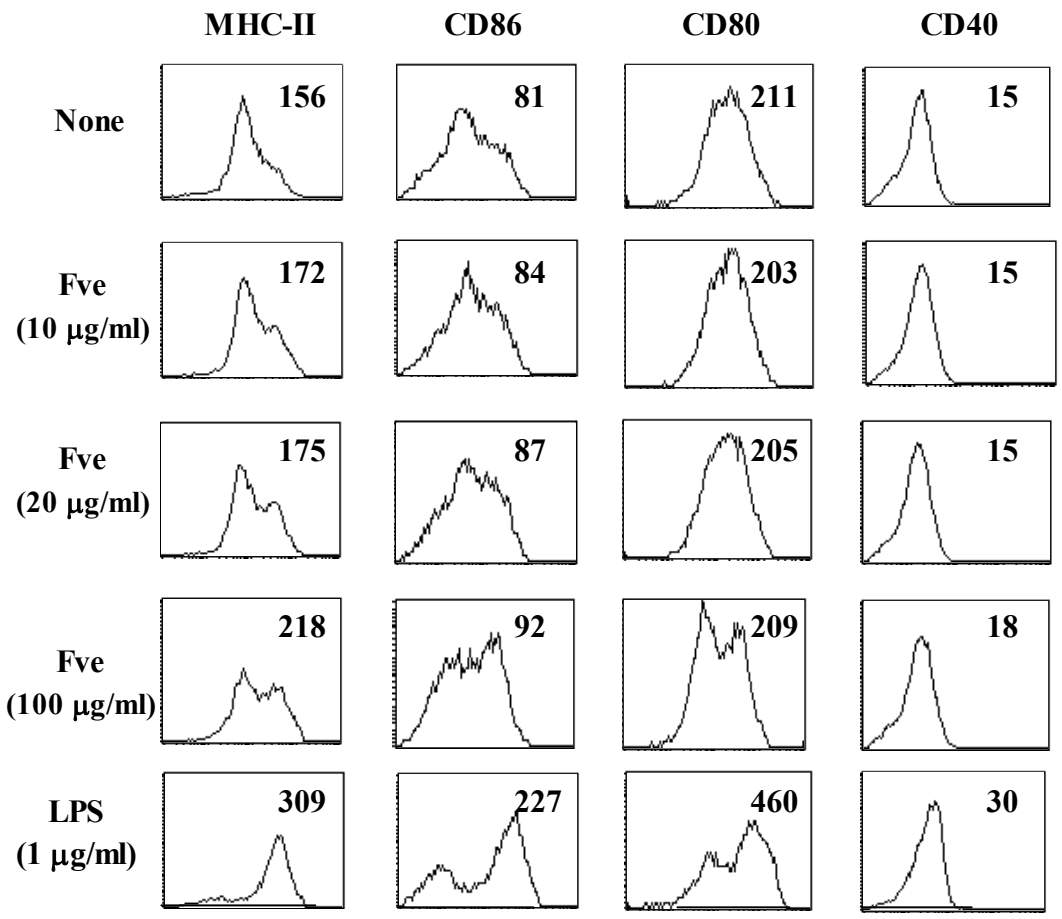


Figure 3.11 Immunofluorecent staining of CD11c$^+$ DC after Fve and LPS treatment. BM-DCs were stimulated with different concentrations of Fve protein, or LPS for 16–18 hrs. The numbers in the histograms represent the mean fluorescence intensities (MFIs) of the surface markers on BM-DCs after stimulation. LPS but not Fve triggered up-regulation of MHC class II, CD86, CD80 and CD40 surface molecules on BM-DCs.

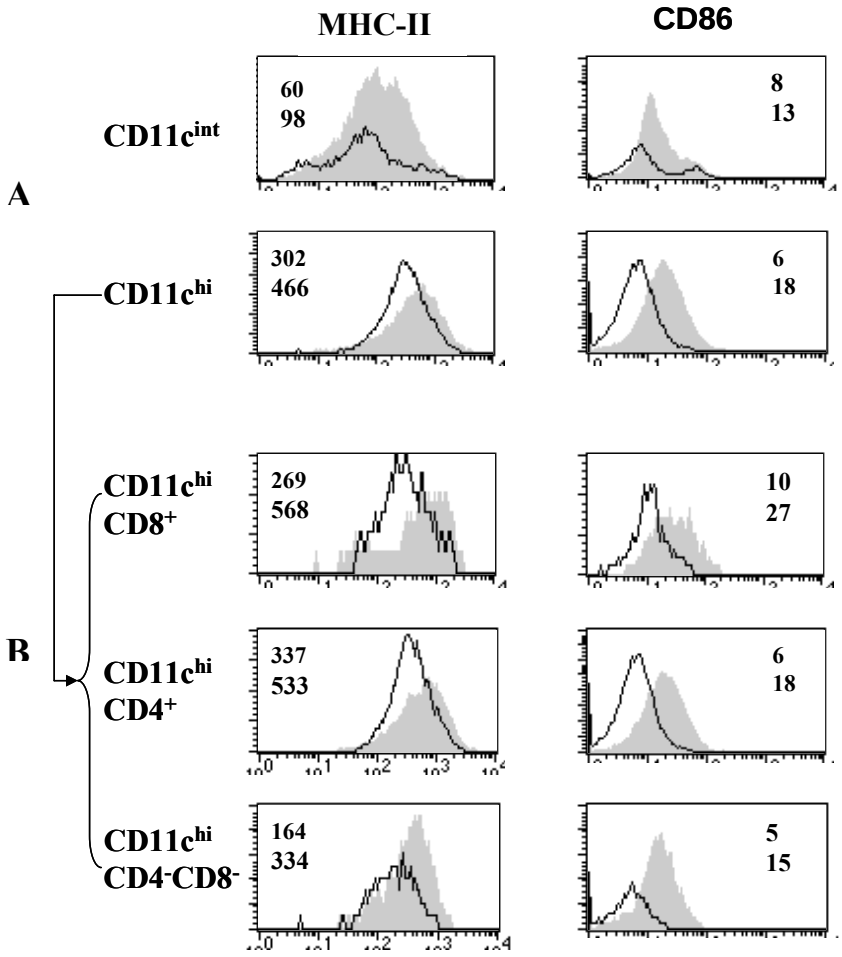
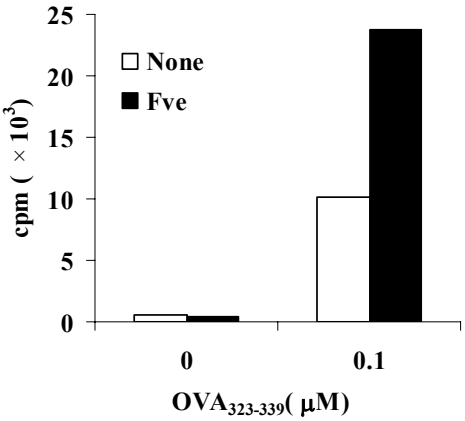


Figure 3.12 Fve induced splenic dendritic cells phenotypic maturation *in vivo*. BALB/c mice (n = 8 mice/group) were injected intraveneously with PBS, Fve, or LPS 12 hrs prior to cell harvest. DCs were enriched from spleen for staining of MHC class II and CD86. Splenic DCs were separated into $CD11c^{int}$ and $CD11c^{hi}$ populations (A) and $CD11c^{hi}$ DCs could be divided further into $CD4^+$, $CD8\alpha^+$ and $CD4^-CD8\alpha^-$ sub-populations (B). Dark line histograms represent PBS-stimulated DCs, filled grey histograms represent Fve-stimulated DCs. The numbers in the histograms indicate the MFIs of surface markers.

A


25
20
15
10
5
0
cpm (× 10³)
None
Fve
0
0.1
OVA323-339(μM)

B

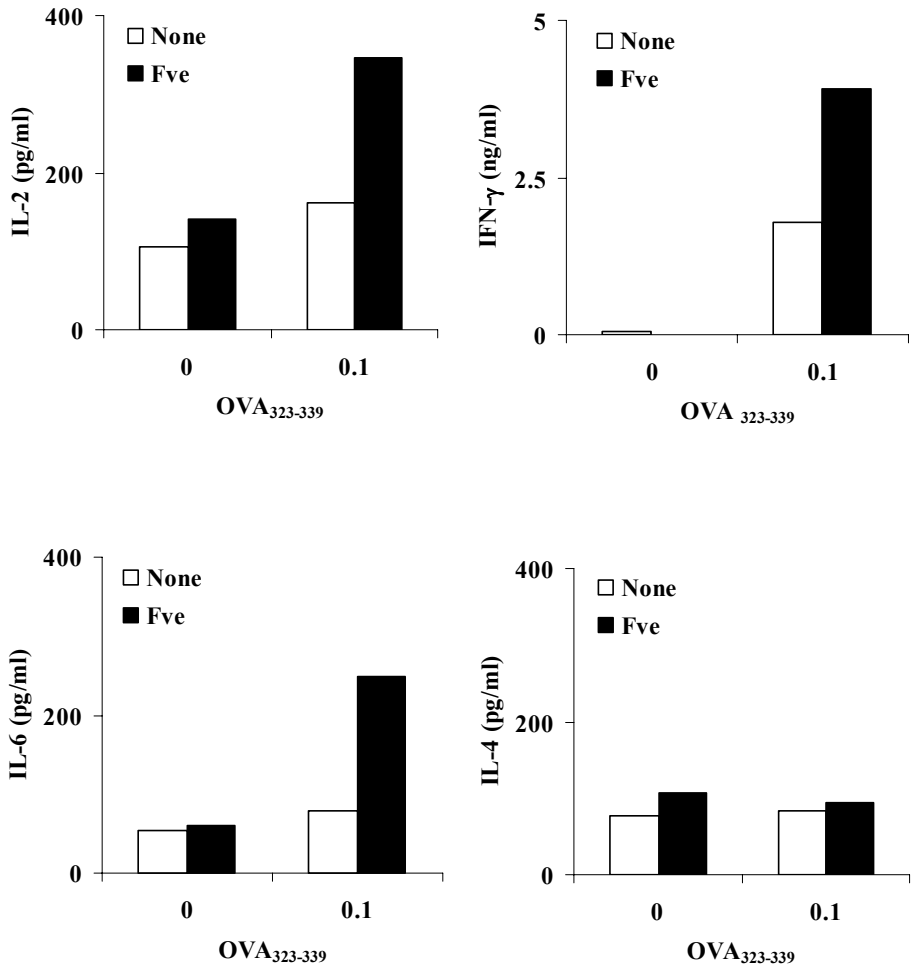

400
200
0
IL-2 (pg/ml)
None
Fve
0
0.1
OVA323-339
5
2.5
0
IFN-γ (ng/ml)
None
Fve
0
0.1
OVA 323-339
400
200
0
IL-6 (pg/ml)
None
Fve
0
0.1
OVA323-339
400
200
0
IL-4 (pg/ml)
None
Fve
0
0.1
OVA323-339

Figure 3.13 Fve-activated splenic DCs induced the antigen-specific $CD4^+$ T cells priming. BALB/c mice (n = 8 mice/group) were injected intraveneously with PBS or Fve, and the DCs from the pool of eight spleens from each group of mice were isolated 12 hrs later. Purified DCs were pulsed with or without 0.1 μM of $OVA_{323-339}$ peptide for 2 hrs at 37°C and washed three times before cocultured with $CD4^+$ T cells from DO11.10 transgenic mice for 72 hrs. The ^{3}H-thymidine was added 18 hrs before cell harvest and thymidine incorporation was measured by liquid scintillation counting after 72 hrs (A). Supernatants were collected and cytokine production was measured by ELISA (B). The experiments were performed three times and representative data from one of these experiments are presented. DC: dendritic cells alone; DC/CD4=1/10: the ratio of DCs to $CD4^+$ T cells is 1 to 10.

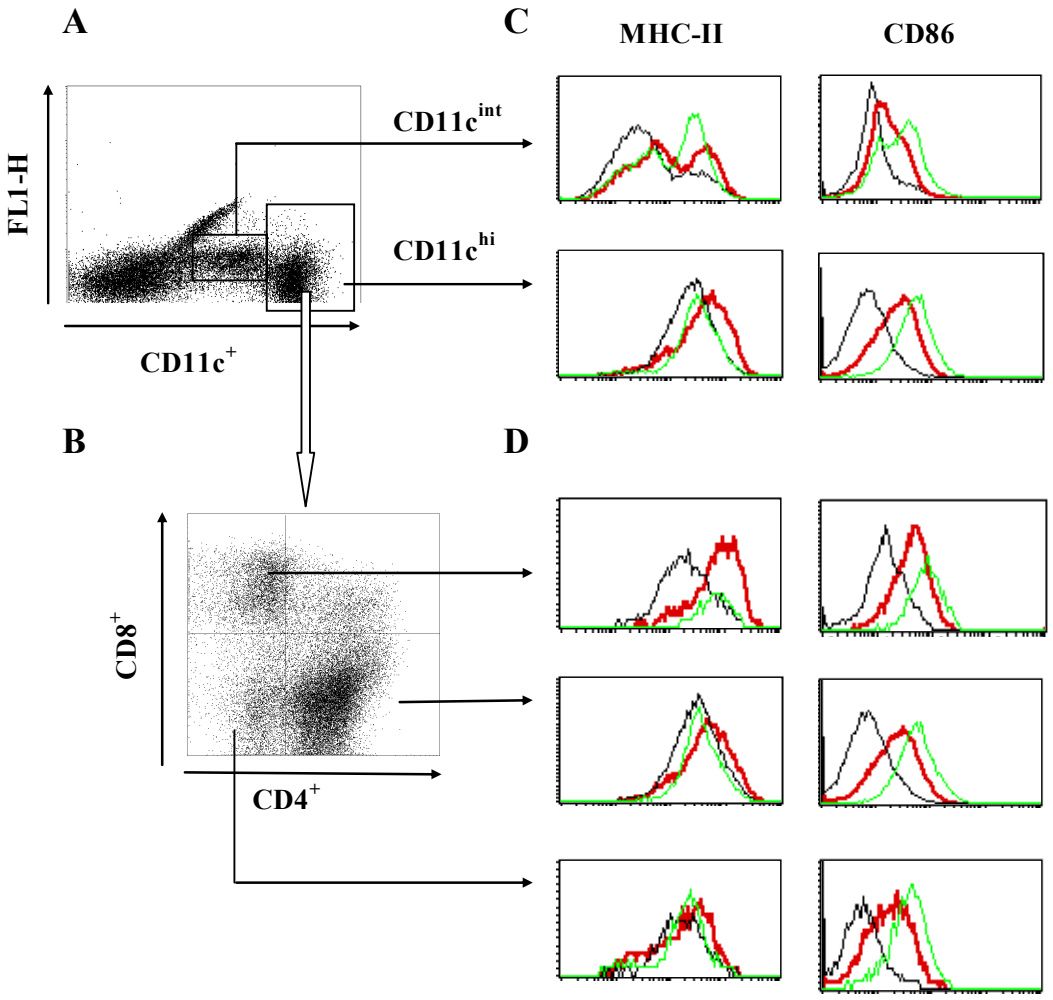
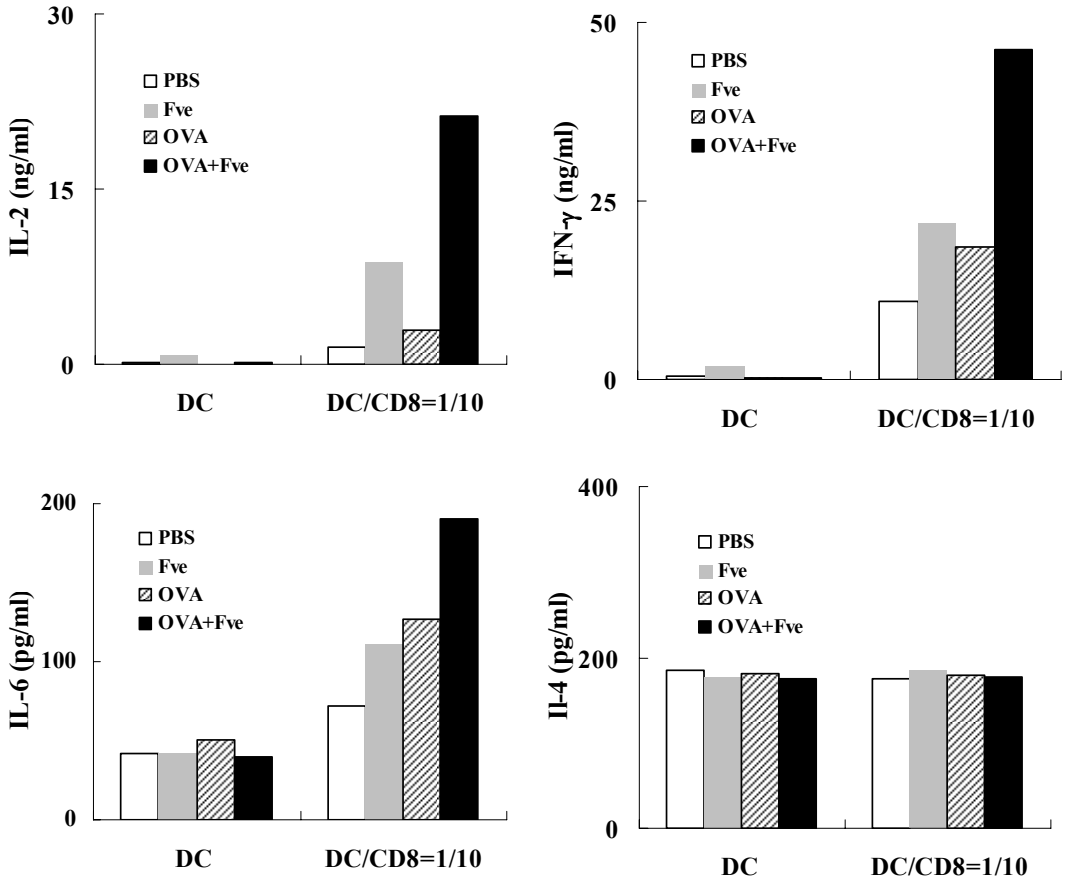


Figure 3.14 Fve induced splenic dendritic cells phenotypic maturation *in vivo*. C57BL/6 mice (n = 8 mice/group) were injected intraveneously with PBS, Fve, or LPS 12 hrs prior to cell harvest. DCs were enriched from spleen for staining of MHC class II and CD86. Splenic DCs were separated into $CD11c^{int}$ and $CD11c^{hi}$ populations (A) and $CD11c^{hi}$ DCs could be further divided into $CD4^+$, $CD8\alpha^+$ and $CD4^-CD8\alpha^-$ sub-populations (B). MHC class II and CD86 up-regulation can be seen in both $CD11c^{int}$ and $CD11c^{hi}$ populations (C) and the three $CD11c^{hi}$ sub-populations of DCs (D) after stimulation by Fve *in vivo*. Dark line histograms represent PBS-stimulated DCs, red line histograms represent Fve-stimulated DCs, and green line histograms represent LPS-stimulated DCs. Data are representative of two independent experiments

A



(To be continued)

B

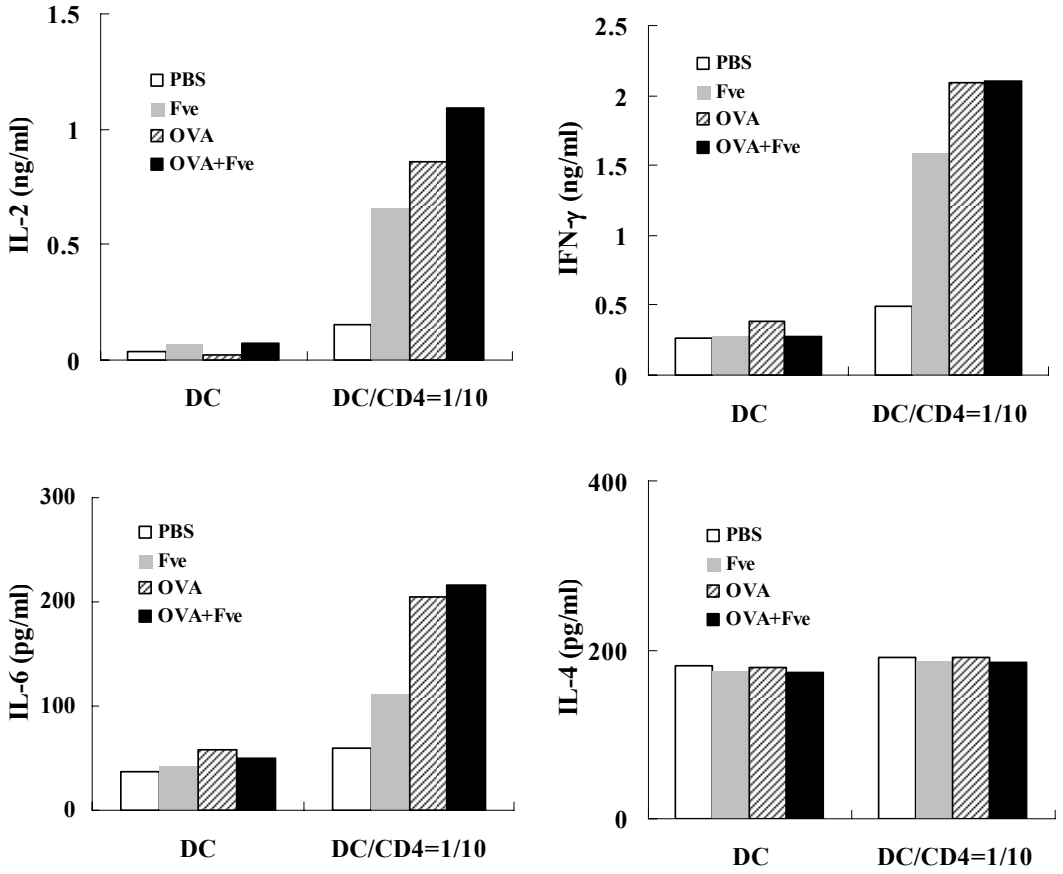


Figure 3.15 Fve greatly enhanced antigen-specific responses of $CD8^+$ T cells. C57BL/6 mice (n = 8 mice/group) were intraveneously injected with PBS (□), OVA (▨), Fve (■), or OVA plus Fve (■), and the DCs from the pool of eight spleens from each group of mice were isolated 24 hrs later. Purified DCs were pulsed with 1 μM of $OVA_{257-264}$ peptide or 1 μM of $OVA_{323-339}$ peptide for 2 hrs at 37°C and washed three times before co-cultured with $CD8^+$ T cells from OT-I mice or $CD4^+$ T cells from OT-II mice, respectively, for 72 hrs. Supernatants were collected and cytokine productions by $CD8^+$ T cells (A) and $CD4^+$ T cells (B) were measured by ELISA. The experiments were performed three times and representative data from one of these experiments are presented. DC: dendritic cells alone; DC/CD8=1/10: the ratio of DCs to $CD8^+$ T cells from OT-I mice is 1 to 10; DC/CD4=1/10: the ratio of DCs to $CD4^+$ T cells from OT-II mice is 1 to 10.

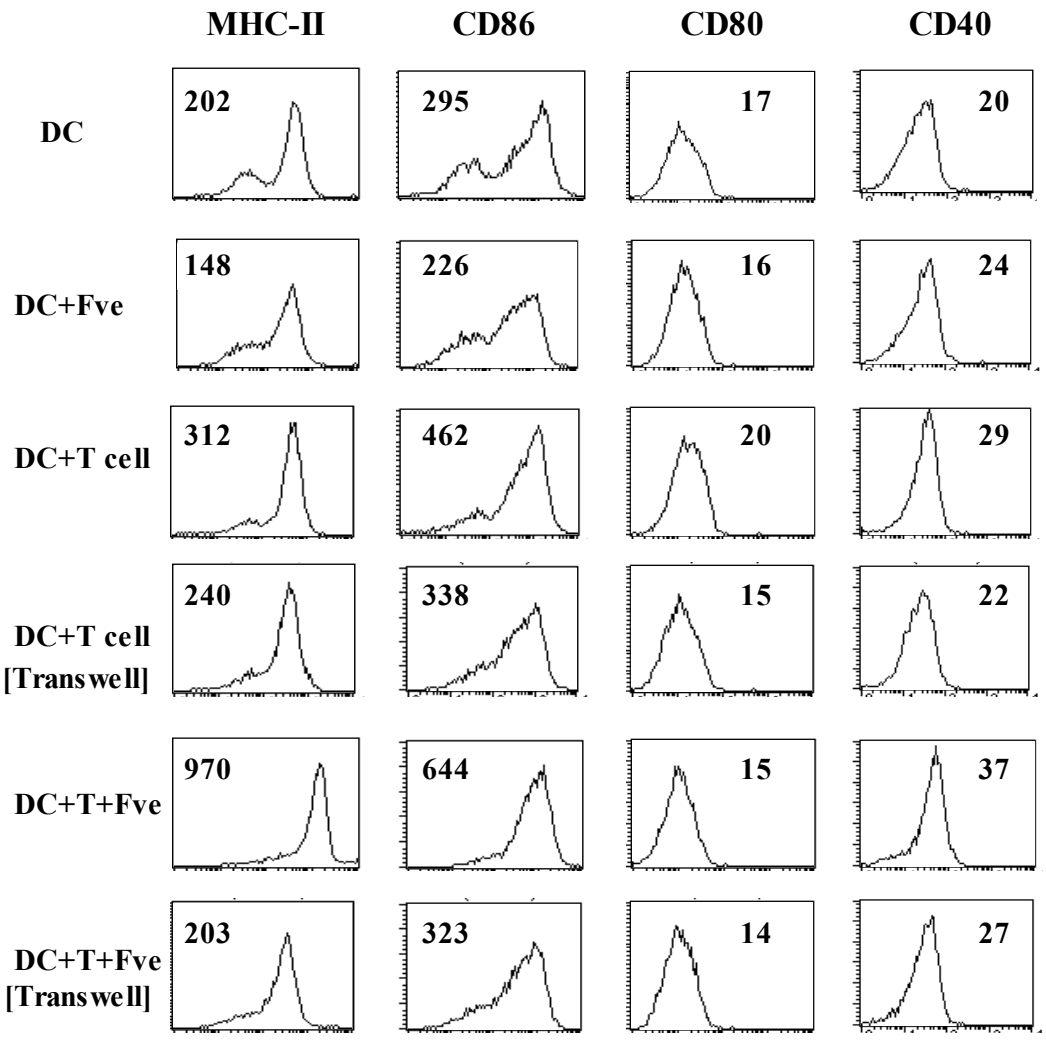


Figure 3.16 T cells helped phenotype maturation of BM-DCs stimulated by Fve in a cell-to-cell contact manner. Purified BM-DCs were incubated alone (DC alone), or with $CD90^+$ T cells at a T cell/ DC ration of 10:1; with $CD90^+$ T cells (T cell/DC = 10:1) separated in trans-well in the presence or absence of Fve protein (20 μg/ml). MHC class II, CD86, and CD40 staining of DCs were performed and represented as dark line histograms. The numbers in the histograms indicates the MFIs of surface markers. DC respresents dendritic cells, T represents T cells.

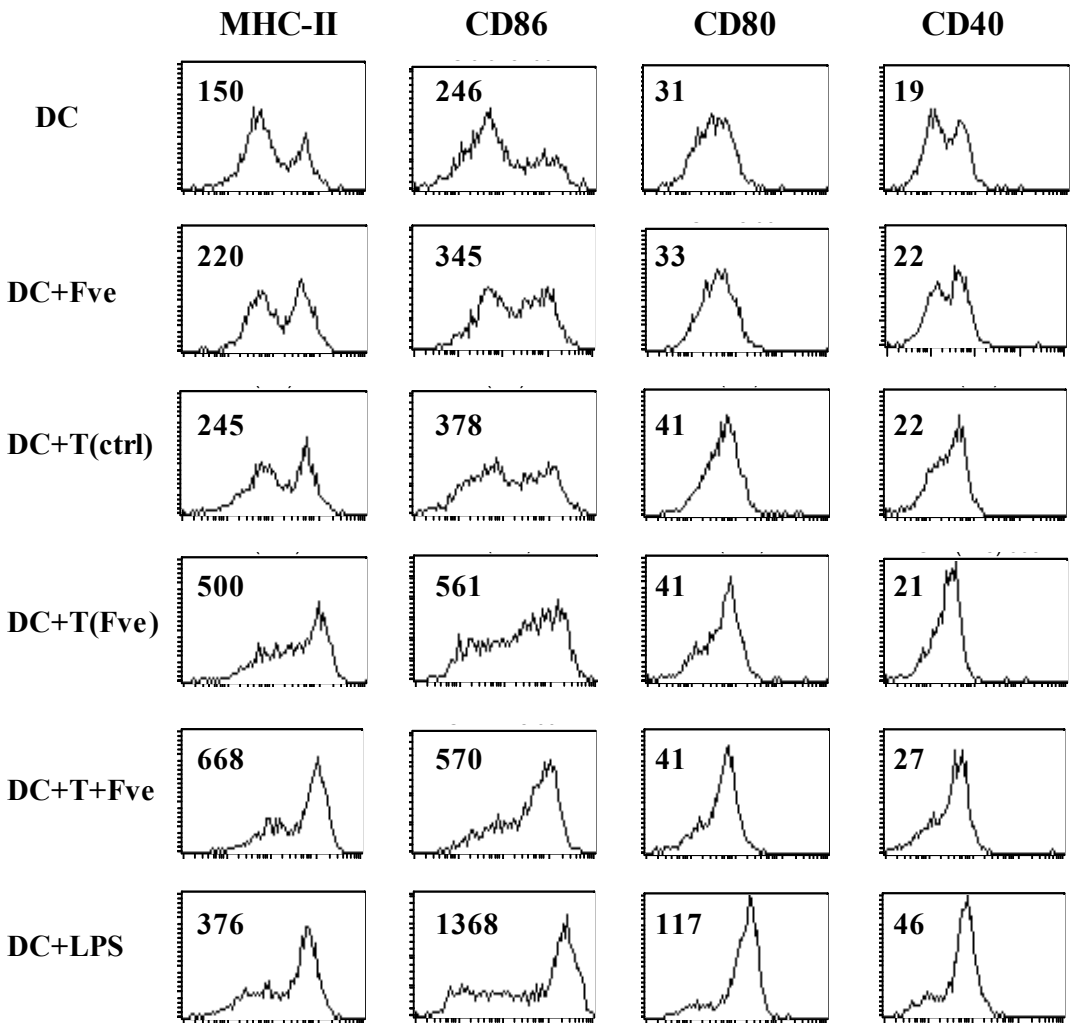


Figure 3.17 Preactivated T cells can help BM-DCs to up-regulate MHC class II and CD86. $CD90^+$ T cells were purified from splenocytes in C57BL/6 mice and stimulated with/without Fve protein (20 μg/ml) for 24 hrs. The purified BM-DCs were incubated with freshly purified T cells or activated T cells (T cell/DC ratio= 10:1) for 24 hrs. The surface markers, such as MHC class II, CD86, and CD80 on BM-DCs were stained and analyzed. The numbers in the histograms represents the MFIs of the surface markers. DC respresents Bone marrow-derived dendritic cells (BM-DCs), T represents T cells. DC+ T (ctrl) indicates non-stimulated T cells co-cultured with BM-DCs. DC+ T (Fve) indicates Fve-preactivated T cells co-cultured with BM-DCs. DC+T+Fve indicates fresh prepared T cells co-culured with BM-DCs in the presence of Fve protein.

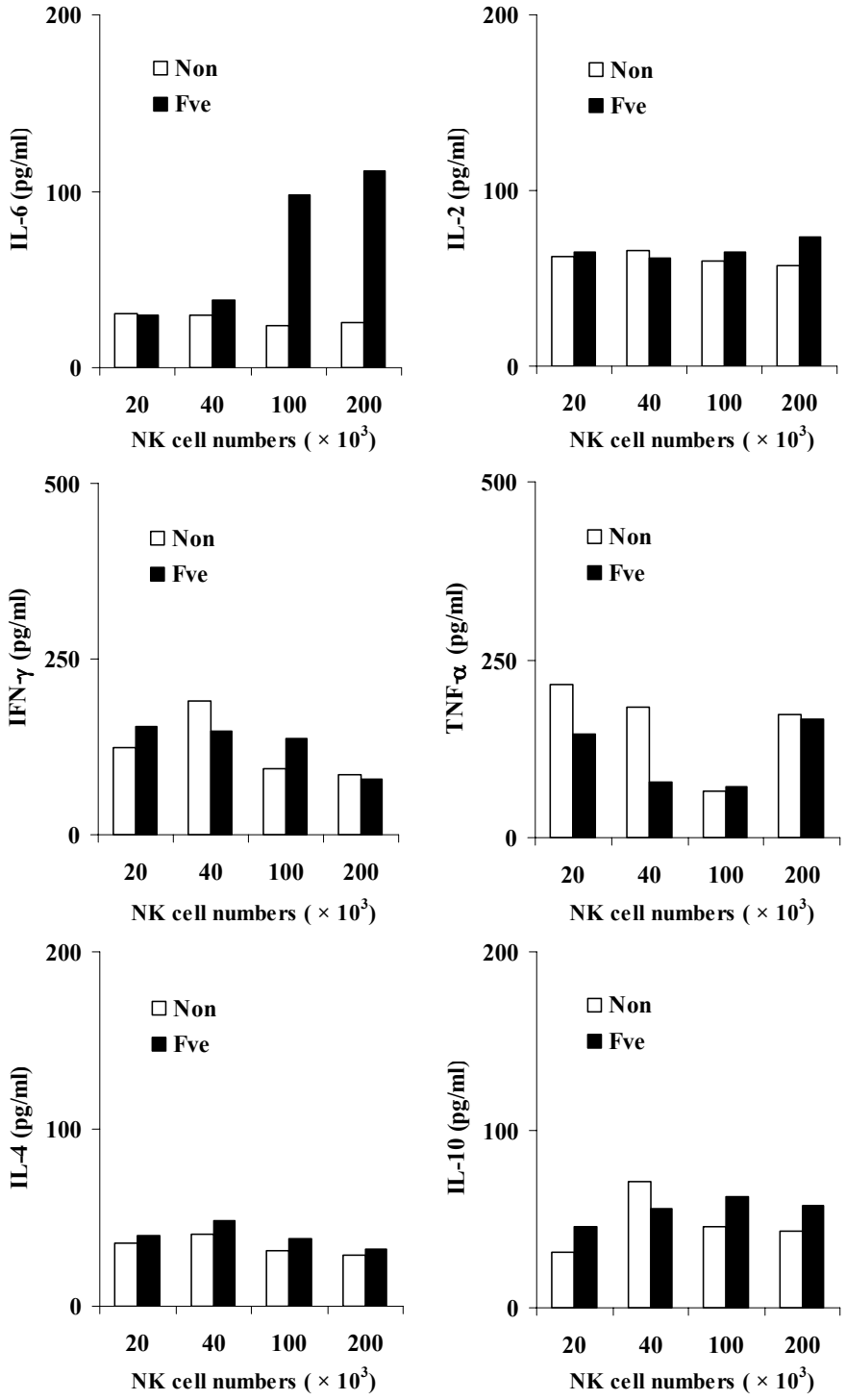


Figure 3.18 Cytokine profile of NK cell stimulated by Fve *in vitro*. Different cell numbers of purified NK cells from BALB/c mice were stimulated with/without 10 μg/ml Fve *in vitro*. After 24 hrs, supernatants were collected for cytokine assay using ELISA. The experiments were performed two times and representative data from one of these experiments are presented.

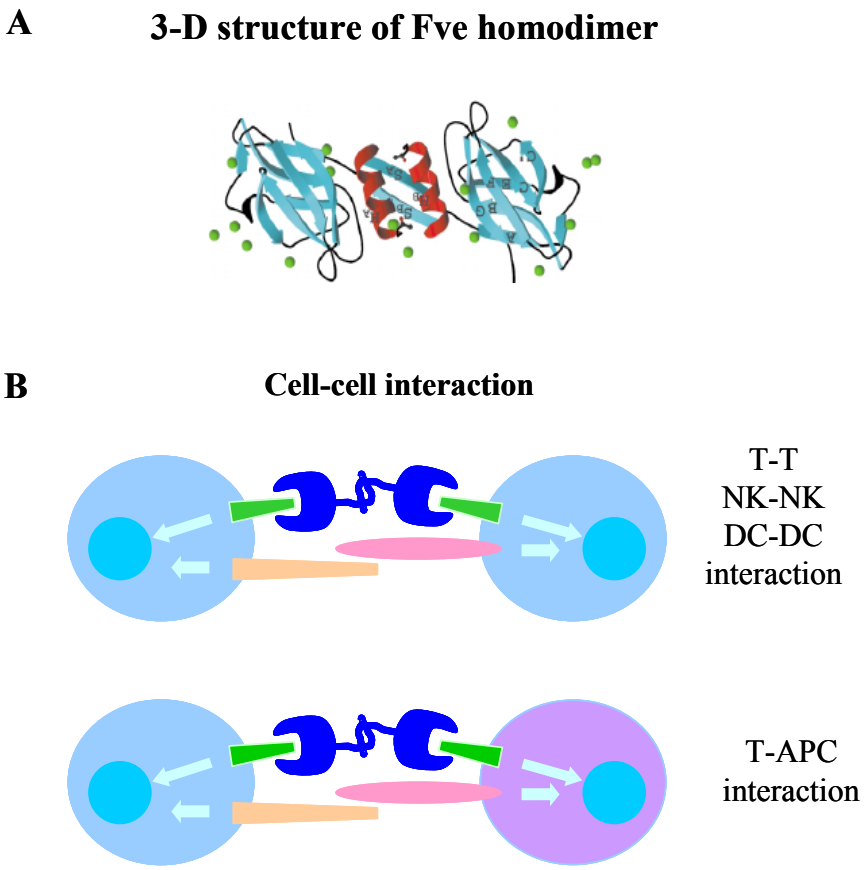


Figure 3.19 Three-dimensional structure of Fve and a proposed model for the interactions between Fve and target cells. (A) Three-dimensional structure of Fve protein. Fve is a homodimer protein and each monomer consists of N-terminal α-helix followed by a FNIII-like domain with Ig fold. (B) In this proposed model, the homodimer structure of Fve protein could act as a bridge to induce cell-cell aggregations that occur via a stepwise manner. (1) The interaction of Fve protein with the cellular receptor(s) probably via binding to the receptor's sugar components could represent the first step to initiate cell-cell aggregation, which results in the up-regulation of CD69, adhesion molecules such as ICAM-1, and other accessory molecules on target cells. (2) The Fve-induced adhesion molecules and accessory molecules expressed on activated target cells, which further enhanced the cell-cell interaction and aggregation. The APC-T cell bidirectional interaction results in: (1) full activation of the T cells for the cytokine production and cell proliferation; (2) the APC maturation.

* ( ) represents the Fve homodimer; (■) represents possible sugar component(s) of receptor(s) constitutively expressed on target cells, including T cells, DCs and NK cells; (■, ■) represent the putative adhesion and accessory molecules expressed on the activated target cells.

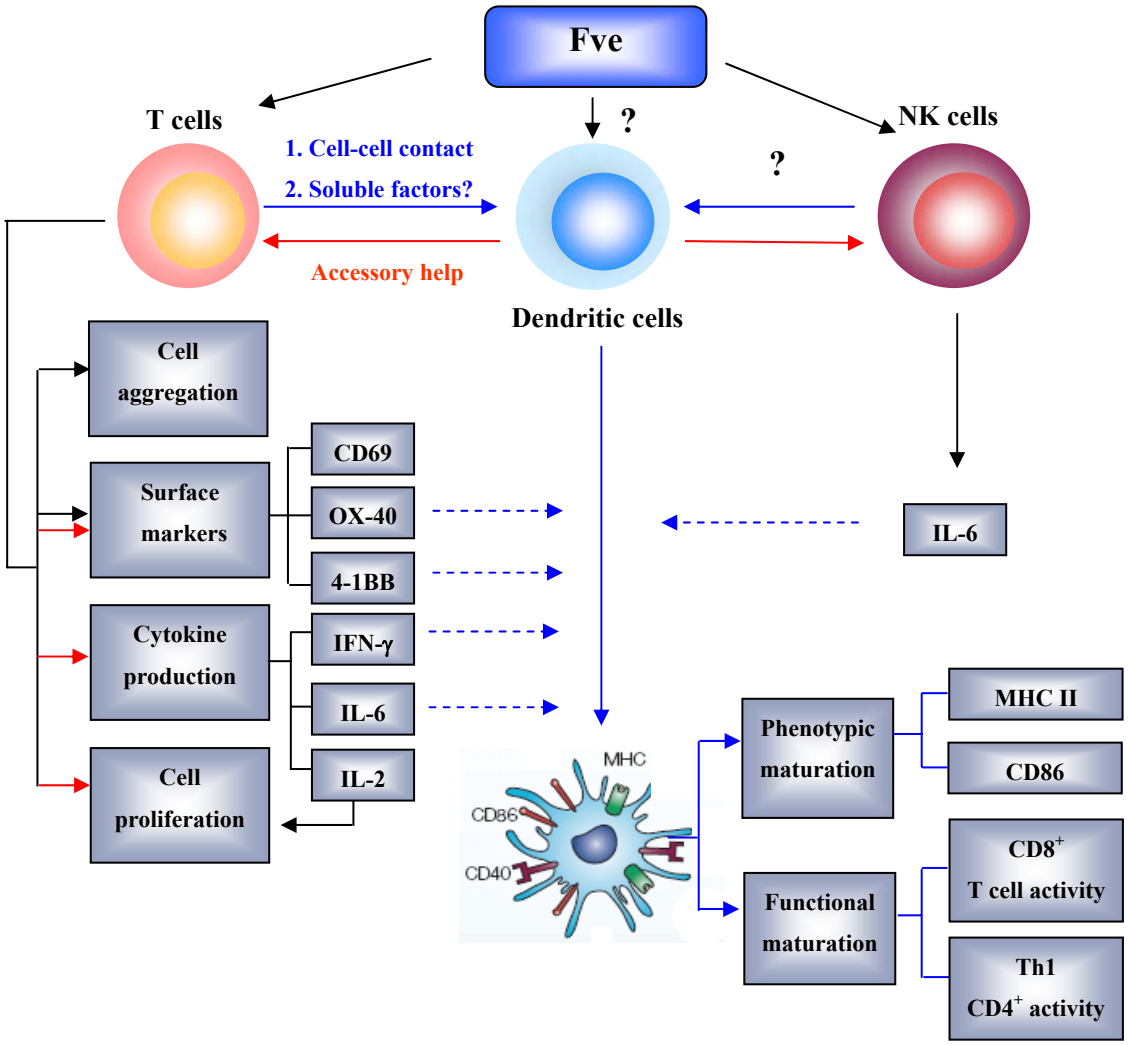


Figure 3.20 Proposed mechanisms by which Fve protein enhanced innate and adaptive immune responses via cooperative interactions between T cells, DCs and NK cells.

3.3 Discussion

This chapter describes the *in vitro* and *in vivo* studies on the immunomodulatory effects of native Fve protein on T subsets, DCs and NK cells. In addition, the interactions between Fve, T and DC and the subsequent immunological outcomes and implications will be described and discussed.

In this study, it was observed that total splenocytes from C57BL/6 mice co-cultured with graded doses of Fve protein showed dose-dependent cell proliferative responses (Fig. 3.2A). After 72 hrs of Fve stimulation, the percentages of $CD4^+$ and $CD8^+$ T subsets in the splenocytes were analyzed by flow cytometry. As shown by the cytometric data in Table 3, there was a greater percentage increase of $CD8^+$ T cells than $CD4^+$ T cells with the corresponding change of CD4/CD8 ratio from 2.0 to 0.9 for after Fve stimulation. The results suggest that Fve can stimulate more $CD8^+$ T cell proliferation than $CD4^+$ T cell proliferation (Table 3), and such an intriguing preferential increase in $CD8^+$ T cells also was observed in the *in vivo* settings, whereby mice received three subcutaneous injections of Fve protein that resulted in a similar increase of the percentage of $CD8^+$ T cells in spleen and draining lymph nodes (unpublished data from our laboratory). The underlying mechanisms for this intriguing Fve-induced preferential increased proliferation of $CD8^+$ T cells remain unclear and need to be explored further.

Subsequently, the effects of Fve protein on purified $CD90^+$ T cells, $CD8^+$ and $CD4^+$ T subsets were analyzed. Microsopic visualization of stimulated cells revealed that

the purified $CD4^+$ and $CD8^+$ T subsets aggregated 2–4 hrs after being co-cultured with 20 μg/ml of Fve protein (Fig. 3.3). Similar T cell aggregation by Fip proteins has been observed in the previous studies, which showed that hPBML aggregation can be induced by Lin Zhi-8[424] and Fve protein[415]. Moreover, the data from our lab also indicate that Fve could induce purified human $CD4^+$ and $CD8^+$ T cell adhesion. Incidentally, such cell aggregations also were observed in Fve-stimulated purified $CD11c^+$ DCs and NK cells (data not shown). Cytometric surface marker analysis of the Fve-stimulated purified T cells showed an up-regulation of CD69, an early activation marker (Fig. 3.4), and adhesion molecule ICAM-1 (data from our laboratory), but there was no cytokine production and cell proliferation by these T cells, suggesting that Fve could not fully activate purified T cells in the absence of accessory cells, although Fve induced the cellular aggregation. Further studies showed that with the help of DCs or B cells as accessory cells, T cells could be fully activated as reflected by further up-regulation of CD69, OX40, 4-1BB expression and cellular proliferation and cytokine production. The importance of accessory cells for T cell activation by mitogens has been reported[443,444] and previous mechanistic studies found that soluble factors (IL-1, IL-6) and physical interaction with T cells of accessory cells were both required for the activation of human peripheral blood T cells or murine T cells[445-448].

The modes of interaction of Fve with its target cells probably could be explained by the unique structural and functional properties of Fve protein. A published 3D-structural study of Fve protein from our laboratory[430] has demonstrated that native

Fve exists as a homodimer protein in natural conditions (Fig. 3.19A, appendix 4). Each monomer consists of an N-terminal α-helix and β-strand followed by a fibronectin type III-like domain with immunoglobulin like-fold. The N-terminal α-helices and the β-strands in both subunits, which run anti-parallel to each other, form secondary structure elements for the protein dimerization, while Ig like domains are responsible for the carbohydrates-binding.

Some preliminary sugar binding studies suggested that Fve could be a unique lectin sharing some functional similarities with other well-known lectins such as PHA, ConA, and PMA, as summarized in appendix 6. Moreover, there are two predicted patches of residues on Fve protein that could be associated with sugar binding properties ([430], appendix 5). On the basis of the characteristic structural features of Fve, A model will be proposed to explain the possible modes of interaction of Fve protein and its target cells, and the subsequent induction of cellular aggregation using the cartoon shown in Figure 3.19B. It is conceivable that Fve could bind to the sugar components of the constitutive expressed surface cellular receptor(s) of its target cells, such as T cells, leading to cellular aggregation that resulted in partial T cell activation. In the settings whereby T cells co-culture with Fve in the presence of DCs or B cells as accessory cells, a multitude cellular interactions could occur through the binding of Fve to all its target cells. For example, Fve-induced DC-DC aggregation may lead to up-regulation of additional adhesion molecules or counterpart ligands for molecules such as OX-40, 4-1BB expressed on Fve-activated T cells, resulting in further enhanced cell-cell interaction and

subsequent mitogenic activation of T cells.

Generally, the activation of T lymphocytes by antigens or mitogens initiates a coordinated up- and down-regulation of the expression of a wide number of known genes during the early phase. The CD69 molecule, designated as activation inducer molecule (AIM)[449], early activation antigen (EA-1)[450], MLR-3, Leu-23[451], is a phosphorylated disulfide-linked 27/33-kD homodimeric protein[452]. CD69 expression is induced *in vitro* on most immune cells, especially on T lymphocytes. In this study, the up-regulation of CD69 on both $CD4^+$ and $CD8^+$ T cells was observed after Fve stimulation (Fig. 3.4A&B). Similar findings have been reported for T cell activation induced by a variety of stimuli, such as Lin Zhi-8[424], anti-CD3/TCR[453] and anti-CD2 mAbs, activators of protein kinase C[454], or PHA[449]. Moreover, CD69 signal has been shown to induce synthesis of different cytokines, such as IL-2, TNF-α, and IFN-γ, and T cell proliferation. In the present study, Fve-induced T cell proliferation (Fig. 3.5), and IL-2 and IFN-γ production (Fig. 3.9) , is an accessory cell-dependent event although CD69 was also up-regulated on T cells without the help of accessory cells, indicating that CD69 expression is insufficient to trigger full activation of T cells and that accessory cells have definitely an important role in helping T cell activation.

Moreover, Fve-activated $CD4^+$ and $CD8^+$ T cells also showed up-regulation of OX-40 (CD134) and 4-1BB (CD137) (Fig. 3.4 A&B). OX-40 and 4-1BB are TNF superfamily molecules that are commonly expressed following T cell activation;

their direct effects on $CD4^+$ and $CD8^+$ T cells have been studied extensively[455,456]. Their counterparts, designated as OX-40L and 4-1BBL, respectively, can be expressed by dendritic cells, B cells, T cells and non-immune cells, such as smooth muscle cells and endothelial cells[457,458]. The signaling of OX-40 and 4-1BB controls the T cell immune response in two ways. First, they positively regulate the T cell division, proliferation, and survive by enhancing the expressions of survivin and aurora B kinase[459], anti-apoptotic molecules (BCL-2 and BCL-XL)[380,460], and inhibiting the expression of the pro-apoptotic molecule BIM[461]. The interactions between OX-40L and OX-40, 4-1BBL and 4-1BB can also expand the antigen-specific memory cell pool. Second, studies using mouse T_{Reg} cells have shown that ligation of the OX-40 affect these T-cell subsets[462]. Signaling triggered by OX-40 has been found to inhibit the development of $FOXP3^+$ T_{Reg} cells from naive $CD4^+$ T cells[463-465] and suppress the differentiation of IL-10-producing $CD4^+$ T_{Reg} cells from naive $CD4^+$ T cells[466]. Furthermore, ligation of OX-40 down-regulated FOXP3 and IL-10 expression in newly differentiated inducible T_{Reg} cells and consequently resulted in their conversion to effector T cells[466]. Although how signaling through 4-1BB affects the induction of peripheral T_{Reg} cells is not yet known, binding of 4-1BB to its ligand has been shown to block the suppressive activity of T_{Reg} cells in culture systems that contained both inducible and natural T_{Reg} cells[467]. Thus, ligation of OX40 and 4-1BB has a dual effect on promoting T-cell responses: it enhances the proliferation of effector T cells and concomitantly blocks the generation of inducible T_{Reg} cells or blocks their

suppressive functions[468].

Besides, results from this study showed that Fve-induced production of IL-2 and IFN-γ by T cells followed different kinetics. IL-2 was produced very rapidly and reached the peak by 24 hrs, whereas the level of IFN-γ increased continuously and was maintained at a high level after 48 hrs (Fig. 3.10). The different kinetics of IL-2 and IFN-γ production may be due to sequential activation of the different signaling pathways in T cells and the characteristics of IL-2 and IFN-γ themselves. Early *in vivo* study indicates that production of IL-2 by activated T cells can be detected as early as 1–2 hrs, maximal secretion achieved within 6–8 hrs, and secretion undetectable by 20–24 hrs[469]. IL-2 production by T cells in this study is somewhat slower than the above *in vivo* study but consistent with the report *in vitro* study[470]. The molecular mechanism underlying the transient nature of IL-2 is less well understood. A classical auto-regulatory feedback loop has recently been described in which IL-2 inhibits its own production by activating several STAT transcription factors and transcriptional repressor B lymphocyte maturation protein-1 (Blimp-1)[470,471]. Moreover, it has been widely accepted that IL-2 is a potent mediator that stimulate the proliferation and promotes survival of activated T cells[472,473]. Lin Zhi-8, which is a homology of Fve, has been shown to induce T cell proliferation. Neutralizing Ab to IL-2 receptor blocks its proliferative response[424]. Based on these observations, it is reasonable to speculate that IL-2 is important in the Fve-induced T cell proliferation. Besides, IL-2 is able to induce the activation of NK cells[474] and facilitates the induction of CTLs, and induces the

proliferation of, and immunoglobulin synthesis by, B cells[148]. IFN-γ has been regarded as a key cytokine in protecting host against microorganism infection and tumor formation[48,62,64-67,73]. The major properties of IFN-γ include stimulation of phagocyte bacteriacidal activity, stimulation of antigen presentation through class I and class II MHC molecules[475], orchestration of leukocyte-endothelium interactions[476], up-regulation of co-stimulatory molecules CD86, CD80 on APCs[477,478], the activation of transcription factors, such as IRF-1/2[479], and the stimulation and repression of a variety of genes[476]. Based on this, induction of IL-2 and IFN-γ production by Fve provided the theoretical bases that led us ot develop Fve-based cancer immunotherapies.

DCs are well recognized for their role in the priming and differentiation of naïve T cells[480]. DCs collect and process antigens for presentation to T cells, but there are many variations on this basic theme. DCs differ in the regulatory signals they transmit, directing T cells to different types of immune response or tolerance. The data from the present study showed that Fve can aggregate BM-DCs after an overnight stimulation (data not shown). However, the up-regulation of the conventional maturation markers such as MHC class II, CD86, CD80, and CD40 on BM-DCs and the enhanced production of cytokine IL-12 and TNF-α by BM-DCs were not observed after *in vitro* co-cultured with Fve (Fig. 3.11). In contrast to the *in vitro* data, Fve induced maturation of DCs *in vivo* as documented by the up-regulation of MHC class II and CD86 (Figs. 3.12&3.14). Notably, it appears that Fve preferentially, although not exclusively, drives maturation of the

$CD8^+$ dendritic cell subset (Figs. 3.12 & 3.14). Compatible with phenotypic maturation, DCs from Fve-injected mice enhanced the OVA-specific $CD4^+$ activity and skewed Th1 immune response (Fig. 3.13). Moreover, *in vitro* functional assay using OVA-specific $CD8^+$ T cells from OT-I mice clearly showed that DCs from mice co-immunized with Fve and OVA greatly enhanced the activation of antigen-specific $CD8^+$ T cells (Fig. 3.15A). It is well known that there are at least three distinct subsets ($CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$) of DCs found in the mouse spleen. Previous studies have shown that these subsets exhibit intrinsic differential capacity to present soluble antigen and activate antigen-specific $CD4^+$ and $CD8^+$ T subsets. The $CD8^-$ subsets show the greatest ability to stimulate antigen-specific MHC class II-restricted $CD4^+$ T cells, whereas the $CD8^+$ DC are much more efficient at cross-presenting antigen and stimulating MHC class I-restricted $CD8^+$ T cells[481,482]. Thus, it is tempting to speculate that Fve could modify the ability of DCs (for example, by up-regulating the $CD8^+$ DC subset to enhance $CD8^+$ T cells via cross presentation pathway) to generate enhanced Ag-specific T cell immune response *in vivo*.

The differential effects of Fve on DC maturation seen in the *in vivo* and *in vitro* experimental settings suggest the possible roles of other cell types to help DC maturation. Moreover, it is well known that immune cells such as NK cells, γδ-T cells and activated T cells can induce DCs maturation[442,483-487]. The mitogenic effects of Fve on T cells activation prompted me to conduct to explore the helper role of activated T cells in DC maturation. Indeed, the data from this study revealed

that Fve-stimulated T cells could induce phenotypic maturation of DC *in vitro* a cell-to-cell contact dependent manner (Figs. 3.16). Similar observations have been reported by a number of laboratories[442,485-487]. McLellan et al. demonstrated that Ag-stimulated T cells positively regulated the expression of costimulatory molecules on DCs[487]. In another study, Muraille et al. found that activated T cells induced by staphylococcal superantigen or anti-CD3 mAbs were required for optimal DC maturation *in vivo*[442]. Collectively, these observations support the notion that T cells can be stimulated rapidly *in vivo* and initiate a cascade of events leading to DC maturation. However, the precise mechanisms by which Fve-induced DC maturation in the *in vivo* setting remain unclear in the present study. Therefore, further studies using $RAG^{-/-}$, $CD4^{-/-}$ or $CD8^{-/-}$ mice can be carried out to confirm the helper role of T cells in DC maturation *in vivo*. Besides, trans-well study showed that T cell-DC direct interaction is a prerequisite for DC maturation. It is well known that CD40 stimulation is important for DC maturation and IL-12 production. In this study, the CD40L expression was not up-regulated in the Fve-stimulatd T cells, indicating CD40-CD40L interaction might not be essential for the T cell-mediated DC maturation. Thus, identification of the surface molecules involved in T-DC interaction is necessary in order to further dissect the mechanisms of T cell-mediated DC stimulation.

Of note, it has been shown that interaction between NK cells and DCs can also induce DC maturation, mediated by NK cell-derived cytokines. Ligation of NKp30 on NK cells by DCs promotes the secretion of TNF-α and IFN-γ, which leads to

DC maturation[488]. Another study showed that induced recruitment of NK cells into lymph nodes provided an early source of IFN-γ for Th1 polarization, which could be a mechanism by which some adjuvants facilitated Th1 responses[489]. Both studies suggested that IFN-γ secreted by NK cells play a pivotal role in the initiation and regulation of innate and adaptive immune responses. In this study, however, it was observed that Fve stimulated NK cells to produce significant levels of IL-6 without concomitant elevated production of IFN-γ, IL-2, IL-4, and IL-10. It appears that Fve-induced NK cells responses could be unique and the implications of such responses DC phenotypic maturation and T cell activation deserve further studies.

The roles of IL-6 in innate and adaptive immunity have been an area of active research in recent years. In this study, it was observed that Fve-stimulated T cells (Fig. 3.9) and NK cells (Fig. 3.18) secreted significant levels of IL-6 that was not reported in other Fve-related studies. IL-6 was first found in 1977 [490] and has multiple well-characterized functions, such as regulating T cell proliferation, survival, and B cell differentiation[491]. The mechanism for maintaining the T cell survival is that IL-6 rescues T cells from entering apoptosis, and protects cells from Fas-mediated cell death[492,493]. More recent studies have documented a series of IL-6 activities that are critical for regulating the innate immunity. For example, at least two *in vitro* studies suggested that IL-6 was involved in skewing the differentiation of human monocytes away from a dendritic lineage to a more macrophage phenotype[494,495] indicating that IL-6 might have some inhibitory

effects on the development of dendritic cells. Moreover, another study reported that IL-6 inhibited NF-kB activity and suppressed CCR7 expression in dendritic cells[496], suggesting that IL-6 may inhibit their maturation or trafficking. In contrast, Pasare et al. found that IL-6 secretion by dendritic cells following TLR activation blocks the immunosuppressive activities of regulatory T cells, indicating IL-6 is essential in the activation of antigen-specific adaptive immune response induced by dendritic cells[497]. The implications of Fve-induced IL-6 production by T and NK cells on the innate and adaptive immunological outcomes have not been addressed in this study. It is conceivable that IL-6, acting cooperatively with other cytokines, is likely to be an important regulatory and determining factor in the initiation and differentiation of antigen-specific immune responses. Therefore, it is an important area to be addressed by more studies.

Taken together, data derived from this study indicate that Fve is a mitogen, targeting immune cells of both innate and adaptive immune systems. Our data also strongly support the notion that Fve is a potent mitogen that predominantly activates T cells, and the full activation of T cells is accessory cells dependent. Furthermore, results from *in vitro* and *in vivo* studies suggested that there is a dynamic cross talk and bi-directional positive feedback between DCs and T cells. It appears that DCs can act as accessory cells to fully activate T cells to produce IL-2, IFN-γ, and IL-6, and induce cellular proliferation. On the other hand, Fve-activated T cells can help DC subsets to acquire phenotypic and functional maturation for the priming and differentiation of antigen-specific $CD4^+$ and $CD8^+$ T cells.

Furthermore, Fve inducs NK cells to secrete IL-6 and probably other unidentified mediators that can have important immunological implications. These multitude interactions of Fve with T, DC, and NK cells, and their possible cooperative interactions and the possble immunological outcomes, are summarized in Figure 3.20.

As we know, successful cancer immunotherapy aims to enhance antigen-specific immune response not only by boosting components of the immune system that produce an effective and memory immune response intrinsically but also by inhibiting components that may induce tolerance. Information generated from this study prompted us to propose that Fve can be used as a potent adjuvant molecule to initiate and potentiate a Th1-skewed antigen-specific $CD4^+$ T cell and $CD8^+$ T cell immune response. Such adjuvant properties can be further exploited for boosting effectiveness of immunotherapeutic vaccines to target cancer. A classic example for this type of immunotherapeutic vaccines against cancer is the vaccine against HPV-associated cervical cancer. The proof of concept work in support of this notion will be described and discussed in the next chapter.

Chapter 4
Enhanced antitumor immunity by coadministration of HPV-16 E7 protein and Fve protein

4.1 Introduction

Cancer immunotherapy is an attractive alternative for the treatment of patients with cancer. The side effects, when used in the absence of radiotherapy or chemotherapy, are lower than those of classical antitumor chemotherapeutics. Identifying and cloning genes encoding tumor-associated antigens recognized by T cells has reopened the never-ending hope of curing cancer[13]. Although several treatments using tumor-associated antigens have been approved for some types of cancers, their efficacies have been variable and generally not sufficient. One important contributory factor for the insufficient and variable responses of immunotherapy is the poor immunogenicity of most tumor antigens, which results in insufficient activation of tumor antigen-specific $CD8^+$ T cells.

Cervical cancer is the second largest cause of cancer deaths in women and kills approximately 274,000 women worldwide each year[498,499]. Epidemiological and laboratory studies strongly support a crucial role for persistent human papillomavirus (HPV) infection and transcription in cervical carcinogenesis[500,501]. Although more than 100 HPV genotypes have been identified, four “high-risk” types of HPV (types 16, 18, 31, 45) are associated with approximately 80 percent

of all cervical cancers[502,503]. Of the high risk types, HPV type 16 is the most common and is responsible for more than 50 percent of all cervical cancers[503]. HPV are double-stranded circular viruses of approximately 8 kb that infect basal and suprabasal layers of stratified epithelium. The early genes, which include E1, E2, E4, E5, E6, and E7, code for proteins involved in viral DNA replication, transcription control, and cellular transformation. Late genes encode the major viral capsid protein, L1 and a minor capsid protein, L2. Infection with high-risk HPV sometimes results in integration of the viral episome into host DNA. If the integration interrupts the viral E2 gene and results in the loss of E2-mediated transcriptional repression, the transformed cells overexpress the E6 and E7 proteins, initiating the malignant transformation process[501,504]. Thus, HPV-16 E7 has been a major target of many prophylactic HPV vaccines in the prevention of HPV infections, as well as of many therapeutic HPV vaccines for the control of existing HPV infections and HPV-associated lesions, such as squamous intraepithelial lesion and cervical cancer. However, the antigen-specific immune responses and antitumor effects generated by HPV-16 E7 alone are weak and insufficient to control tumor growth. Several strategies have been developed to increase the potency of HPV-16 E7 vaccine; for example, immune modulators, such as cytokines[505,506], heat shock proteins[507,508], non-toxic bacteria toxins[509], or CpG[270] have been incorporated as adjuvant to enhance the HPV-16 E7-specific immunity. These have proven to be effective in animal models, but their potency in humans

has yet to be assessed, and active research to develop novel adjuvant molecules still is ongoing.

Fve has been shown to have the ability to trigger the proliferation of murine T cells and production of IL-2, IFN-γ (from my data in chapter 3, [415,423]). Moreover, Fve can activate the innate immunity by stimulating the DCs maturation and further enhance the antigen-specific $CD8^+$ T cells activity. Previous study also found that coadministration of Fve with antigen could drive strong Th1-skewed immune responses[431]. Taken together, information generated from this study prompted us to propose that Fve could be a potent adjuvant molecule to initiate and potentiate Th1-skewed antigen-specific immune responses, and such adjuvant properties could be exploited further to boost the immunogenicity and effectiveness of immunotherapeutic vaccines targeted at inducing immune responses specific to the cancer.

Therefore, in this chapter, the potential role of Fve as an adjuvant for cancer immunotherapy was explored. Using HPV type 16 E7 as a model tumor antigen and TC-1 cell-induced tumor model, a series of proof-of-concept studies were carried out to show the effectiveness of Fve as an adjuvant to enhance both humoral and cellular responses of the antitumor therapy *in vivo*.

4.2 Results

4.2.1 Production of recombinant E7 protein

The recombinant E7 protein containing 98 amino acid residues of the full-length E7 gene was expressed and purified in *E. coli* as described previously[510]. A total 4 mg of the recombinant E7 protein was obtained from 6L of bacterial culture. The predicted molecular weight of the HPV 16 E7 protein was calculated to be 11 kDa. However, the E7 protein migrated at 18 kDa in SDS-PAGE (Fig. 4.2A), at a higher molecular mass than predicted. This dissimilarity between experimental and theoretical molecular weight could be explained based on its amino acidic composition that had been reported earlier[510-512]. The recombinant E7 was further confirmed by E7-specific monoclonal antibodies using western blot (Fig. 4.2B).

4.2.2 Co-administration of HPV-16 E7 plus Fve increased HPV-16 E7-specific humoral immune response

To investigate the effects of Fve on the induction of HPV-16 E7-specific humoral immunity, C57BL/6 mice were either immunized with PBS, Fve, E7, or E7 plus Fve at day 0, 14, 28. The HPV-16 E7-specific antibodies in the sera of different immunized mice were measured by ELISA (section 2.2.9). As shown in Figure 4.3 A&B, mice immunized with HPV-16 E7 plus Fve produced significantly higher levels of IgG1 and IgG2c as compared to those immunized with HPV-16 E7 alone. HPV-16 E7-specific IgG1 and IgG2c in HPV-16 E7 plus Fve immunized mice were

7-fold and 33-fold higher respectively than those of mice immunized with HPV-16 E7 alone at day 28. Negligible levels of HPV-16 E7-specific IgG1 and IgG2c antibodies were detectable in the PBS control group over the same studied period (Fig. 4.3 A&B). Furthermore, the BALB/cJ mice were immunized with the same regimen, and the E7-specific IgG1 and IgG2a were measured by ELISA from day 0 to day 63 after the first immunization. Consistent with the data from C57BL/6 mice, E7 plus Fve vaccination enhanced IgG1 and IgG2a production significantly higher than E7 immunizaiton alone. HPV-16 E7-specific IgG1 and IgG2a in HPV-16 E7 plus Fve immunized mice were 4-fold and 27-fold higher respectively than those of mice immunized with HPV-16 E7 alone at day 35 (Fig. 4.4). These results indicate that Fve could enhance strong HPV-16 E7-specific humoral immune responses.

4.2.3 Coadministration of HPV-16 E7 and Fve enhanced IFN-γ production by E7-specific $CD4^+$ and $CD8^+$ T cells

To evaluate the E7-specific T cell responses in the immunized mice, splenocytes from C57BL/6 immunized mice were collected and stimulated with different concentrations of E7 protein from 1 to 20 μg/ml *in vitro* (section 2.2.9). The culture supernatants were collected at 72 hrs and the cytokine production were measured by ELISA. As shown in Figure 4.5, IFN-γ production was significantly enhanced in splenocytes from HPV-16 E7 plus Fve immunized mice as compared to those from HPV-16 E7 or PBS immunized mice. There was no statistical difference for the

production of TNF-α, TGF-β, and IL-10 in splenocytes between E7 plus Fve immunized mice and E7 immunized mice. No IL-4 production was detected in the splenocytes of all the three groups.

To investigate the effect of Fve on E7-specific T cell immune response in immunized mice, the splenocytes from immunized mice were cultured with E7 protein and IL-2 for nine days. Then, short-cultured T cells were restimulated with different concentrations of E7 protein *in vitro*, and the cytokine production was measured at 72 hrs (section 2.2.10). As shown in Figure 4.6, the IFN-γ production was significantly enhanced in T cells from HPV-16 E7 plus Fve immunized mice as compared to those from HPV-16 E7 or PBS immunized mice. There was no statistical difference for the production of TNF-α, TGF-β, and IL-10 in T cells between E7 plus Fve immunized mice and E7 immunized mice. To further explore which subset of T cells are responsible for the enhanced IFN-γ production, short-cultured T cells were re-stimulated with 5 μg/ml of anti-CD3 and 2 μg/ml of anti-CD28 antibody for 12 hrs. IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells were stained with specific monoclonal antibodies and analyzed by flow cytometry (section 2.2.12). Flow cytometry analysis revealed that 18.7 percent of IFN-γ^+ producing cells within the $CD4^+$ T subset were induced in the HPV-16 E7 plus Fve co-immunized mice, whereas only 4.9 percent and 5.7 percent of IFN-γ^+ cells were produced in HPV-16 E7 and PBS mice, respectively (Fig. 4.7 upper panel). Similarly, 37.8 percent of IFN-γ^+ cells among the $CD8^+$ T subset were induced in the HPV-16 E7 plus Fve co-immunized mice whereas only 11.4 percent and 12.5

percent of IFN-γ^+ T cells were produced in HPV-16 E7 and PBS mice, respectively (Fig. 4.7 lower panel).

In summary, these results indicate that Fve could significantly increase HPV-16 E7-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells.

4.2.4 Coadministration of HPV-16 E7 and Fve enhanced protection of mice against tumor growth

Immunization with HPV-16 E7 plus Fve led to an enhanced HPV-16 E7-specific immunity prompted us to explore the antitumor potential of Fve *in vivo*. Mice immunized with PBS, HPV-16 E7, Fve, or HPV-16 E7 plus Fve were challenged with TC-1 cells subcutaneously and the tumor growth was monitored (Fig. 4.8A). Results showed that 60 percent of mice co-immunized with HPV-16 E7 plus Fve remained tumor free for up to 167 days after the tumor challenge whereas only 20 percent of mice remained tumor free ($p < 0.05$) in the HPV-16 E7 immunized group (Fig. 4.8B). Mice immunized with PBS or Fve alone developed tumors rapidly within 10 and 15 days after tumor challenge, respectively (Fig. 4.8B). Interestingly, mice immunized with Fve alone generally showed a reduction in tumor sizes as compare to the PBS control mice (Fig. 4.8C&D). This suggests that Fve protein alone could confer partial suppression of tumor growth. However, such suppressive effect was insufficient to protect mice against tumor formation in the absence of HPV-16 E7-specific immune responses, as only mice in the group immunized with HPV-16 E7 plus Fve exhibited low tumor burden or had remained

tumor-free over the total duration of this study.

To determine whether long-term HPV-16 E7-specific immunity could be established in the immunized mice, splenocytes from tumor-free mice (HPV-16 E7 alone or HPV-16 E7 plus Fve groups) were collected and stimulated with HPV-16 E7 protein *in vitro* 167 days after tumor challenge. Splenocytes from naïve mice were used as the negative control. Results showed that upon HPV-16 E7 antigen stimulation, splenocytes from HPV-16 E7 plus Fve co-immunized mice still produced higher levels of IFN-γ as compared to those from HPV-16 E7-immunized mice (Fig. 4.9). Hence, Fve enhanced HPV-16 E7-specific immunity persisted in the co-immunized mice and may account for the long-term protection against tumor formation.

In addition, tumor protection assays were performed using the metastasis tumor model as described previously[513]. Mice pre-immunized with the same immunization regimen were challenged with TC-1 cells intravenously (Fig. 4.10A). Results showed that mice immunized with E7 plus Fve had longer survival as compared to mice immunized with E7, Fve, and PBS (Fig. 4.10B).

Taken together, these data indicate that immunization with Fve plus HPV-16 E7 is not only more effective than immunization with HPV-16 E7 alone; it also is able to confer long-term protection to the mice against tumor formation and metastasis.

4.2.5 Therapeutic immunization of HPV-16 E7 and Fve suppressed the tumor growth and prolonged the survival of tumor bearing mice

Whether coadministration of HPV-16 E7 and Fve was equally effective in suppressing the growth of the established tumor was further determined by therapeutic tumor assay. In this set of experiments, TC-1 cells were inoculated in the left flank of mice three days prior to regular treatments with PBS, HPV-16 E7, Fve, and HPV-16 E7 plus Fve, respectively (Fig. 4.11A). Results showed that mice treated with HPV-16 E7 plus Fve had the longest survival (Fig. 4.11A).

Further investigations then were carried out in a tumor metastasis model established by injecting TC-1 cells intraveneously into the tail vein of each mouse at day 0. Mice then were treated with same regimens (Fig. 4.12A). As shown in Figure 4.12B, all the mice in the groups immunized with PBS, Fve, or HPV-16 E7 alone did not survive beyond 55 days, while mice treated with HPV-16 E7 plus Fve showed significantly prolonged survival for up to 120 days. These results indicate that Fve could significantly enhance the HPV-16 E7-specific antitumor activity therapeutically.

In summary, the results from these series of proof-of-principle experiments strongly support the notion that Fve could enhance an antigen-specific immune response that not only confers long-term protection against tumor growth but also retards tumor growth at the early and advanced stages of tumor development.

4.2.6 Both $CD4^+$ and $CD8^+$ T cell subsets and IFN-γ were essential for the tumor protection

Since Fve significantly increased IFN-γ-secreting T cells and enhanced HPV-16 E7-specific antitumor immunity, *in vivo* antibody depletion assay was performed to determine the roles of the T cell subsets and IFN-γ in the antitumor effects induced by combined vaccination (Fig. 4.13A). As expected, 60 percent of the HPV-16 E7 plus Fve co-immunized mice without any depletions remained tumor free throughout the duration of this part of the study (Fig. 4.13B), while the tumors developed in the remaining 40 percent of these mice were dramatically reduced in size (Fig. 4.13B). In contrast, all the mice depleted of $CD4^+$, $CD8^+$ T cells, or IFN-γ developed tumors within 27 days. Interestingly, mice depleted of $CD8^+$ T cells had similar tumor size as PBS control, whereas tumor growth was retarded in the mice depleted of $CD4^+$ T cells and of IFN-γ (Fig. 4.13C). These data suggested that while $CD8^+$ T cells, $CD4^+$ T cells, and IFN-γ are essential for the antitumor protection generated in mice co-immunized with HPV-16 E7 plus Fve; $CD8^+$ T subset plays a more dominant role in these antitumor effects.

4.2.7 Adoptively transfer T cells from co-immunized mice retarded the tumor growth

To investigate further the roles of T cells in the antitumor effects seen in the HPV-16 E7 plus Fve co-immunized mice, a T cell-adoptive transfer experiment was set up. The immunization and tumor challenge regimen was as detailed in

Figure 4.14A. Mice received T cells from co-immunized mice showed significant reduction in tumor growth (Fig. 4.14B) as compared to those adoptively transferred with T cells from donor mice immunized with HPV-16 E7, Fve and PBS, respectively. These data support the notion that T cells play a pivotal role in the therapeutic antitumor effects and such effects probably are directly correlated to the efficacy and magnitude of the HPV-16 E7-specific T cell responses as demonstrated in Figure 4.5-7.

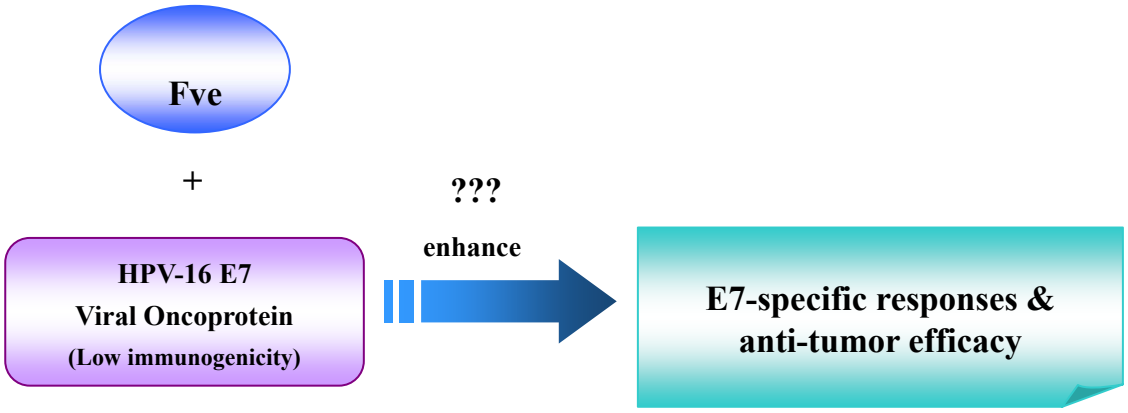


Figure 4.1 The schematic diagram showing the strategy for HPV related cancer immunotherapy using Fve as adjuvant.

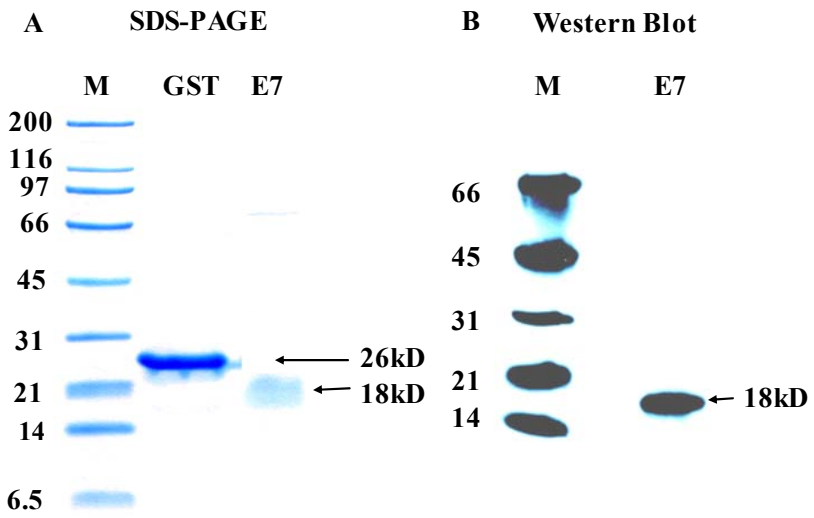


Figure 4.2 Analysis of recombinant E7 protein by SDS-PAGE and western blot. The recombinant GST-E7 proteins were expressed in *E. coli* and purified using GST-affinity column. After thrombin cut, E7 protein was eluted and run at 18 kDa in 7.5 % of Tricine SDS-PAGE (A). E7 was further examined by western blot (B).

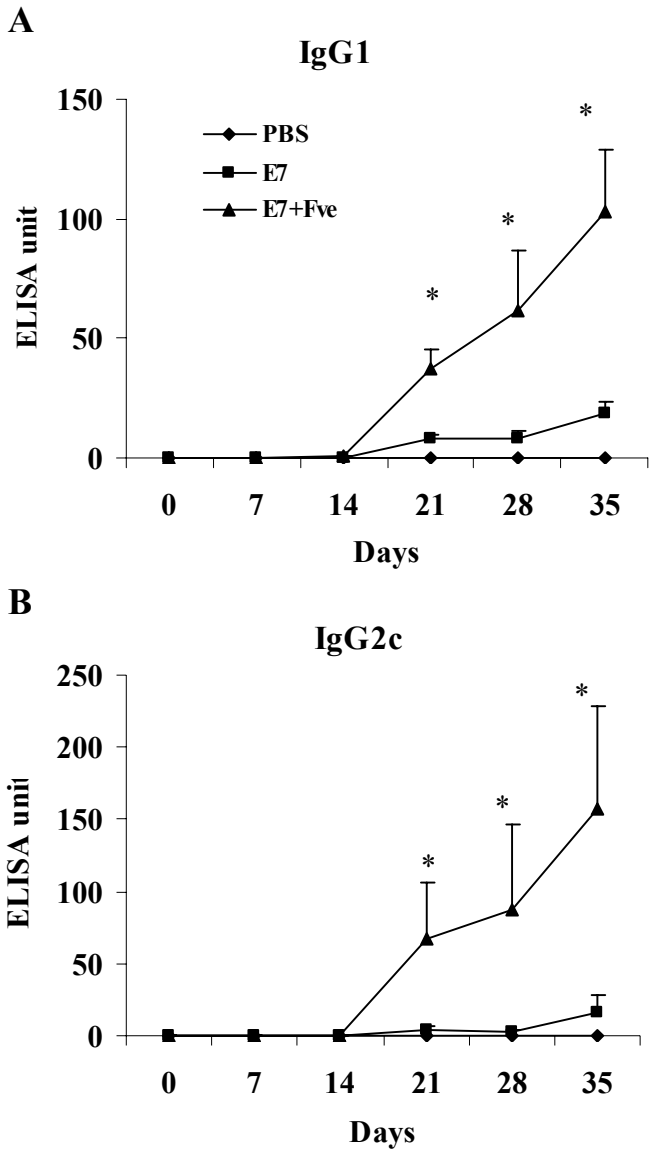


Figure 4.3 Fve enhanced E7-specific antibodies production in C57BL/6 mice. Mice (n = 5 per group) were subcutaneously immunized with PBS (♦), 20 μg of E7 (■) or 20 μg of E7 plus 20 μg of Fve (▲) at day 0, 14 and 28. Sera were collected weekly for measurement of E7-specific IgG1 (A) and IgG2c (B) by ELISA. Data are representative of three independent experiments. Bars represent means ±SEM, *, $p < 0.05$ (E7 vs E7 plus Fve).

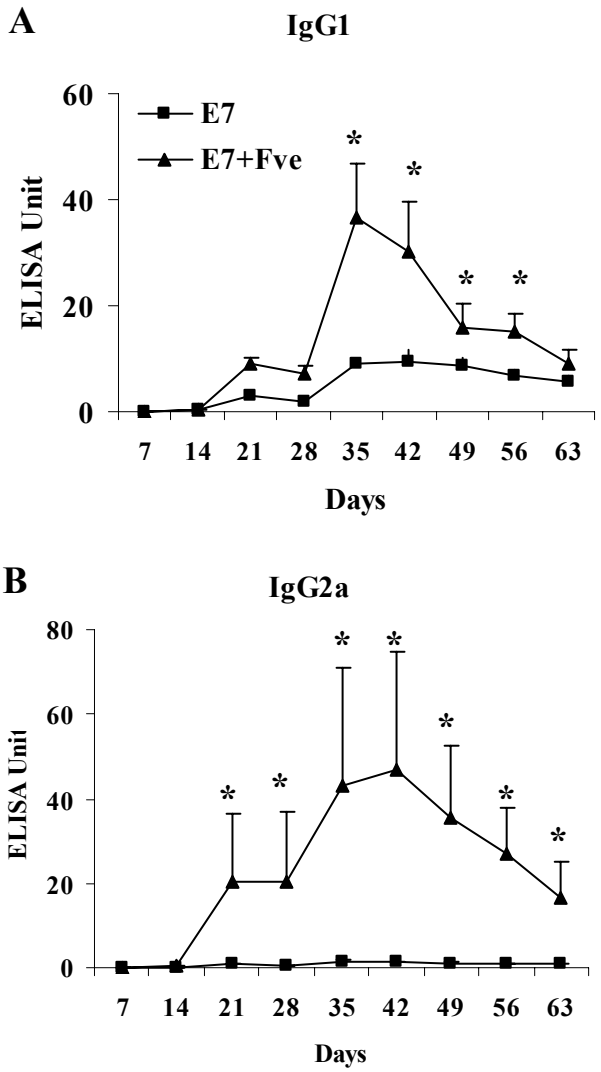


Figure 4.4 Fve enhanced E7-specific antibodies production in BALB/cJ mice. BALB/cJ mice (n = 5 per group) were subcutaneously immunized with 20 μg of E7 (■) or 20 μg of E7 plus 20 μg of Fve (▲) at day 0, 14 and 28. Sera were collected weekly for measurement of E7-specific IgG1 (A) and IgG2a (B) by ELISA. Data are representative of two independent experiments. *Bars*, SEM. *, $p < 0.05$ (E7 vs E7 plus Fve).

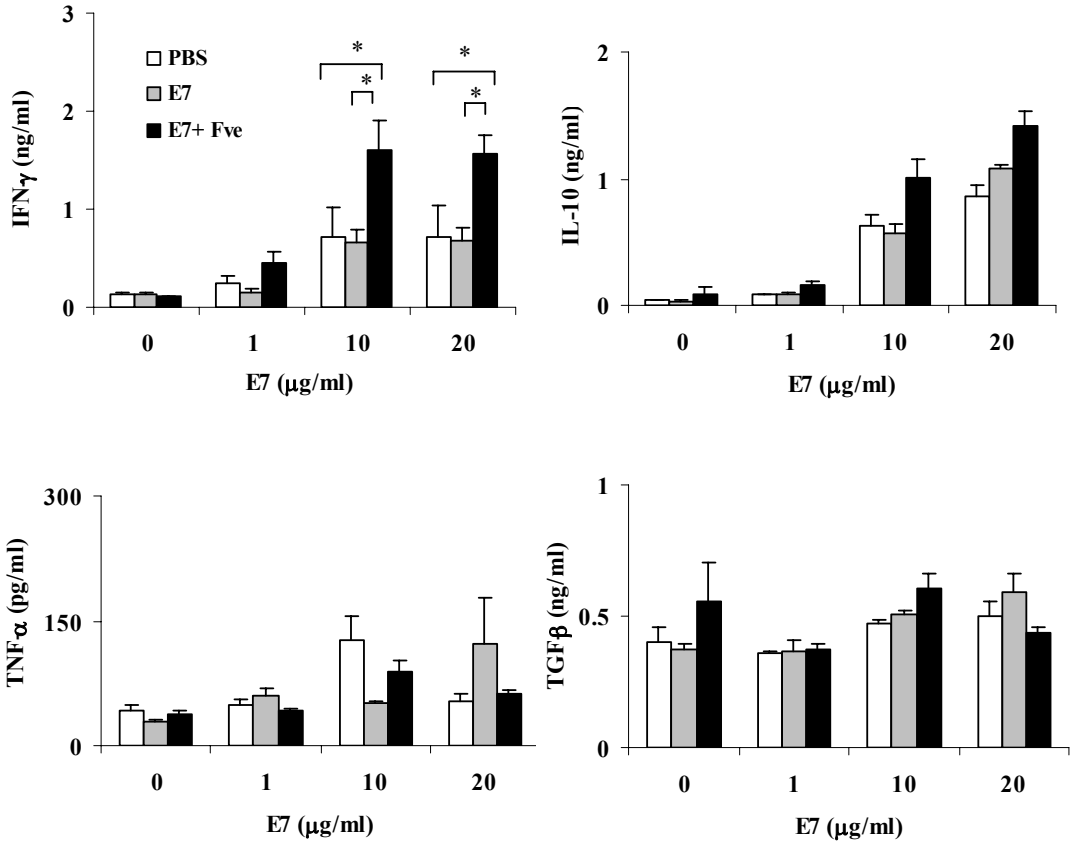


Figure 4.5 Cytokine profile by splenocytes from immunized C57BL/6 mice. Mice (n = 5 per group) were subcutaneously immunized with PBS (□), 20 μg of E7 (■), and 20 μg of E7 plus 20 μg of Fve (■) at day 0 and 14. Splenocytes were collected at day 28 and cultured with 1, 10, or 20 μg/ml of E7. Supernatants were collected at 72 hrs and cytokine production was measured by ELISA.

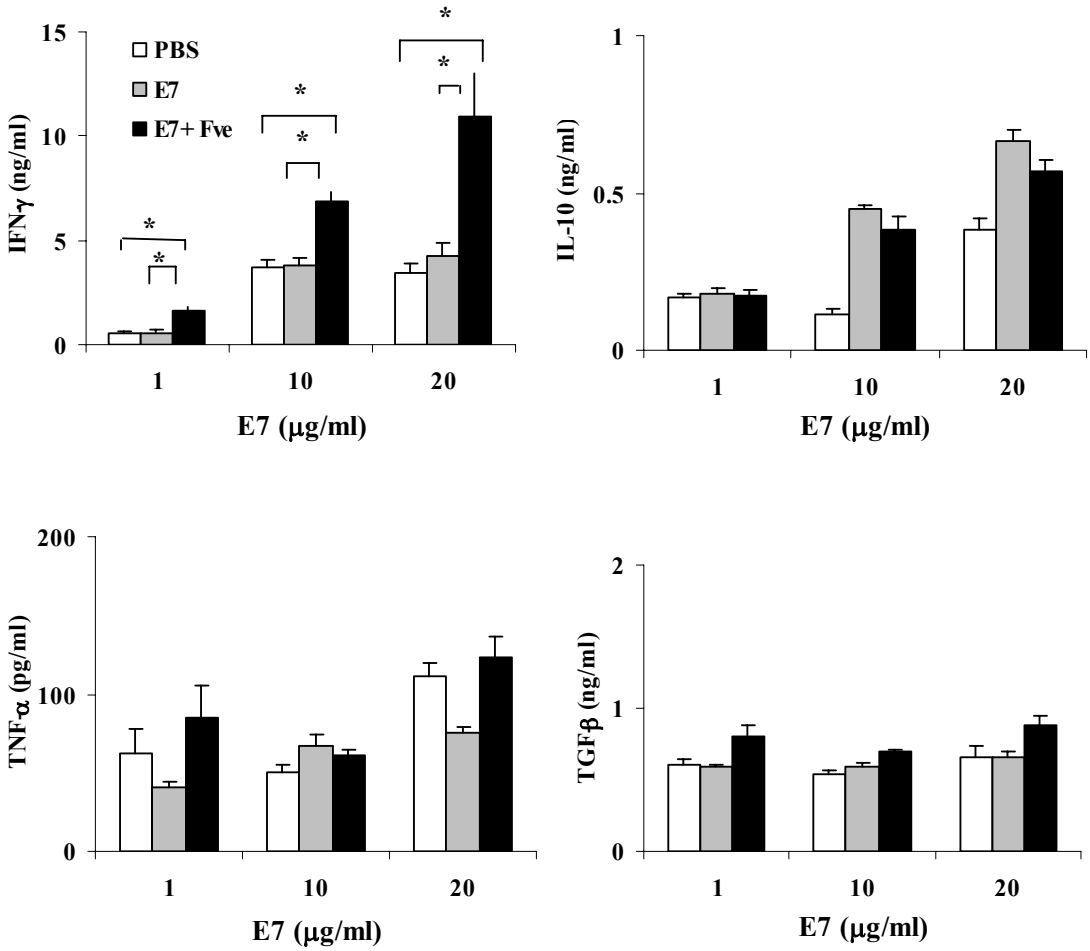


Figure 4.6 Cytokine profile by short-term cultured T lymphocytes from immunized C57BL/6 mice. Mice (n = 5 per group) were subcutaneously immunized with PBS (□), 20 μg of E7 (▒), and 20 μg of E7 plus 20 μg of Fve (■) at day 0 and 14. Splenocytes were collected from immunized mice at day 28 and then cultured with E7 protein and IL-2 for 9 days. Short-term cultured T cells were then stimulated with 1, 10 or 20 μg/ml of E7 for 72 hrs. Culture supernatants were collected and cytokines were measured by ELISA. Bars represent means±SEM.

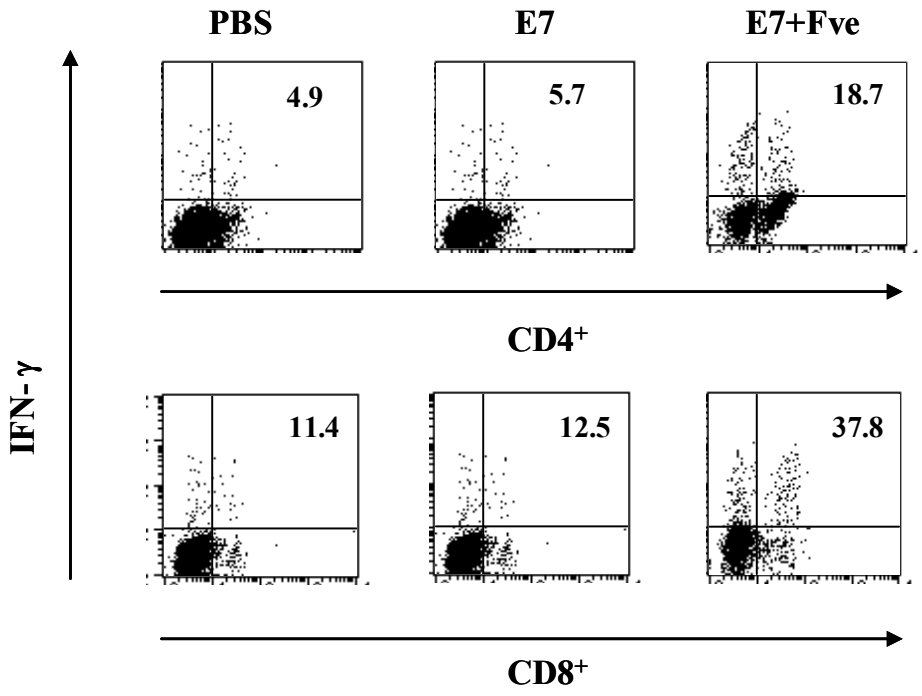
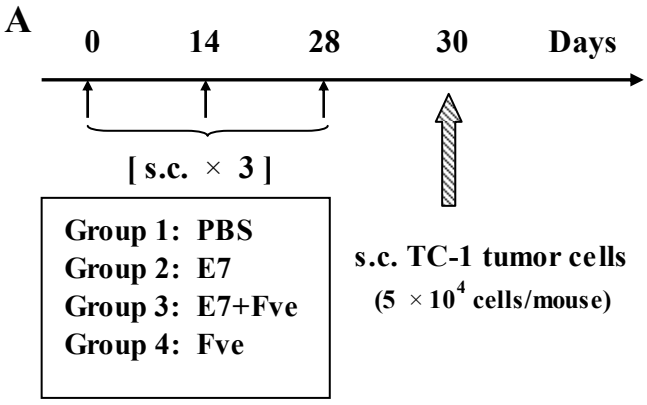
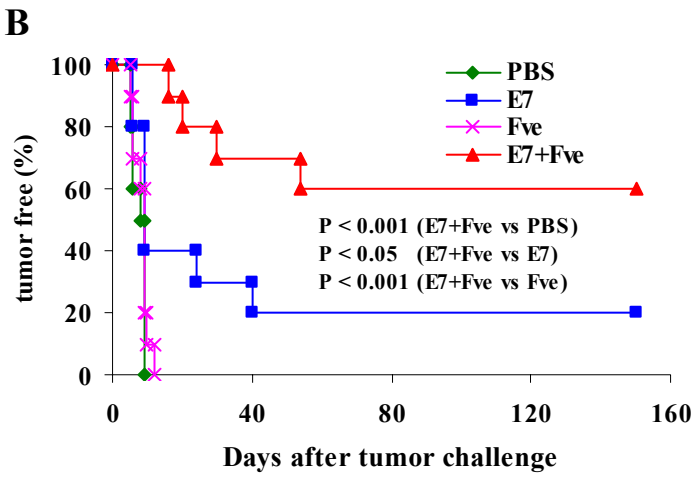


Figure 4.7 ICCS analysis of short-term cultured T lymphocytes from immunized C57BL/6 mice. Mice (n = 5 per group) were subcutaneously immunized with PBS, 20 μg of E7, and 20 μg of E7 plus 20 μg of Fve at day 0 and 14. Splenocytes were collected at day 28 and then cultured with E7 protein and IL-2 for 9 days. For intracellular staining, short-term cultured T cells were stimulated with anti-CD3 and anti-CD28 antibodies for 12 hrs. Cytokine-secreting $CD4^+$ T cells and $CD8^+$ T cells were then analyzed by flow cytometry. The numbers in the dot plots indicate the percentages of $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ T cells in the total T cells.





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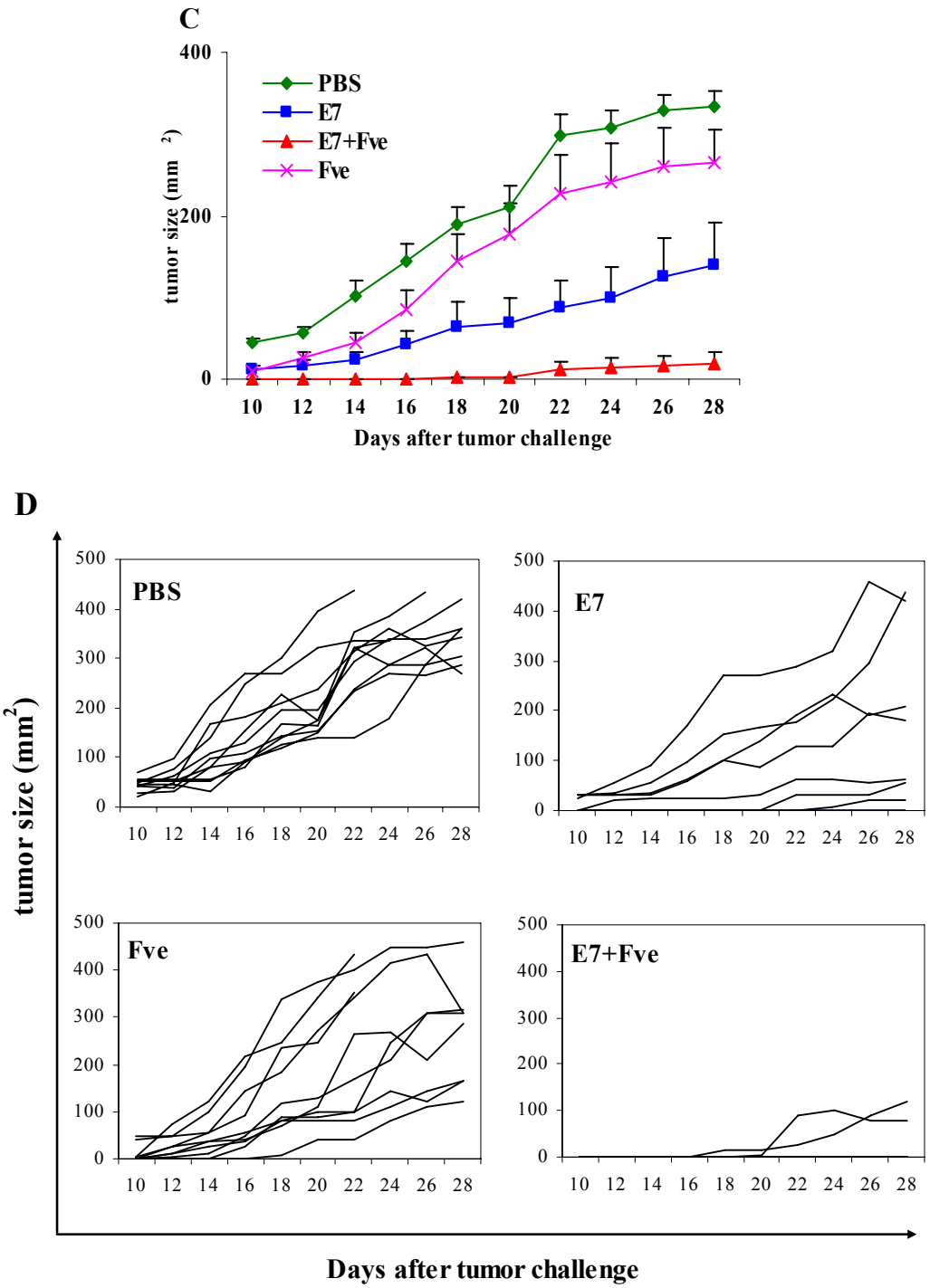


Figure 4.8 Co-immunization of E7 and Fve enhanced protection against the growth of TC-1 tumors. Mice (n = 10 per group) were immunized with PBS (♦), 20 μg of E7 (■), 20 μg of Fve (×) or 20 μg of E7 plus 20 μg of Fve (▲) at day 0, 14, 28 and then inoculated subcutaneously with TC-1 cells at day 30 (A). The mice were monitored daily for tumor growth by palpation (B) and the tumor size was measured every two days (D). Mean tumor size was calculated (C). Data are representative of two independent experiments. Bars represent means±SEM..

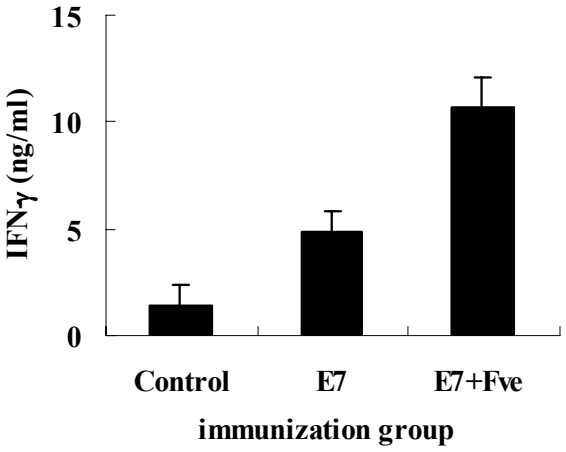


Figure 4.9 IFN-γ production by splenocytes in tumor-free mice of day167 after tumor challenge. Mice (n = 10 per group) were immunized with PBS (♦), 20 μg of E7 (■), 20 μg of Fve (×) or 20 μg of E7 plus 20 μg of Fve (▲) at day 0, 14, 28 and then inoculated subcutaneously with TC-1 cells at day 30. One hundred and sixty-seven days after the tumor challenge, spleens were collected from the tumor-free mice and cultured with 20 μg/ml of E7 protein *in vitro*. Supernatants were collected at 72 hrs and IFN-γ levels were analyzed. Bars represent means± SEM.

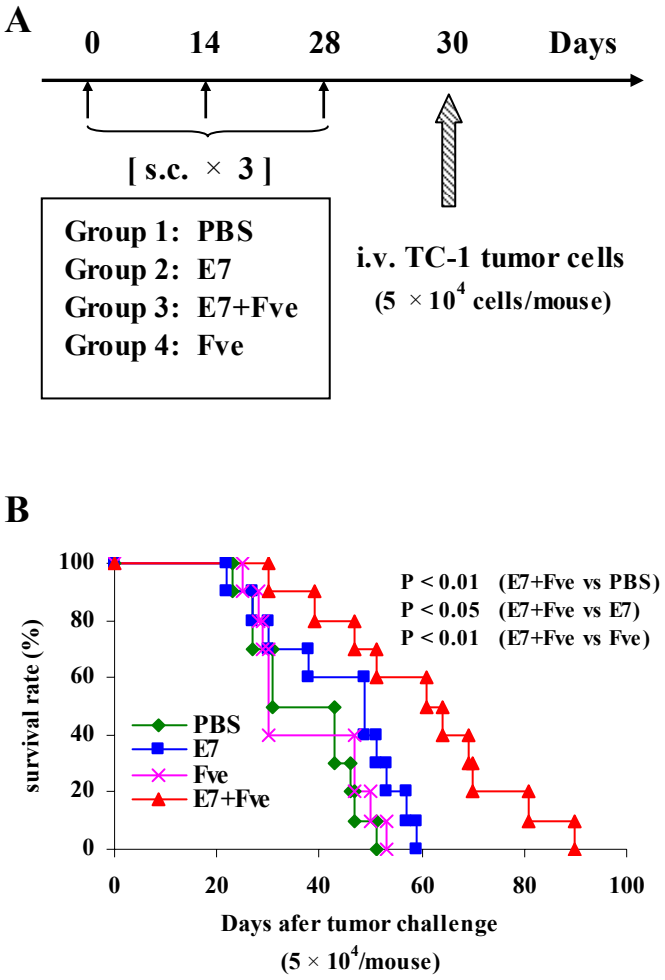
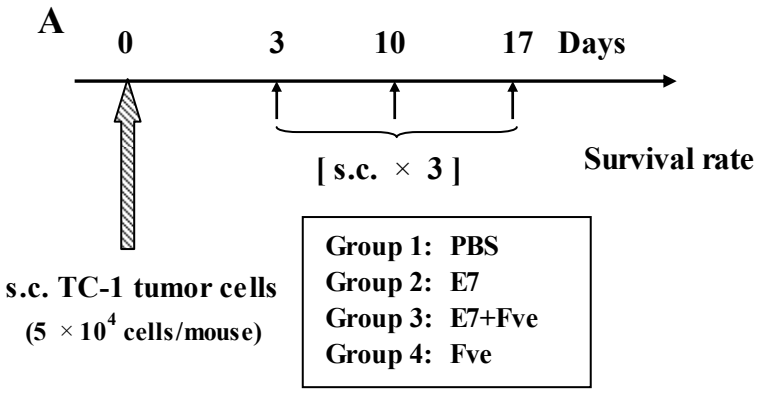
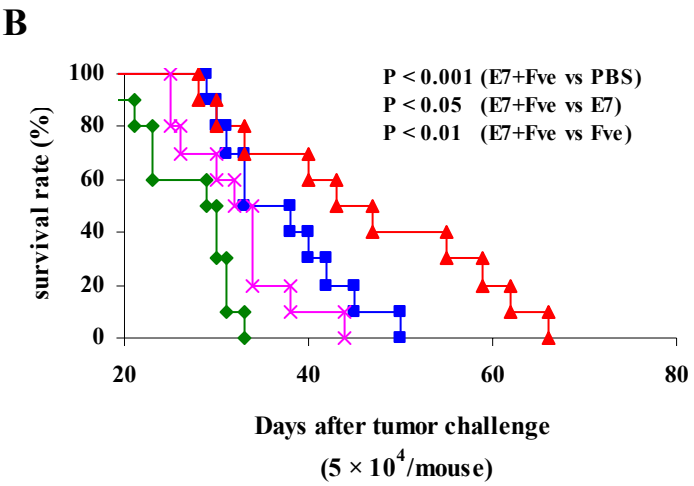


Figure 4.10 E7 plus Fve co-immunization extended the survival of mice in metastatic prevention tumor model. Mice (n = 10 per group) were immunized with PBS (♦), 20 μg of E7 (■), 20 μg of Fve (×) or 20 μg of E7 plus 20 μg of Fve (▲) at day 0, 14, 28 and then were intravenously injected with TC-1 cells at day 30 (A). The survival of mice was monitored and the survival curves were analyzed by log-rank test. Data are representative of two independent experiments.





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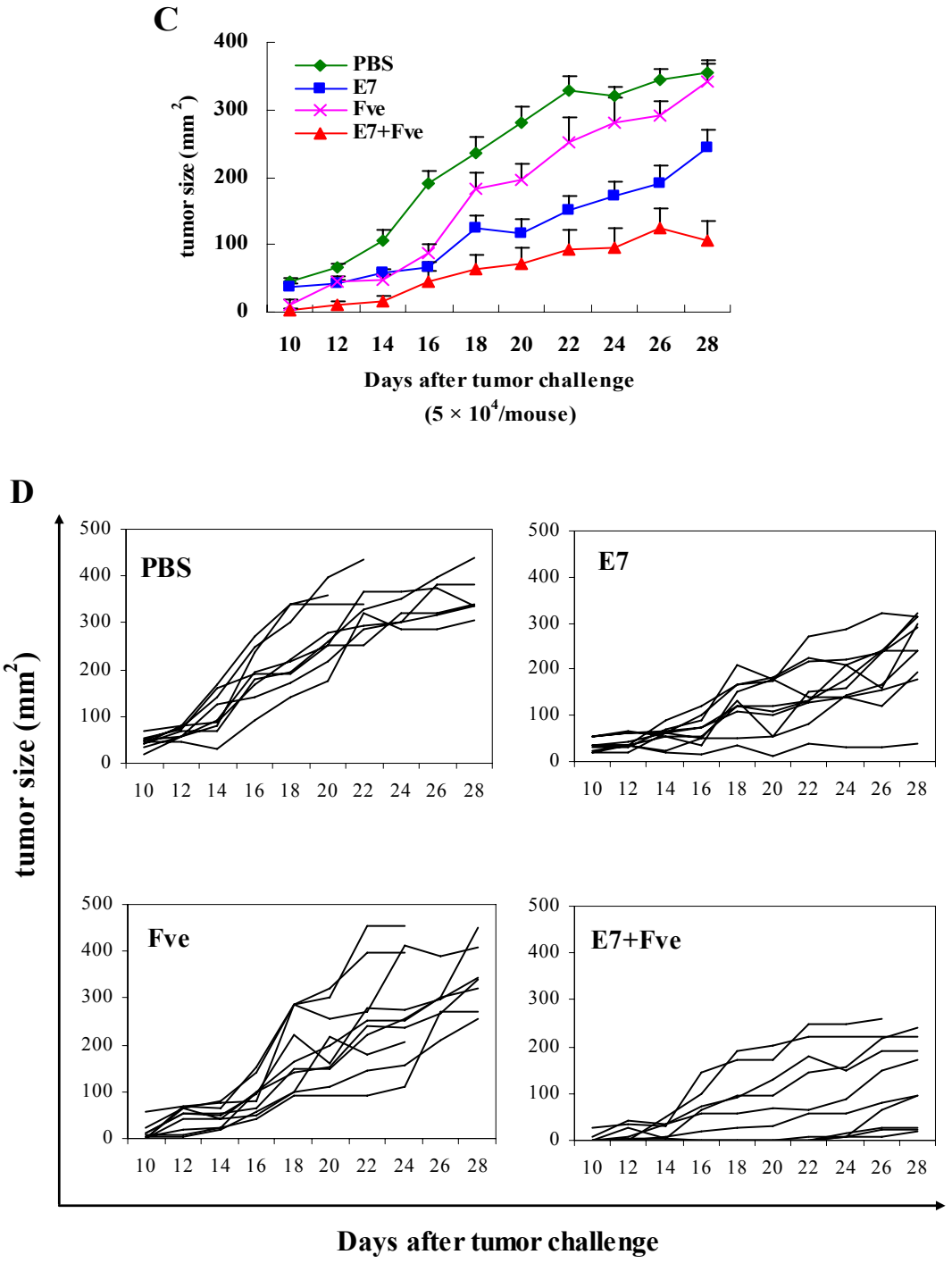


Figure 4.11 E7 plus Fve co-immunization therapeutically reduced tumor growth. Mice (n = 10 per group) were inoculated subcutaneously with TC-1 cells on day 0 and then treated with PBS (♦); 20 μg of E7 (■), 20 μg of Fve (×) or 20 μg of E7 plus 20 μg of Fve (▲) at day 3, 10, 17 (A). The tumor size was measured every two days (D) and the survival was monitored daily (B). The mean tumor size was calculated (C).The survival curves were analyzed by log-rank test. Data are representative of two independent experiments. *Bars*, SEM.

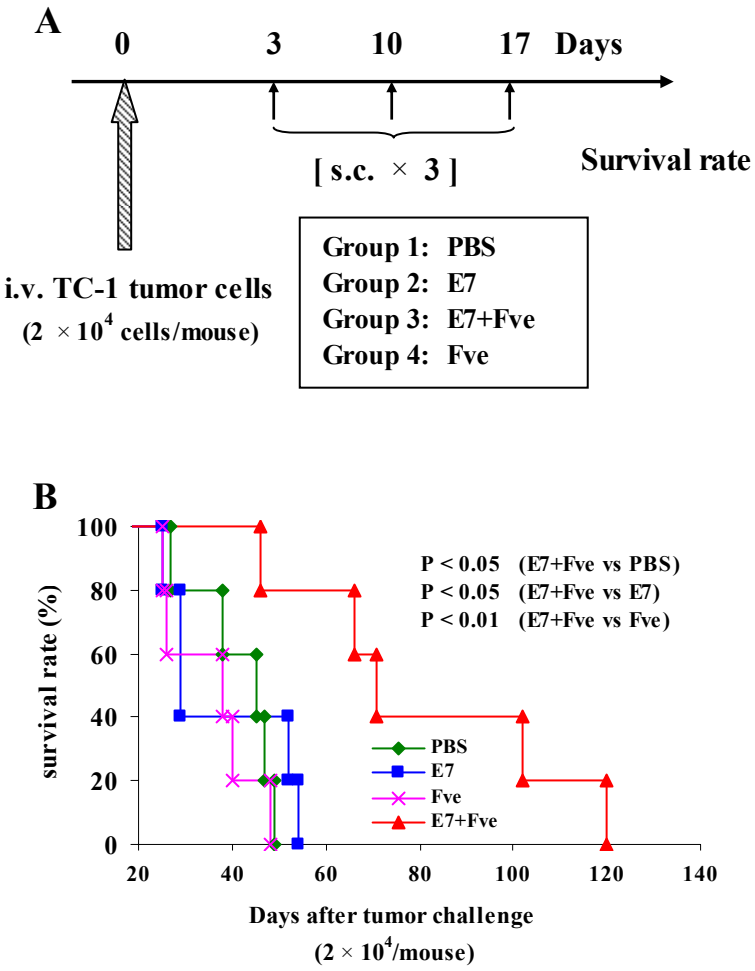
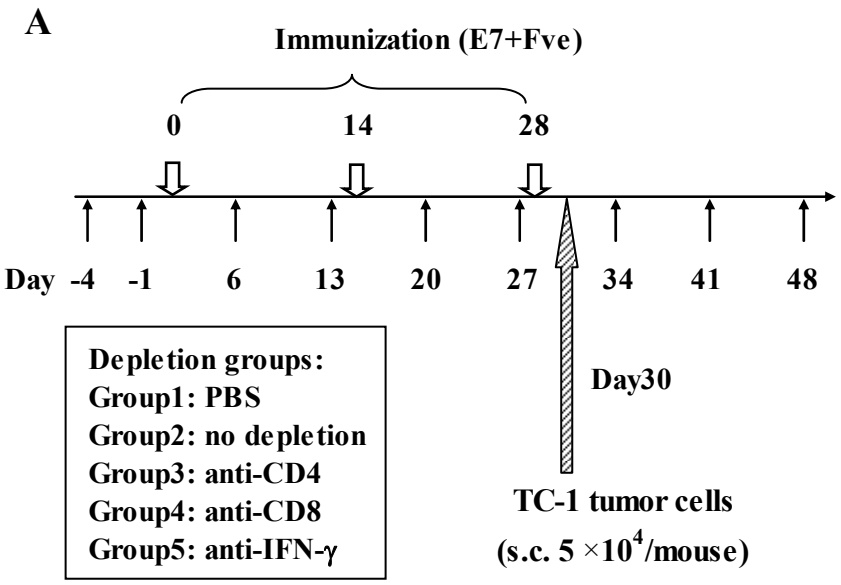
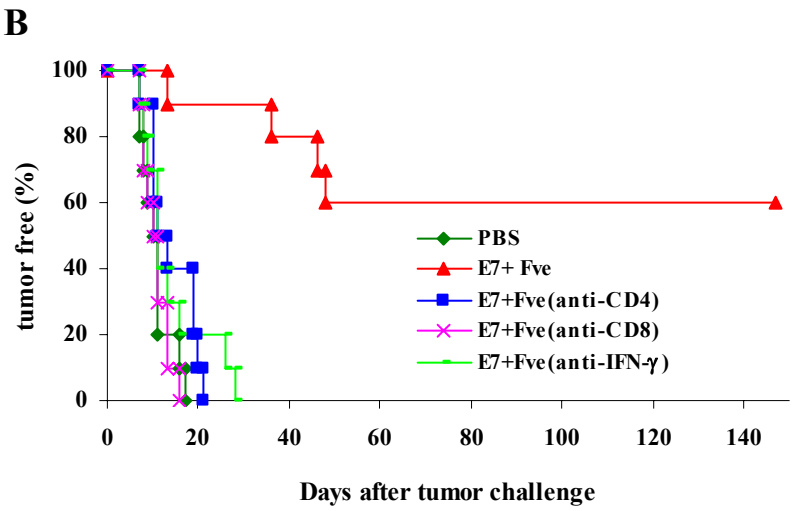


Figure 4.12 E7 plus Fve co-immunization extended the survival of mice in metastatic therapeutic tumor model. Mice (n = 5 per group) were intravenously injected with TC-1 cells on day 0 and then treated with PBS (♦); 20 μg of E7 (■), 20 μg of Fve (×) or 20 μg of E7 plus 20 μg of Fve (▲) at day 3, 10, 17 (A). The survival of mice was monitored and the survival curves (B) were analyzed by log-rank test. Data are representative of two independent experiments. *Bars*, SEM.





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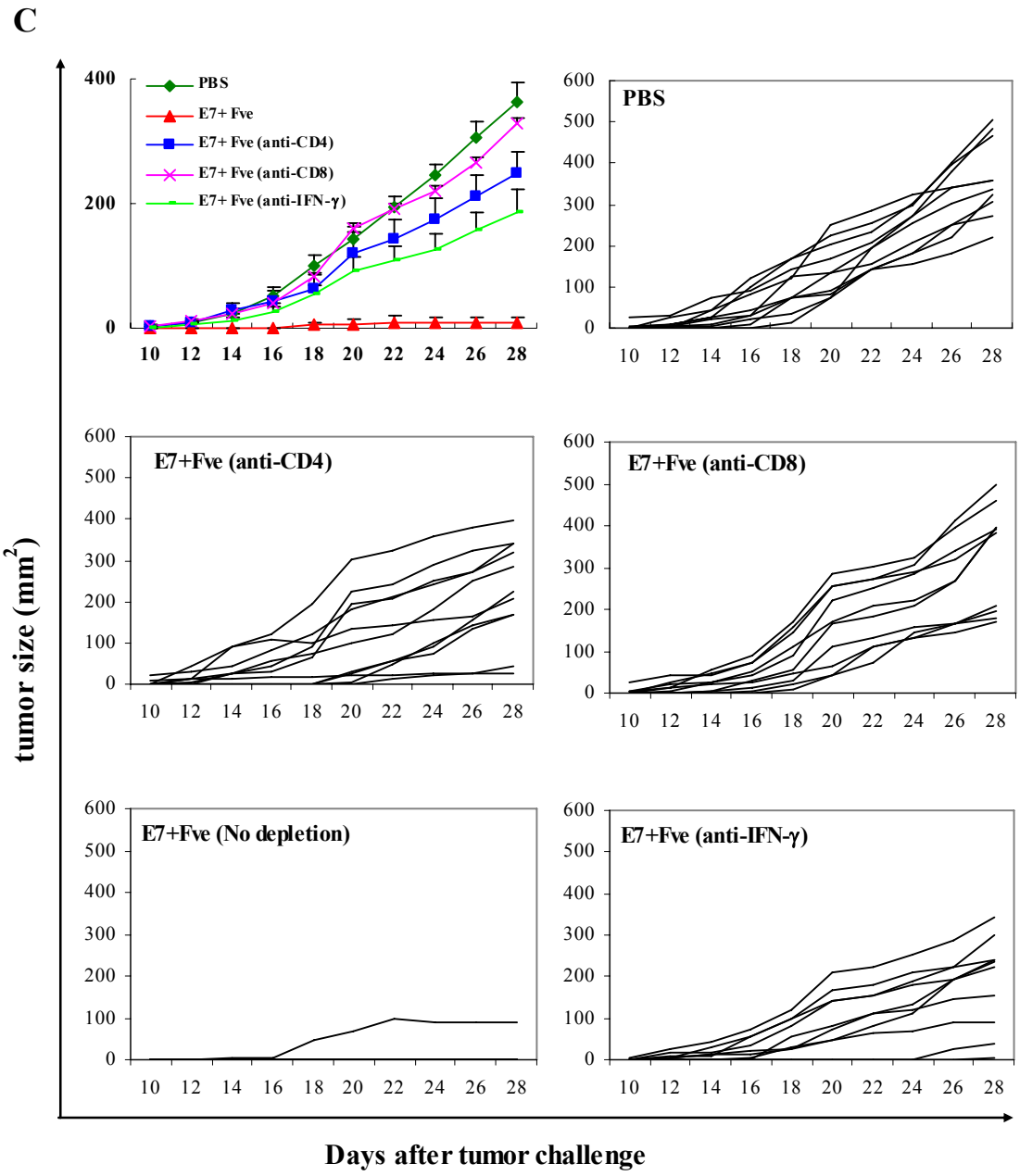

Figure 4.13 CD4$^+$, CD8$^+$ T cells and IFN-γ were essential for the tumor protection in E7 plus Fve immunized mice. Mice (n = 10 per group) were immunized with PBS or 20 μg of E7 plus 20 μg of Fve at day 0, 14, 28. 5 × 10^4 of TC-1 cells were inoculated subcutaneously into the right flank at day 30. To deplete the CD4$^+$ (■), CD8$^+$ (×) T cells or IFN-γ (–), E7 plus Fve immunized mice were injected intraperitoneally with α-CD4, α-CD8, α-IFN- γ mAbs respectively at day -4, -1, 6, 13, 20, 27, 34, 41, 48. PBS (♦) and E7 plus Fve immunized mice without depletion (▲) were control groups (A). The mice were monitored daily for tumor growth by palpation (B). Tumor size was measured every two days (C) and the mean tumor size of each groups was calculated (C). *Bars*, SEM.

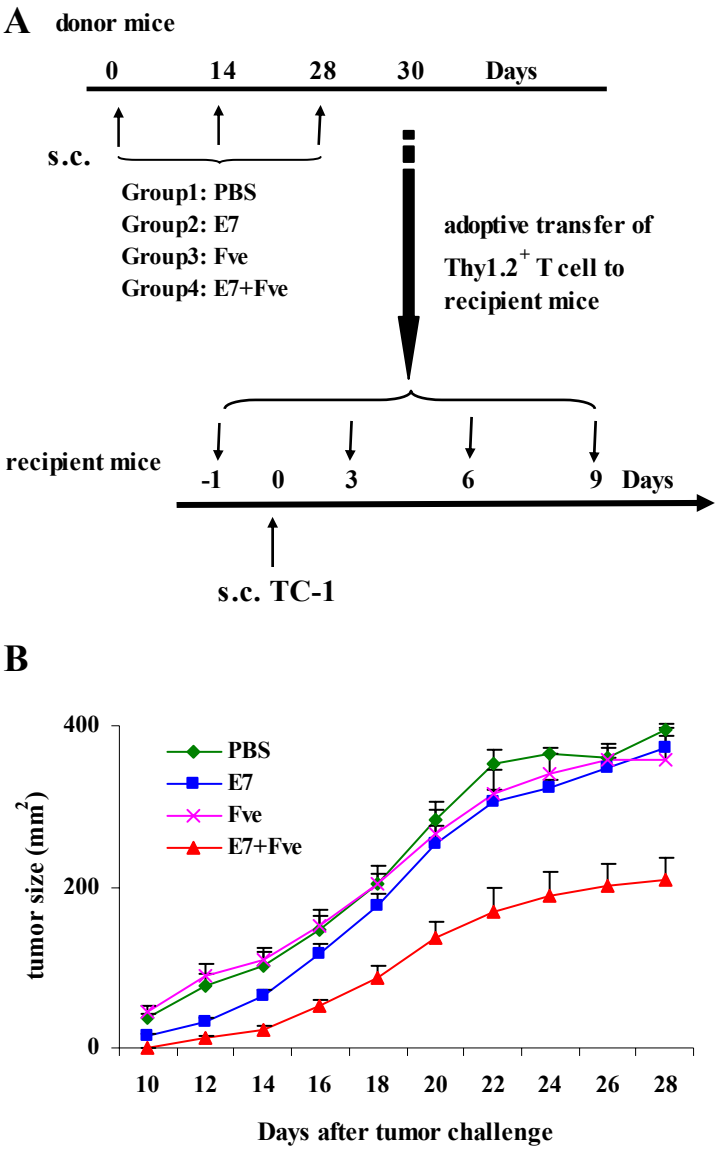

Figure 4.14 Adoptive transfer of T cells from the E7 plus Fve immunized mice retarded tumor growth. Eight million of T cells purified from the mice immunized with PBS (♦), HPV-16 E7 (■), Fve (×) or HPV-16 E7 plus Fve (▲) were adoptively transferred to recipient mice at days -1, 3, 6, and 9. Recipient mice (n = 10 per group) were inoculated subcutaneously with 5×10^4 of TC-1 cells at day 0 (A). Ten days after the tumor challenge, the size of the tumor formed was measured on alternate days. Data are presented as mean ± SEM (B).

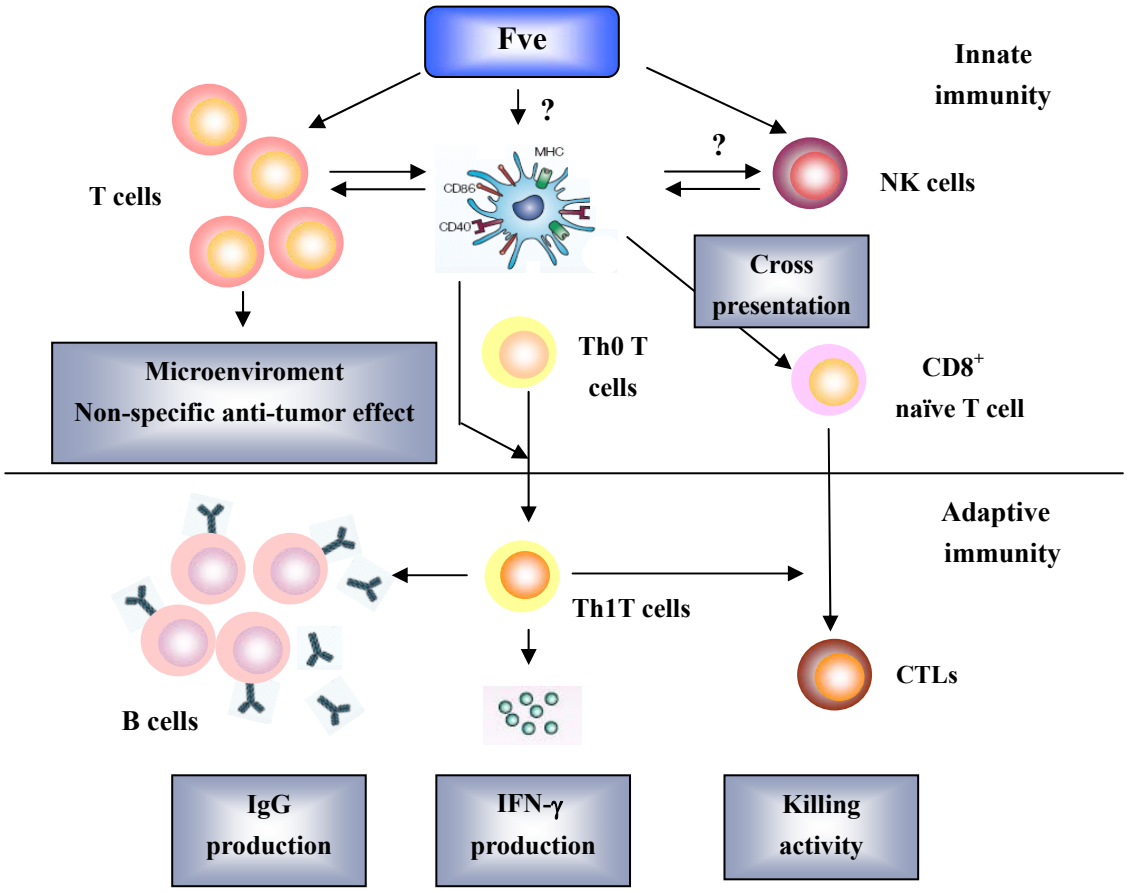

Figure 4.15 Proposed mechanisms by which Fve protein facilitates innate and adaptive immune responses for tumor immunotherapy. Fve protein stimulates mouse dendritic cells *in vivo*, activating the innate immune response to form a foundation on which antigen-specific adaptive immunity is based. In particular, by improving the function of professional APCs, Fve facilitate the generation of Th1 humoral and cellular antigen-specific immunity. Moreover, it is speculated that Fve could enhance the antigen-specific $CD8^+$ T cell immunity through cross-presentation by stimulating the $CD8^+$ DCs. These enhanced antigen-specific immune responses play key roles in the tumor immunotherapy. Besides, the cytokine productions (such as IFN-γ and IL-6) secreted from Fve-stimulated T cells and NK cells create a positive microenvironment to confer partial antitumor effects.

4.3 Discussion

Antigen-specific immunotherapy is a promising strategy to eradicate systemic tumors at multiple sites while conferring the advantage of specific discrimination between neoplastic and non-neoplastic cells. However, a major hurdle for the development of such vaccines for treatment and prevention of cancer is the poor immunogenicity of tumor-associated antigens. An attractive strategy to overcome this problem is the use of an immune modulator as an adjuvant to boost the antigen specific immunity and enhance the efficacy of tumor vaccines[514].

Our *in vivo* tumor protection results showed that 60 percent of the mice remained tumor free from the HPV-16 E7 plus Fve co-immunization after tumor challenge as compared to only 20 percent of mice immunized with HPV-16 E7 (Fig. 4.8B). More importantly, the enhanced antitumor effects induced by HPV-16 E7 plus Fve co-immunization were also observed in therapeutic tumor model (Fig. 4.11). These data indicate that HPV-16 E7 plus Fve co-immunization is more efficacious to protect mice against tumor challenge and to eradicate established tumors. It is conceivable that enhancing HPV-16 E7-specific T cell immunity and IFN-γ production may contribute, at least in part, to the Fve augmentation of antitumor effects observed in this study.

It is well known that antigen-specific T cell immunity plays a critical role in tumor immunotherapy[515]. Investigators using other adjuvants such as heat shock protein 65[507], bacteria exotoxin[509], IL-12[505], CpG[270], or MPL mixed with QS1[516] had found

that $CD4^+$ and/or $CD8^+$ cells play major roles in protecting animals from challenge with HPV-16 E7-expressing TC-1 cells. In this study, by depletion of $CD4^+$ and $CD8^+$ T cells, we showed that both T cell subsets are essential for the inhibition of tumor growth, and $CD8^+$ T cells appear to play a more dominant role in tumor protection. These results concur with the conventional dogma that $CD8^+$ T cells are pivotal and highly specialized for cytolytic function and thus have been the main focus in cancer immunotherapy, whereas $CD4^+$ T cells confer helper functions in antitumor effect by providing activation signals to $CD8^+$ T cells[517,518] and contributing to the survival maintenance of $CD8^+$ T memory cells[519-523]. Recent studies, however, found that tumor-specific $CD4^+$ T cells are able to eliminate a wide variety of tumors that were resistant to CD8-mediated rejection[524,525], providing new supporting evidence for the hypothesis that $CD4^+$ T cells may play a broader role in antitumor responses. Moreover, the HPV-16 E7 plus Fve immunization-protected tumor-free mice recalled higher levels of IFN-γ production at 167 days after tumor inoculation (Fig. 4.9), indicating that Fve can enhance HPV-16 E7-specific memory immunity to protect mice against tumor growth. These findings are important because the capacity to elicit an effective long-term memory immune response is essential to the success of a vaccination strategy[526-528].

To further address the importance of T cells in mediating the antitumor effects seen in this study, total T cells from HPV-16 E7 plus Fve immunized mice were adoptively transferred into tumor-bearing recipient mice. Results indicated that T cells from HPV-16 E7 plus Fve immunized mice were more efficacious in

suppressing tumor growth as compared to those from HPV-16 E7 immunized mice (Fig. 4.14B). This enhanced efficacy correlated with the increased number of HPV-16 E7-specific effector T cells induced by co-immunization. However, other possibilities such as increased HPV-16 E7-specific T cell avidity cannot be excluded as antigen-specific $CD8^+$ T cells with high avidity are known to produce stronger antitumor effects in vaccinated mice than low-avidity $CD8^+$ T cells[529,530]. In recent years, adoptive transfer of antigen-specific T cells into patients has emerged as a promising new approach to cancer treatments[198,531,532]. T cell transfer data from this study suggest that Fve protein could be a good immunotherapeutic vaccine adjuvant to enhance the antitumor immunity mediated by tumor antigen-specific T cells, thereby providing a promising new strategy to improve the efficacy of the adoptive cell therapy approach.

Besides, HPV-16 E7 plus Fve co-immunization could significantly up-regulate the HPV-16 E7-specific antibodies especially IgG2c in C57BL/6 (Fig. 4.3B) and IgG2a in BALB/cJ mice (Fig. 4.4B), which was consistent with previous finding that Fve can increase the Th1 skewed humoral OVA-specific immune response[431]. Previous reported studies using NY-ESO-1 tumor antigen as model antigen have shown that antigen-specific IgG2a antibodies contribute to DC maturation and cross-priming of $CD8^+$ T cells, probably through a mechanism mediated by antigen-antibody immune complexes[533,534]. Although there is no direct evidence showing that antibody-mediated responses play an important role in controlling HPV-associated malignancies, the possible role of higher production of HPV-16 E7-specific

antibody in improving the antitumor activity cannot be ruled out and deserves more studies.

IFN-γ has been shown to inhibit tumor growth *in vivo* by up-regulation of MHC class I, inducing inflammation at tumor site as well as eliciting an angiostatic effect[48,73,476,535-537]. This study also demonstrated that IFN-γ was critical for generating potent antitumor effects against TC-1 tumor challenge. The data showed that the tumor protection effect of HPV-16 E7 plus Fve vaccine was significantly attenuated in IFN-γ-depleted mice (Fig. 4.13B&C). These results are consistent with previous studies demonstrating the important role of IFN-γ in the antitumor effect[48,63,536,538]. Interestingly, It was found that the mean tumor size of HPV-16 E7 plus Fve immunized mice with IFN-γ-depletion was smaller than that of PBS immunized mice (Fig. 4.13C), suggesting that additional IFN-γ independent mechanisms may also contribute towards the suppression of tumor growth observed.

It is worth noting that Fve protein alone conferred some antitumor effects as compared to those treated with PBS (Fig. 4.8C&D). This interesting observation could be explained by the fact that Fve protein mitogenically expands T cell pool and induces high levels production of IFN-γ (Fig. 3.9), creating a positive and conducive microenvironment to confer partial antitumor effects. This may also represent an additional beneficial effect of using Fve as an adjuvant for antitumor immunotherapeutic vaccines.

In summary, this study has demonstrated that an immunomodulatory protein Fve exhibited effective adjuvant effects in enhancing robust and long-lasting adaptive antigen-specific immune responses that conferred strong prophylactic and therapeutic antitumor effects. Notably, it appears that by targeting at DCs, Fve could effectively enhance antigen-specific $CD8^+$ T cell immune responses. Prophylactic vaccines aiming to induce neutralizing antibodies have been successfully translated into clinical applications for HPV associated malignancies [539,540]. However, the future challenge is to combine prophylactic approach together with a therapeutic immunization, for example, combining E7 to HPV capsid protein L1 or L2 to develop chimeric vaccines[541-543]. Overall, Fve could potentially be an effective adjuvant for such HPV chimeric vaccine. It could also be exploited to develop other anti-cancer and anti-viral vaccines.

Chapter 5

Conclusions and future perspectives

5.1 Conclusions

A group of fungal proteins defined by amino acid sequence similarity and their actions on immunological responses are classified into a distinct family, fungal immunomodulatory protein (Fip). The molecular masses of Fips were estimated to be approx. 15 kDa by SDS-PAGE and their major biological activities resembled the mitogenic activity of lectins towards mouse spleen cells and hPBMCs. In the present study, Fve, a fungal immunomodulatory protein isolated from edible mushroom *Flammulina velutipes*, was shown to induce adhesion/aggregation, activation and proliferation of mouse lymphocytes. This protein stimulated partial activation of both purified $CD4^+$ and $CD8^+$ T cells by up-regulating the expression of CD69, OX-40 and 4-1BB in the absence of accessory cells. In the presence of accessory cells, the $CD4^+$ T cells and $CD8^+$ T cells were fully activated to proliferate and secrete high levels of IL-2, IFN-γ, and IL-6 accompanied by further up-regulation of CD69, OX-40 and 4-1BB. Transwell studies showed that accessory cell-T cell direct interaction was important to the full activation of T cells. Production of IFN-γ and IL-2 but not IL-4 by Fve-stimulated T cells suggests that a type I development and differentiation process of T cells occurred after Fve

stimulation. Moreover, ^{3}H-thymidine cell proliferation assay showed that B cells and dendritic cells both can act as accessory cells for Fve-stimulated T cell activity. Besides, Fve could stimulate NK aggregation and produce high level of IL-6 but not comcomitant elevated production of IFN-γ. It appears that Fve-induced NK cell responses could be unique and the implications of such responses to DC phenotypic maturation and T cell activation deserve further studies.

In vitro co-culture experiments showed that Fve failed to induce BM-DC phenotypic maturation. In contrast, *in vivo* studies in wild type mice revealed intraveneously injected Fve could drive splenic DCs to full maturation as shown by the up-regulation of MHC class II molecules and CD86 expression on DCs. Functional assays using DO11.10 and OT-II mice showed that such Fve-stimulated splenic DCs could polarize T cells to antigen-specific Th1 cells. Furthermore, splenic DCs isolated from OVA plus Fve proteins co-injected C57BL/6 mice co-cultured with T cells from OT-1, showed that Fve enhanced the production of IL-2 and IFN-γ by antigen-specific $CD8^{+}$ T cells. The differential *in vitro* and *in vivo* effects of Fve on DC maturation imply that the Fve-induced DC maturation *in vivo* may involve accessory help by other cell types. Indeed, it was found that Fve-activated T cells could help to induce phenotypic maturation of DC in cell contact-dependent manner.

Taken together, my data demonstrated that Fve can drive Th1-skewed polarization and enhance $CD8^{+}$ T cells activity in antigen-specific manner. In view of this, it

was hypothesized that Fve could act as a vaccine adjuvant which can enhance the immunogenicity of the co-administered antigens. To verify this hypothesis, the proof-of-concept experiments were carried out with HPV type 16 E7 protein as a model antigen in tumor animal models induced by the cervical cancer-related E7-expressing TC-1 tumor cells.

Mice co-administered with Fve plus E7 proteins showed enhanced E7-specific antibody production as well as E7-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells as compared to that in mice immunized with E7 alone. Tumor protection assays showed that 60 percent of mice co-immunized with E7 plus Fve remained tumor free for up to 167 days after tumor cells challenge compared to only 20 percent of the E7-immunized group. Tumor therapeutic assays showed that E7 plus Fve treatment significantly prolonged the survival of tumor bearing mice as compared to those treated by E7 alone. *In vivo* cell depletion assay and adoptive transfer of T cells illustrated that the $CD4^+$, $CD8^+$ T cells and IFN-γ play critical roles in conferring the antitumor effects.

In summary, this study has demonstrated that fungal immunomodulatory protein-Fve, a homodimer with immunoglobulin superfamily-like fold, has strong mitogenic stimulatory effects on predominantly $CD4^+$ and $CD8^+$ T cells. Moreover, activated T cells helped DC maturation. In addition, Fve could drive Th1-skewed T cell polarization and enhance $CD8^+$ T cells activity in an antigen-specific manner. Tumor model studies demonstrated that Fve enhanced robust and long-lasting

adaptive antigen-specific immune responses that mediated the strong prophylactic and therapeutic antitumor effects.

5.2 Future perspectives

Based on the current study, several aspects require further investigation:

5.2.1 Delineation of the proposed carbohydrate binding site of the Fve protein

Fve is structurally predicted to be a saccharide-binding protein. The 3-D structural analysis indicates that Fve has two potential carbohydrat-binding pockets[430]. The first patch, consisting of residues Tyr11, Arg50, Asp57, Phe94, Glu104, Glu105, Tyr106 and Glu110 is found on the same side of the dimer (Appendix 5A). The second patch, continuous with the first, consists of Tyr11, Trp24, Arg26, Asp87, Glu110 and Trp111. This patch lies on opposite sides of the dimmer (Appendix 5B). In addition, microscopic visualization in this study found that purified $CD4^+$ T cells, $CD8^+$ T cells, DCs, and NK cells can aggregate after Fve stimulation indicating Fve is probably a lectin with specificity for cell surface carbohydrates binding. Therefore, it is worthwhile to delineate the carbohydrate binding site on Fve by further studies. PCR-based site-directed mutagenesis will be used to generate a panel of Fve point mutants. Functional screening of these point mutants will be performed by the assays as follows: glycan binding assay, cytokine production profiling by ELISA, and cell proliferation assay by [^{3}H]-thymidine uptake.

5.2.2 Identification of cellular receptor (s) involved in Fve interaction

The signal transduction pathways involved in Fve-induced cellular activation still need to be elucidated at the molecular levels. It is necessary to identify the cellular receptor (s) involved in Fve interaction. The 3D- structure of Fve protein showed that Fve is a homodimer, each monomer consists of an N-terminal α-helix and β-strand followed by a fibronectin type III-like domain with immunoglobulin like-fold structure (Appendix 4). The swapping of N-terminal α-helix links with hydrogen bonding and this α-helical pair is essential for protein dimerization and stabilization[430]. The presence of FNIII-like domain (immunoglobulin superfamily-like domain, also the first FNIII domain found in products of fungal origin) indicates that Fve shares structural similarity with human fibronectin domain, which is known to be involved in integrin binding. Therefore, it is speculated that Fve may interact with integrin-like proteins or with the proteins of the immunglobulin superfamily, which are involved in cell adhesion, costimulation, and activation[544-546]. The preliminary data from our lab showed that Fve-stimulated proliferation of hPBMC was greatly inhibited in the presence of blocking antibodies for CD2 or LFA-1. To examine whether there is a direct interaction between Fve to CD2 and LFA-1, *in vitro* pull-down assay can be performed. Moreover, co-immunoprecipitation will be performed to identify the potential receptors for Fve in the target cells such as T cells and DCs.

5.2.3 Functional characterization of Fve-induced OX-40 and 4-1BB expressing T cells.

OX-40 and 4-1BB were inducible in the Fve-activated murine T cells. As discussed in chapter 3, the signaling of OX-40 and 4-1BB controls T cell immune response by: (a) enhancing the expression of anti-apoptotic molecules; (b) inhibiting the development and activation of T_{Reg} cells. Further studies are required to confirm whether Fve-induced OX-40 and 4-1BB exhibit such regulatory functions to enhance T cell immune response.

Moreover, in the context of cancer immunotherapy as described in chapter 4, the expansion of antigen-specific effector T cell and inhibition of Treg cells are important antitumor mechanisms. Therefore it is worthwhile to investigate whether mice co-immunized with tumor antigens such as E7 antigen with Fve protein could up-regulate the expression of OX-40 and 4-1BB on antigen-specific T cells and increase T cell functions.

5.2.4 Clarification of the underlying mechanisms of Fve-activated T cell-dependent DC maturation

DCs are professional antigen-presenting cells with the unique ability to take up and process antigens in the peripheral blood and tissue. Immature DCs are particularly efficiant in ingestion and processing antigen to activate T cells. DCs must undergo maturation to to fully activated DCs, which express high levels of cell surface

MHC class I/II and costimulatory molecules. Data from this study revealed that Fve can induce DCs phenotypic and functional maturation under *in vivo* but not *in vitro* settings, but the underlying mechanism is unclear. *In vitro* co-culture studies showed that Fve-induced DC phenotypic maturation is activated T cell-dependent indicating that Fve-activated T cells may constitute a novel form of immune signal, functionally linked to DC maturation. Therefore, further studies using $RAG^{-/-}$, $CD4^{-/-}$ or $CD8^{-/-}$ mice can be carried out to confirm the helper role of T cells in DC maturation *in vivo*. Besides, trans-well study showed that T cell-DC direct interaction is a prerequisite for DC maturation. Identification of the surface molecules involved in such interaction is necessary in order to further dissect the signaling pathway for DC maturation. Furthermore, soluble mediator(s) responsible for T cell-dependent DC maturation cannot be ruled out. To this end, blocking experiments with mAbs against TNF-α, IFN-γ, IL-6, OX-40, 4-1BB or CD40 should be performed.

5.2.5 Further studies on the function of Fve-induced splenic $CD8^+$ DC subsets.

In vivo data from the current study showed that Fve preferentially drive maturation of splenic $CD8^+$ DCs. It has been well documented the $CD8^+$ DC are much more efficient in activating MHC class I - restricted $CD8^+$ T cells by cross-priming[481,482] whereas the $CD8^-$ subsets preferentially stimulate MHC class II-restricted $CD4^+$ T cells. Further functional studies should be performed using TAP-1 knockout mice to determine whether the Fve-induced $CD8^+$ DC subsets are responsible for the

cross-priming of antigen-specific $CD8^+$ T cells.

5.2.6 Exploration of the potential effects of Fve on NK cells

NK cells share a common biopotential progenitor with T cells and share a common killing mechanism with cytotoxic T cells. Previous *in vivo* studies from our laboratory revealed that with Fve treatment increased NK cells in the spleen from 0.85 percent to about 4 percent (unpublished data), suggesting that Fve may have direct or indirect effects on NK cells *in vivo* leading to an expansion of this cell type. NK cells are effector cells of innate resistance that are able to lyse tumor and virus-infected cells spontaneously in the absence of Ag-specific recognition and clonal expansion[547]. Furthermore, the cytolytic activity of NK cells can be augmented by IL-2 and IFN-γ. In view of this, it is possible that Fve-induced production of IL-2 and IFN-γ could enhance the cytolytic functions of NK cells *in vivo*, this warrant more research work to test this hypothesis. Taken together, the data suggest that it will be worthwhile to explore the use of Fve to expand the NK cells population and to increase NK cell functions to treat cancer and viral infections.

5.2.7 Characterization of the possible interaction between Fve and other immune cells.

B cells and macrophages are important cells in both innate and adaptive immunities[548-550]. B cells can act as APC for T cell activation or tolerance.

Macrophages are known to be involved in angiogenesis by producing a series of cytokines/growth factors[551,552]. Moreover, macrophages facilitate B cell activation *in vivo* by collecting and displaying native antigen[549,553]. Thus, the immunomodulatory effects of Fve on these cells should not be overlooked and therefore deserve further exploration.

5.2.8 Optimization strategies to enhance the adjuvant effects of Fve for cancer immunotherapy.

The proof of study described in chapter 4 demonstrated the potential use of Fve as an adjuvant in cancer immunotherapy. The results showed that Fve was capable of increasing the immunogenicity of a tumor antigen, which is a promising strategy to induce specific antitumor response. However, that the use of Fve significantly inhibited tumor cell growth but did not completely eradicate tumor from host. It could be due to limited efficacy of the vaccines against tumor cells. To optimize the effects of Fve may increase the efficacy of the vaccine. To increase the numbers of immunizations and T regulatory cells depletion should be explored

5.2.9 Safety of Fve for human use.

Several safety concerns may be raised when Fve is prepared for clinical use. These include the possibility that Fve protein might (1) enhance the immunogenicity of self antigens, thereby triggering the development of organ specific or systemic autoimmune disease, or (2) increase susceptibility of the host to pathogenic agents

that cause toxic shock. To clarify the magnitude of these safety concerns, *in vivo* experiments should be carried out in which mice are repeatedly injected with different doses of Fve protein. The histochemical staining of lymphoid tissue and organs should be done. And morbidities and mortality of the Fve-treated animals should be evaluated.

Lastly, I conclude this study by citing the following quote:

"With science having largely demystified the 'witchcraft' of immune response, immunologists are turning to the next challenge: putting their new knowledge to clinical use in taming pathological immune responses. Successes are still mostly on the horizon."

— Annual Review of Immunology, Vol. 23: 1-2

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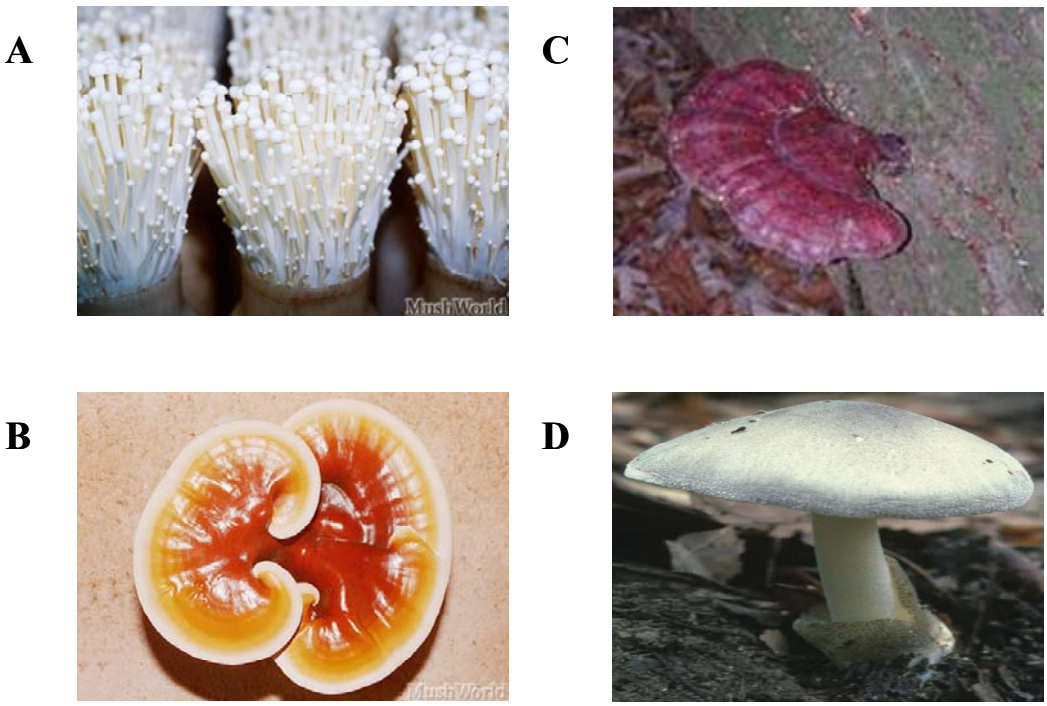
APPENDICES

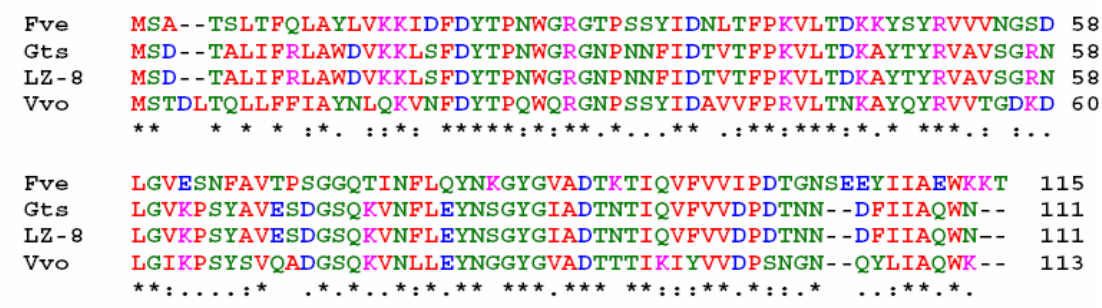


Appendix 1: Fungal immunomodulatory protein (FIP) family.
A family of fungal immunomodulatory proteins (Fips) which include Fip-Fve (A), Fip-LZ-8 (B), Fip-Gts (C), and Fip-Vvo (D) purified from *Flammulina velutipes, Ganoderma lucidium*, *Ganoderma tsugae*, and *Volvariella volvacea*, respectively.

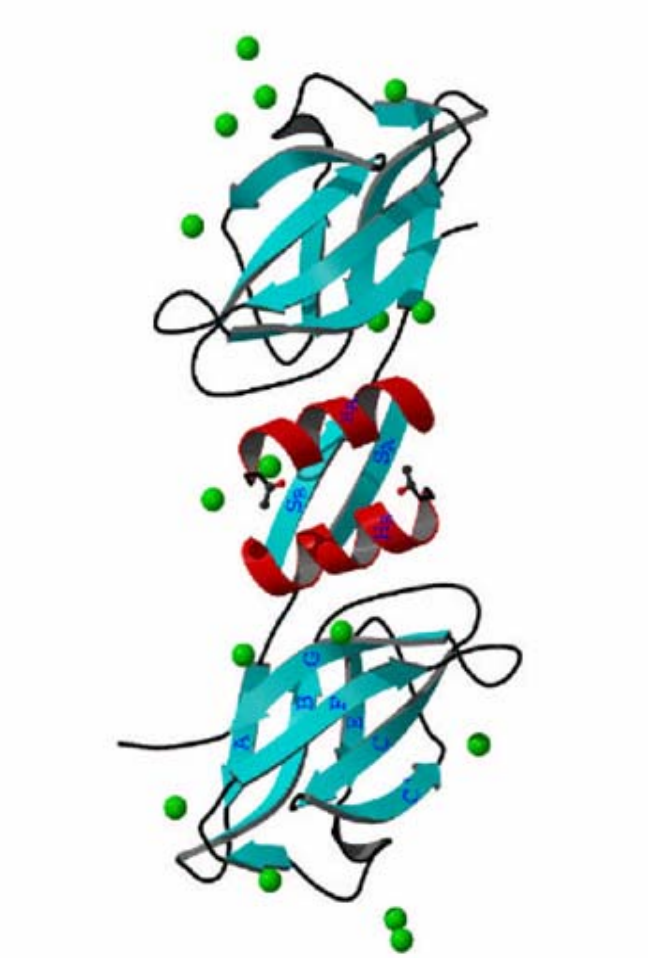
A

B

Appendix 2: Wild type and cultivated form of *Flammulina velutipes*.
Wild type *flammulina velutipes* are often found in clusters on hardwood stumps in forest, frequently in the winter season (A). Cultivated form of *Flammulina velutipes* (also known as golden needle mushroom) shows whitish-yellow color and non-velvety stem (B).

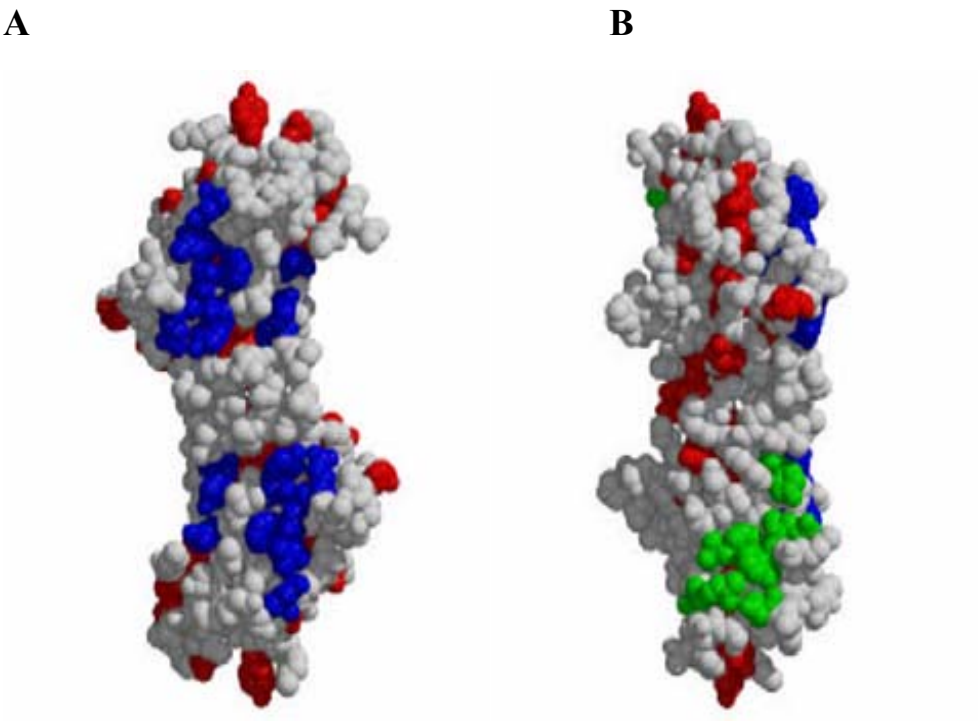


Appendix 3: Alignment of amino acid sequences of Fip-Fve, Fip-Gts, Fip-LZ-8, and Fip-Vvo, immunomodulatory proteins.
Identical amino acids are represented with [*], while partial identical amino acids are indicated with [:] and [.] respectively.



Appendix 4: The overall three dimensional structure of Fve dimer solved by SAD of a NaBr-soaked Fve crystal.

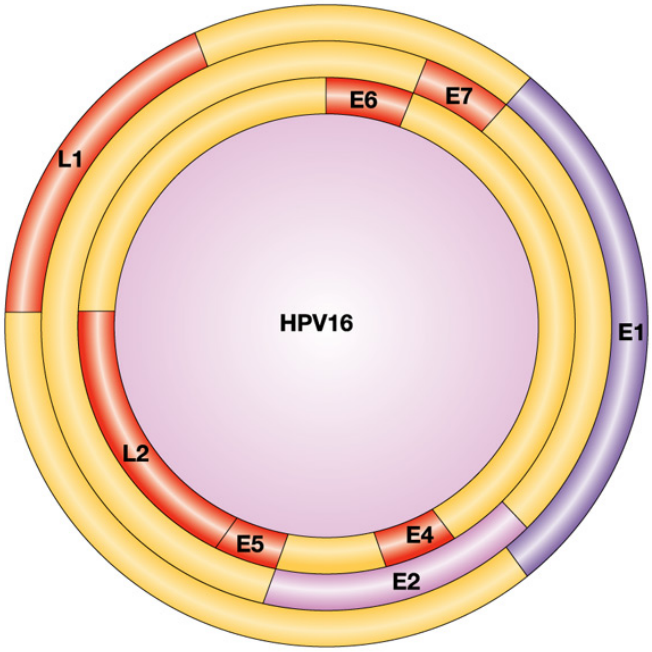
Each monomer consists of an FNIII-like domain with an N-terminal α-helix and β-strand. The α-helices (HA and HB in red color) interact via hydrophobic interactions while the β-strands (SA and SB in blue color) form a β-sheet. The FNIII domain consists of a sandwich of two sheets, I and II, formed by β-strands E-B-A and G-F-C-C', respectively. The Br atoms are colored green. The Fve ID in protein data bank is 1 osy.



Appendix 5: Potential carbohydrate-binding sites of Fve.
All residues with strong propensity to be in the carbohydrate-binding site of proteins (see the text) are shown in red. Among these, there are two pairs of contiguous surfaces, which could potentially bind sugars. (a) The first pair (colored blue), consisting of Tyr11, Arg50, Asp57, Phe94, Glu104, Glu105, Tyr106 and Glu110 are found on the same side of the dimmer (A). The second patch (colored green), which is continuous with the first (and hence shares common residues), consists of Tyr11, Trp24, Arg26, Asp87, Glu110 and Trp111. This pocket lies on opposite sides of the dimmer (B).

Mitogens	ConA	PHA	PWM	Fve
structure	Dimer β sheet no α helix	Tetramer β sheet no α helix	Dimer β sheet no α helix	Dimer α helix, β sheet
origin from	jack bean	French bean	Poke weed	golden needle mushroom
glycoprotein	No (25.5 kD)	Yes (120 kD)	Yes (22.5-38 kD)	No (12.5 kD)
binding ligand	carbohydrate	carbohydrate	carbohydrate	unknown
clinical applicactions	toxic	1.tumor therapy 2.infectious disease	tumor therapy	Unknown
effect cells	T cells	T cells	mast cells, monocytes	APCs ? T, NK cells
hemagglutinating effect	yes	yes (PHA-L4, no)	yes	yes

Appendix 6: Structure and biological properties of mitogens.



Appendix 7: Schematic representation of the human papillomavirus 16 (HPV-16) genome showing the arrangement of the major non-structural and capsid genes. The three circles correspond to the three reading frames in which the sense strand can be translated. There are no known gene products produced from the antisense strand. Viral protein early 4 (E4) is encoded by a messenger RNA transcript that includes the initial amino acids of the E1 gene. The region between late 1 (L1) and E6 is an important transcriptional regulatory region — the mRNAs encoding most nonstructural (E6, E7, E1, E2, E4 and E5) and capsid (L1 and L2) genes originate in this region. Most papillomavirus genomes resemble HPV16 in general organization.

Appendix 8: Publications

Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Coadministration of the fungal immunomodulatory protein FIP-Fve and a tumour-associated antigen enhanced antitumour immunity

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Summary

Fve is a fungal protein isolated from the golden needle mushroom *Flammulina velutipes* and has previously been reported to trigger immunological responses in both mouse and human lymphocytes. In this study, we evaluated the potential application of Fve as an adjuvant for tumour immunotherapy and examined the underlying mechanism(s). When the human papillomavirus (HPV)-16 E7 oncoprotein was used as a model antigen, mice coimmunized with HPV-16 E7 and Fve showed enhanced production of HPV-16 E7-specific antibodies as well as expansion of HPV-16 E7-specific interferon (IFN)-γ-producing $CD4^+$ and $CD8^+$ T cells as compared with mice immunized with HPV-16 E7 alone. Tumour protection assays showed that 60% of mice coimmunized with HPV-16 E7 plus Fve, as compared with 20% of those immunized only with HPV-16 E7, remained tumour-free for up to 167 days after challenge with the tumour cells. Tumour therapeutic assays showed that HPV-16 E7 plus Fve treatment significantly prolonged the survival of tumour-bearing mice as compared with those treated only with HPV-16 E7. *In vivo* cell depletion and adoptive T-cell transfer assays showed that $CD4^+$ and $CD8^+$ T cells and IFN-γ played critical roles in conferring the antitumour effects. Interestingly, Fve could stimulate the maturation of splenic dendritic cells *in vivo* and induce antigen-specific $CD8^+$ T-cell immune responses. In summary, Fve has potent adjuvant properties that enhance T helper type 1 antigen-specific humoral and cellular immune responses which confer strong antitumour effects. The use of Fve as an adjuvant could be an attractive alternative to the current vaccination strategy for cancer immunotherapy.

Keywords: adjuvant; dendritic cells; fungal immunomodulatory protein Fve; human papillomavirus-16 E7; immunotherapy

Introduction

Cancer immunotherapy is an attractive alternative for the treatment of patients with cancer. When immunotherapy is used in the absence of radiotherapy or chemotherapy, there are fewer side effects than found with classical antitumour chemotherapeutics. The identification and cloning of genes encoding tumour-associated antigens recognized by T cells have renewed hopes that it may be possible to develop cures for some cancers.[1] Although several treatments using tumour-associated antigens have been approved for some types of cancer, their efficacies have been variable and generally insufficient. One important factor contributing to insufficient and variable responses to immunotherapy is the poor immunogenicity of most tumour antigens, which results in insufficient activation of tumour antigen-specific $CD8^+$ T cells.

Cervical cancer is the second highest cause of cancer deaths in women and kills approximately 274 000 women worldwide each year.[2,3] Epidemiological and laboratory studies strongly support a crucial role for persistent human papillomavirus (HPV) infection and transcription in cervical carcinogenesis.[4,5] Among over 100 HPV genotypes, HPV type 16 is the most common and is responsible for more than 50% of all cervical cancers.[6] HPV-16 E7, one of its oncoproteins, is essential for the induction and

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maintenance of cellular transformation.[5] Thus, HPV-16 E7 has been a major target of many prophylactic HPV vaccines for the prevention of HPV infections as well as many therapeutic HPV vaccines for the control of existing HPV infections and HPV-associated lesions, such as squamous intraepithelial lesions and cervical cancer. However, the antigen-specific immune responses and antitumour effects generated by HPV-16 E7 alone are weak and insufficient to control tumour growth. Several strategies have been developed to increase the potency of the HPV-16 E7 vaccine; for example, various immune modulators, such as cytokines,[7,8] heat shock proteins,[9,10] non-toxic bacterial toxins,[11] and CpG[12] have been incorporated as adjuvants to enhance HPV-16 E7-specific immunity. These have proved to be effective in animal models, but their potency in humans has yet to be assessed and active research to develop novel adjuvant molecules is still ongoing.

Fve, or fungal immunomodulatory protein (FIP)-fve, is a major fruiting body protein isolated from the edible golden needle mushroom, *Flammulina velutipes*.[13–15] It belongs to an FIP family, sharing sequence similarity with LinZhi-8 from *Ganoderma lucidum*,[16] Fip-gts from *Ganoderma tsugae*[17] and Fip-vvo from *Volvariella volvacea*.[18] The Fve protein is an acetylated protein consisting of 114 amino acid residues with an estimated molecular weight of 12.7 kDa.[13] Fve has been shown to have the ability to trigger the proliferation of mouse splenocytes and human peripheral mononuclear cells and to enhance the production of interleukin (IL)-2, IFN-γ and tumour necrosis factor (TNF)-α.[13,19] Moreover, it has been suggested that coadministration of Fve with antigen may drive strong T helper type 1 (Th1)-skewed immune responses.[20]

In this study we explored the potential role of Fve as an adjuvant for cancer immunotherapy. Using HPV type 16 E7 as a model tumour antigen and the TC-1 cell-induced tumour model, we carried out a series of proof-of-concept studies to show the effectiveness of Fve as an adjuvant to enhance both humoral and cellular responses to antitumour therapy *in vivo*.

Materials and methods

Mice and the tumour cell line

Six- to eight-week-old female C57BL/6 mice were purchased from the Laboratory Animal Center (Sembawang, Singapore). Breeding pairs of OT-I and OT-II mice with transgenic Vα2Vβ5 T-cell receptors (TCRs) specific for the ovalbumin $(OVA)_{257–264}$ epitope in the context of H-2K^b and the $OVA_{323–339}$ epitope in the context of I-A^b were originally acquired from The Jackson Laboratory (Bar Harbor, ME) and were maintained and bred in the Animal Holding Unit of the National University of Singapore. All animal procedures were performed according to approved protocols and in accordance with the Institutional Animal Care and Use Committee of The National University of Singapore. The maintenance of TC-1 cells (kindly provided by Dr T. C. Wu, Johns Hopkins University, Baltimore, MD) has been described previously.[21] On the day of tumour challenge, TC-1 cells were harvested by trypsinization and washed three times with phosphate-buffered saline (PBS), and the designated numbers of cells for tumour inoculation were resuspended in 200 μl of PBS.

Reagents

All antibodies were purchased from BD PharMingen (San Diego, CA) unless otherwise stated. For the detection of antigen-specific immunoglobulin G1 (IgG1) in mouse sera, the rat anti-mouse Igκ light chain (clone 187.1) and biotin-conjugated rat anti-mouse IgG1 monoclonal antibody (mAb) (clone LO-MG1-2; Serotec Ltd., Oxford, UK) were used. The purified mouse IgG1 (clone 107.3) was used as the standard. For the cytokine enzyme-linked immunosorbent assay (ELISA), mAbs of rat anti-mouse IFN-γ (clone R4-6A2), biotin-conjugated rat anti-mouse IFN-γ (clone XMG1.2), rat anti-mouse IL-2 (clone JES6-IA20), biotin-conjugated rat anti-mouse IL-2 (JES6-5H4), rat anti-mouse IL-4 (clone, BVD6-24G2) and biotin-conjugated rat anti-mouse IL-4 (clone BVD4-1D11) were used. Rat anti-mouse TNF-α (clone AF-410-NA) and biotinylated rat anti-mouse TNF-α (clone BAF410) were purchased from R&D Systems (Minneapolis, MN). Rat anti-mouse CD3ε (clone 145-2C11) and CD28 (clone 37.51) mAbs and recombinant mouse IL-2 were used for stimulation of T cells *in vitro*. Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8β (clone 53-5.8), biotin-conjugated rat anti-mouse CD4 (clone GK1.5), streptavidin-PerCP and allophycocyanin (APC)-conjugated rat-anti-mouse IFN-γ (clone XMG-2.1) were used for intracellular cytokine staining. Rat anti-mouse CD16/CD32 (clone D34-485), biotin-conjugated rat anti-mouse CD8α (clone 53-6.7), APC-conjugated rat anti-mouse CD4 (clone, RM4-5), PE-conjugated rat anti-mouse CD11c (clone HL3), FITC-conjugated anti-I-A^b (clone AF6-120.1), FITC-conjugated anti-CD86 (clone GL1) and streptavidin-conjugated PerCP were used for surface marker staining for splenic dendritic cells (DCs).

Monoclonal anti-CD4 (clone, GK1.5), anti-CD8 (clone, 2.43) and anti-IFN-γ (clone, R4-6A2) antibodies for the *in vivo* depletion experiment were purified from the supernatants of hybridoma cells (American Type Culture Collection, Bethesa, MD) by passage through protein G columns (Amersham Biosciences AB, Uppsala, Sweden).

Production of Fve and recombinant HPV-16 E7 proteins

The purification of Fve protein from crude extracts of *F. velutipes* (golden needle mushroom) has been described

previously.[13,15] The purified Fve was treated with the polymyxin B agarose and the endotoxin level of the Fve protein was determined using the LAL assay kit according to the manufacturer's instructions (BioWhittaker, Walkersville, MD). There was no detectable level of endotoxin in the purified Fve protein.

The cDNA of HPV-16 E7 (a gift kindly provided by Dr S. W. Chan, Institute of Molecular and Cellular Biology, ASTAR, Singapore) was subcloned into the pGEX-4T1 expression vector (Amersham Biosciences AB). The open reading frame of HPV-16 E7 was amplified by polymerase chain reaction using a set of primers: E7-F 5′-TTGTTGGATCCCATGGAGATACACCTACATTG-3′ and E7-R 5′-TTACTGAATTCTTATGGTTTCTGAGAACAGATG-3′. The amplified DNA was digested with *Bam*HI and *Eco*RI, and the resulting fragment was then cloned into the *Bam*HI and *Eco*RI sites of the pGEX-4T1 vector. The pGEX-HPV-16 E7 recombinant plasmid was transformed into *Escherichia coli* TG-1 for protein expression. The HPV-16 E7 protein was purified from GST-HPV-16 E7 fusion proteins after thrombin treatment.

Preparation of DCs

Bone marrow-derived dendritic cells (BM-DCs) were generated with granulocyte–macrophage colony-stimulating factor (GM-CSF) according to a method previously described.[22] In brief, bone marrow cells were harvested from femurs and tibias of normal C57BL/6 mice and washed with PBS. The cells (4×10^6 to 6×10^6) were resuspended in complete RPMI-1640 medium containing recombinant mouse GM-CSF (20 ng/ml; BD PharMingen) and cultured in 100-mm-diameter Petri dishes. On day 3 of culture, half of the medium was replaced with fresh medium supplemented with GM-CSF (10 ng/ml). On day 5 of culture, immature DCs were harvested for purification.

The splenic DCs were purified as previously described[23] with some modifications. Spleens (from eight mice) were minced with scissors and digested in 10 ml of Hanks' balanced salt solution (HBSS) with Ca^{2+} and Mg^{2+} (Sigma-Aldrich, St Louis, MO) containing collagenase D (400 U/ml; Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at 37°. Next, 1 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA) was added at room temperature for 5 min to disrupt cell adhesion. The digested tissue samples were filtered through a 40-μm nylon mesh to remove undigested fibrous material. All subsequent steps were performed at room temperature using HBSS without Ca^{2+} and Mg^{2+} (Sigma-Aldrich). Cells in the filtrates were recovered by centrifugation, resuspended in 1·068 g/cm^3 OptiPrep® density gradient medium (Sigma-Aldrich) and centrifuged at 600 ***g*** for 15 min. The low-density fraction was collected (2–4% of the total) and resuspended in magnetic antibody cell sorting (MACS) running buffer [PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA] for subsequent purification.

Purification of DCs and T cells

CD11c (N418), CD90.2 (Thy1.2), CD4 (L3T4) and CD8 (Ly-2) microbeads were used for the isolation of the splenic DCs, BM-DCs from cell cultures, Thy1.2$^+$ T cells, and CD4$^+$ and CD8$^+$ T cells from spleens, respectively, according to the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Briefly, cells were labelled with 10 μl of microbeads per 1×10^7 cells at 4° for 20 min and washed twice. The labelled cells were subsequently separated using an autoMACS™ separator (Miltenyi Biotec GmbH). The purities of the various cell populations were determined by flow cytometry analysis. The purity of DCs and Thy1.2$^+$ T cells was above 97% and 98%, respectively. The purity of OT-II CD4$^+$ T cells and OT-I CD8$^+$ T cells was above 95% and 85%, respectively (data not shown).

In vitro *cell proliferation and cytokine production assays*

Thy1.2$^+$ T cells were purified from spleen cells of C57BL/6 mice by magnetic cell sorting. The purity of Thy1.2$^+$ T cells was above 95%. The purified Thy1.2$^+$ T cells were seeded in triplicate (1×10^5 per well) into a 96-well U-bottom plate in the presence or absence of 2×10^4 BM-DCs treated with mitomycin C (Roche Diagnostics GmbH, Mannherim, Germany). BM-DCs alone were used as a control. All cells were treated with or without Fve (20 μg/ml) and pulsed with [^{3}H]-labelled thymidine for the last 18 hr of cultures. The cells were harvested and [^{3}H]thymidine incorporation was measured by liquid scintillation counting at 72 hr. Supernatants were collected and cytokine production was measured by ELISA.

ELISA for anti-HPV-16 E7 antibodies

Mice were subcutaneously immunized with PBS, 20 μg of HPV-16 E7 or 20 μg of HPV-16 E7 plus 20 μg of Fve at days 0, 14 and 28. Sera were collected weekly for antibody analysis by ELISA. For IgG1 analysis, a 96-well plate was coated with HPV-16 E7 protein (5 μg per well) and incubated at 4° overnight. The wells were then blocked with blocking buffer (PBS containing 0.05% Tween-20 and 1% BSA). Diluted sera were added and incubated at 4° overnight. The plate was then incubated with biotin-conjugated anti-mouse IgG1 for 1 hr, followed by the addition of ExtrAvidin®-alkaline phosphatase (Sigma-Aldrich) for another hour. The signal was developed by adding *p*-nitrophenyphosphate substrate (Sigma-Aldrich) and read

with a microplate reader at 405 nm (Tecan Group Ltd, Männedorf, Switzerland). It has been well established that C57BL/6 mice express the *Igh1-b* gene, which encodes the IgG2c isotype rather than IgG2a.[24] Thus, the levels of antigen-specific IgG2c in the mouse sera were detected using an ELISA kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocol.

Intracellular IFN-γ production in T cells of Fve-immunized mice

To investigate the HPV-16 E7-specific cellular immune response, mice were subcutaneously immunized with PBS, 20 μg of HPV-16 E7, or 20 μg of HPV-16 E7 plus 20 μg of Fve at days 0 and 14 and splenocytes were collected at day 28. To determine the cytokine production profiles of splenocytes in primary cultures, 5×10^5 splenocytes per well were stimulated with HPV-16 E7 protein for 72 hr and culture supernatants were collected for cytokine assays by ELISA. To determine the cytokine production profiles of T subsets by intracellular staining, splenocytes were first cultured with 10 μg/ml HPV-16 E7 for 9 days, followed by secondary stimulation with anti-CD3 and anti-CD28 antibodies prior to intracellular staining with cytokine-specific antibodies. Recombinant mouse IL-2 was added to the primary splenocyte cultures at days 3 and 6 to a final concentration of 10 U/ml to maintain the cells up to 9 days; these cultured cells from mice within the same group were then pooled to set up secondary cultures. Briefly, 2×10^5 cells were restimulated with 5 μg/ml anti-mouse CD3 and 2 μg/ml anti-mouse CD28 mAbs for 12 hr. Monensin (Sigma-Aldrich) was added 6 hr before harvesting. Cells were surface-stained with anti-CD8β and anti-CD4 mAbs, fixed with paraformaldehyde, permeabilized with saponin (Sigma-Aldrich) and then stained intracellularly with anti-mouse IFN-γ mAb. Flow cytometric analysis was performed using a FACSCalibur with CELL QUEST software (BD Biosciences, San Jose, CA).

In vivo *tumour protection and depletion assays*

Mice were subcutaneously immunized with PBS, 20 μg of HPV-16 E7, 20 μg of Fve, or 20 μg of HPV-16 E7 plus 20 μg of Fve at days 0, 14 and 28. TC-1 cells (5×10^4) were inoculated into the right flank of a mice at day 30. To deplete $CD4^+$ T cells, $CD8^+$ T cells or IFN-γ, mice were intraperitoneally injected with 800 μg of anti-CD4, 500 μg of anti-CD8 or 500 μg of anti-IFN-γ mAbs respectively at days −4, −1, 6, 13, 20, 27, 34, 41 and 48. Tumour size was measured every 2 days in two perpendicular dimensions and expressed as length × width (mm^2) and the survival was monitored. The depletion of $CD4^+$ and $CD8^+$ T cells was assessed at day 6 after the first immunization, and the depletion in the spleen was > 95% as determined by flow cytometric analysis. Scientists performing the analyses of tumour size and survival rates were blinded to the treatments.

In vivo *tumour therapeutic assay*

Mice were subcutaneously inoculated with 5×10^4 TC-1 cells in the right flank at day 0. Mice were then subcutaneously immunized with PBS, 20 μg of HPV-16 E7, 20 μg of Fve, or 20 μg of HPV-16 E7 plus 20 μg of Fve at days 3, 10 and 17. For tumour metastasis therapeutic assay, mice were intravenously injected with 2×10^4 TC-1 cells in the tail vein at day 0. Mice were then subcutaneously immunized with the same regimen at days 3, 10 and 17. The survival rates were monitored daily.

T-cell adoptive transfer

Splenocytes from immunized mice were collected and Thy 1.2^+ T cells were isolated using Thy1.2 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for cell transfer experiments. The purity of T cells was above 98% as determined by flow cytometric analysis. Freshly purified Thy1.2^+ T cells (8×10^6) were adoptively transferred into recipient mice at days −1, 3, 6 and 9. Recipient mice were inoculated subcutaneously in the right flank with 5×10^4 TC-1 cells at day 0. The tumour size was measured on alternate days from day 10 onwards.

Analysis of in vivo *activation of DCs*

Mice were intravenously injected with PBS or 20 μg of Fve. Twelve hours later, enriched splenic DCs (1×10^6 cells in 100 μl) were blocked with anti-CD16/CD32 mAb at 4° for 20 min prior to incubation with mAbs against CD4, CD8, CD11c and I-A^b and with mAbs against CD4, CD8, CD11c and CD86 at 4° for 25 min. Flow cytometric analysis was performed using a FACSCalibur with CELL QUEST software (BD Biosciences).

Analysis of DC-directed $CD4^+$ and $CD8^+$ T-cell activation

Naïve C57 BL/6 mice were intravenously injected with PBS, 100 μg of ovalbumin (OVA), 20 μg of Fve, or OVA (100 μg) plus Fve (20 μg) 24 hr prior to isolation of the $CD11c^+$ DCs. Purified $CD11c^+$ DCs were pulsed with 1 μM of $OVA_{323–339}$ peptide or 1 μM of $OVA_{257–264}$ peptide, respectively (AnaSpec, Inc., San Jose, CA), for 2 hr at 37° and then washed extensively. Naïve $CD4^+$ and $CD8^+$ cells were purified from OT-II and OT-I transgenic mice, respectively. DCs (5×10^3 cells) were incubated with 5×10^4 $CD4^+$ T cells or 5×10^4 $CD8^+$ T cells, respectively, in U-bottom 96-well plates in 200 μl of

complete RPMI-1640 medium in triplicate for 72 hr. Supernatants were collected and cytokine production was determined by ELISA.

Statistical analysis

Results for tumour sizes are presented as mean ± standard error of the mean (SEM) and were analysed by one-way analysis of variance (ANOVA). Differences in antibody and cytokine production between groups were analysed by Student's *t*-test. In tumour protection, therapeutic and adoptive transfer experiments, the tumour-free and survival analyses were carried out using the Kaplan–Meier analysis and the log-rank test. $P < 0{\cdot}05$ was considered as statistically significant.

Results

Fve stimulated mouse T-cell proliferation and IFN-γ production *in vitro*

To examine the immunostimulatory effect of Fve *in vitro*, mouse splenocytes were cultured in the presence of increasing concentrations of the Fve protein. As shown in Fig. S1(a and b), Fve induced the proliferation of splenocytes in a dose-dependent manner and significant production of IFN-γ and TNF-α. Fve predominately stimulated the proliferation of T cells in the splenocytes as cellular proliferation was drastically reduced in T-cell-depleted splenocytes (data not shown). Fve-induced T-cell proliferation and IFN-γ production were accessory-cell-dependent (Fig. 1 and Fig. S1c).

Coadministration of HPV-16 E7 plus Fve increased HPV-16 E7-specific B-cell and T-cell activities

We next investigated the effects of Fve on the induction of HPV-16 E7-specific immunity. Firstly, we measured HPV-16 E7-specific antibodies in the sera of the various groups of differentially immunized mice. As shown in Fig. 2a,b, mice immunized with HPV-16 E7 plus Fve produced significantly higher levels of IgG1 and IgG2c as compared with those immunized with HPV-16 E7 alone. HPV-16 E7-specific IgG1 and IgG2c in mice immunized with HPV-16 E7 plus Fve were 7-fold and 33-fold higher, respectively, than those of mice immunized with HPV-16 E7 alone at day 28 (Fig. 2a,b). These results indicate that Fve could enhance strong HPV-16 E7-specific humoral immune responses.

Consistently, IFN-γ production was significantly enhanced in splenocytes from mice immunized with HPV-16 E7 plus Fve as compared with those from mice immunized with HPV-16 E7 or PBS (Fig. 2c and Fig. S3). In order to elucidate the IFN-γ-producing T subsets, the HPV-16 E7 cultured primary cells were restimulated with anti-CD3 and anti-CD28 antibodies for 12 hr and cytokine production was examined by intracellular staining. Flow cytometry analysis revealed that 18·7% of IFN-γ-producing cells within the $CD4^+$ T subset were induced in the HPV-16 E7 plus Fve co-immunized mice, whereas only 4·9% and 5·7% of IFN-γ^+ cells were induced in HPV-16 E7 and PBS mice, respectively (Fig. 2d, upper panel). Similarly, 37·8% of the $CD8^+$ T cells in the HPV-16 E7 plus Fve co-immunized mice were IFN-γ^+ cells, whereas only 11·4% and 12·5% of IFN-γ^+ $CD8^+$ T cells were detected in HPV-16 E7 and PBS mice, respectively (Fig. 2d, lower panel). These results indicate that Fve could significantly increase HPV-16 E7-specific IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells.

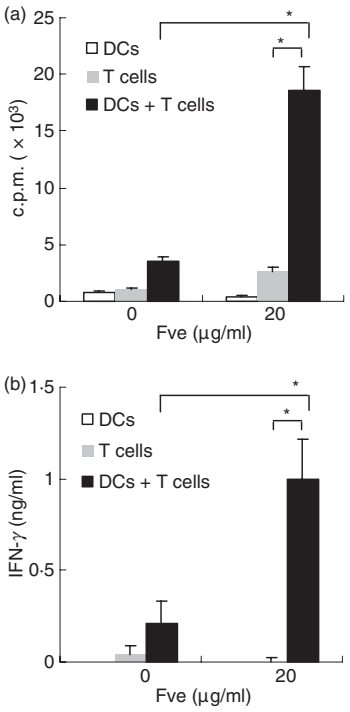

Figure 1. Fve stimulates mouse splenic T-cell activation in an accessory-cell-dependent manner. Purified $CD90^+$ T cells (1×10^5 cells/well) were co-cultured with mitomycin C-treated bone marrow-derived dendritic cells (BM-DCs) (2×10^4 cells/well) in the presence or absence of Fve (20 μg/ml) in triplicate using the 96-well plate. The DCs and $CD90^+$ T cells alone were included for comparison. The cultures were pulsed with [^{3}H]thymidine for the last 18 hr of the co-culture. The cells were harvested and thymidine incorporation was measured by liquid scintillation counting at 72 hr (a). The culture supernatants were collected at 72 hr and interferon (IFN)-γ production was measured by enzyme-linked immunosorbent assay (ELISA) (b). *$P < 0{\cdot}05$. c.p.m., counts per minute.

Coadministration of HPV-16 E7 and Fve enhanced protection of mice against tumour growth

The finding that immunization with HPV-16 E7 plus Fve led to enhanced HPV-16 E7-specific immunity prompted us to explore the antitumour potential of such an immu-

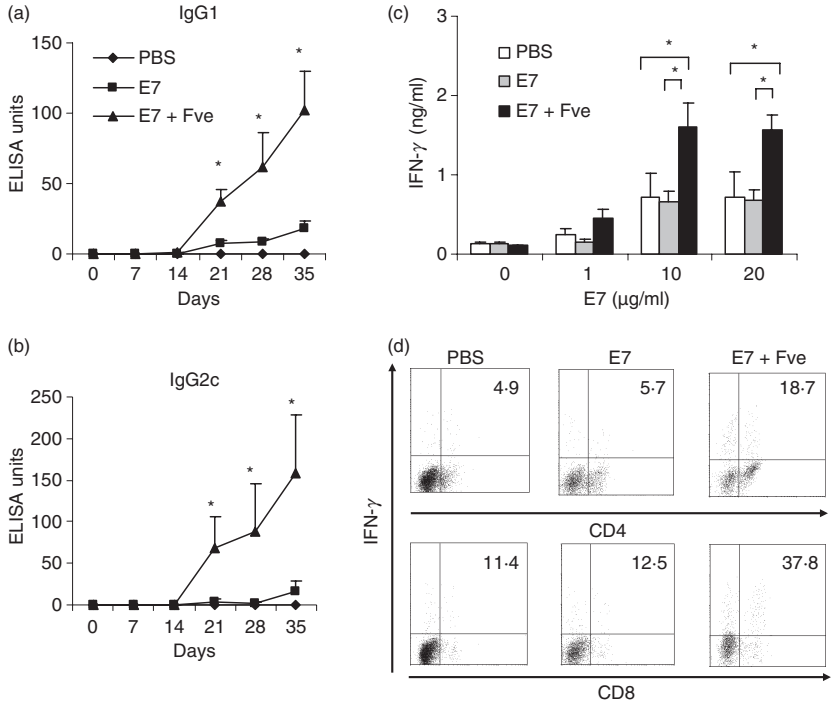

Figure 2. Fve enhanced human papillomavirus (HPV)-16 E7-specific antibody production and up-regulated interferon (IFN)-γ production of *in vitro* HPV-16 E7-stimulated T cells. For the HPV-16 E7-specific humoral immune response, mice ($n = 5$ per group) were immunized with phosphate-buffered saline (PBS) (◆), HPV-16 E7 (■) or HPV-16 E7 plus Fve (▲) at days 0, 14 and 28. Sera were collected weekly for the measurement of HPV-16 E7-specific (a) immunoglobulin G1 (IgG1) and (b) IgG2c by enzyme-linked immunosorbent assay (ELISA). For the T-cell immune response, mice ($n = 5$ per group) were immunized with PBS (◆), HPV-16 E7 (■), and HPV-16 E7 plus Fve (▲) at days 0 and 14. Splenocytes were collected at day 28 and cultured with 1, 10 or 20 μg/ml HPV-16 E7. Supernatants were collected at 72 hr and IFN-γ levels were measured by ELISA (c). For intracellular staining, the HPV-16 E7 cultured primary T cells were restimulated with anti-CD3 and anti-CD28 antibodies for 12 hr. The numbers in the upper right quadrant indicate the percentage of IFN-γ^+ cells among the gated $CD4^+$ cells and $CD8^+$ cells, respectively (d). Data are representative of three independent experiments. Error bars represent the standard error of the mean. $^*P < 0{\cdot}05$.

nization regimen *in vivo*. Mice immunized with PBS, HPV-16 E7, Fve, or HPV-16 E7 plus Fve were challenged with TC-1 cells subcutaneously and tumour growth was monitored. Results showed that 60% of mice co-immunized with HPV-16 E7 plus Fve remained tumour-free for up to 167 days after the tumour challenge, whereas only 20% of mice remained tumour-free ($P < 0{\cdot}05$) in the group immunized with HPV-16 E7 (Fig. 3a). Mice immunized with PBS or Fve alone developed tumours rapidly within 10 and 15 days after tumour challenge, respectively (Fig. 3a). Interestingly, mice immunized with Fve alone generally showed a reduction in tumour size as compared with the PBS control mice (Fig. 3b). This suggests that Fve protein alone could confer partial suppression of tumour growth. However, such a suppressive effect was insufficient to protect mice against tumour formation in the absence of HPV-16 E7-specific immune responses as only mice in the group immunized with HPV-16 E7 plus Fve exhibited a low tumour burden or remained tumour-free over the total duration of this study.

To determine whether long-term HPV-16 E7-specific immunity could be established in the immunized mice, splenocytes from tumour-free mice (HPV-16 E7 alone or HPV-16 E7 plus Fve groups) were collected and stimulated with HPV-16 E7 protein *in vitro* 167 days after tumour challenge. Splenocytes from naïve mice were used as the negative control. Results showed that, upon HPV-16 E7 antigen stimulation, splenocytes from HPV-16 E7 plus Fve co-immunized mice still produced higher levels of IFN-γ as compared with those from HPV-16 E7-immunized mice (Fig. 3c). Hence, Fve-enhanced HPV-16 E7-specific immunity persisted in the co-immunized mice and may account for the long-term protection against tumour formation. Taken together, these data indicate that immunization with Fve plus HPV-16 E7 is not only more effective than immunization with HPV-16 E7 alone; it is also able to confer long-term protection against tumour formation in the co-immunized mice.

Therapeutic immunization of HPV-16 E7 and Fve suppressed tumour growth and prolonged the survival of tumour-bearing mice

We also determined whether coadministration of HPV-16 E7 and Fve was equally effective at suppressing the growth of the established tumour. In this set of

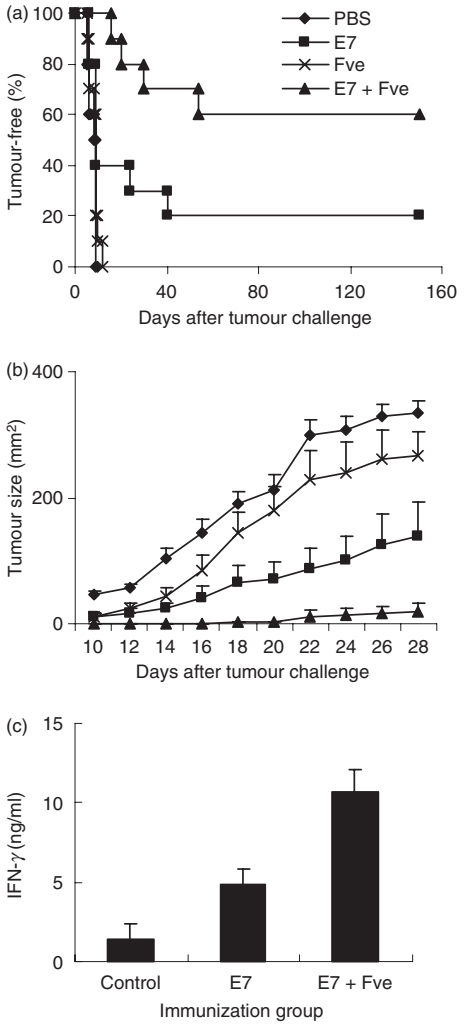

Figure 3. Co-immunization with human papillomavirus (HPV)-16 E7 and Fve enhanced protection against the growth of TC-1 tumours. Mice (n = 10 per group) were immunized with phosphate-buffered saline (PBS) (◆), 20 μg of HPV-16 E7 (■), 20 μg of Fve (×) or 20 μg of HPV-16 E7 plus 20 μg of Fve (▲) at days 0, 14 and 28 and then inoculated subcutaneously with TC-1 cells at day 30. The mice were monitored daily for tumour growth by palpation (a) and the tumour size was measured every 2 days (b). One hundred and sixty-seven days after tumour challenge, splenocytes were collected from the tumour-free mice and cultured with HPV-16 E7 protein *in vitro*. Supernatants were collected at 72 hr and interferon (IFN)-γ levels were analysed (c).

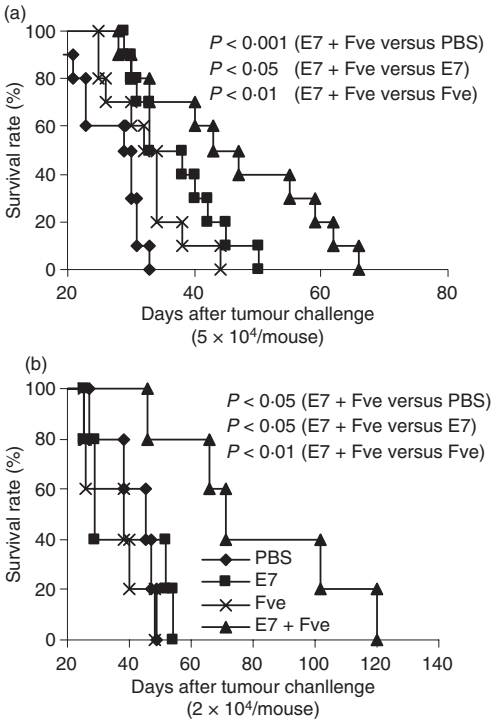

Figure 4. Human papillomavirus (HPV)-16 E7 plus Fve co-immunization therapeutically extended the survival of tumour-bearing mice. For the therapeutic assay (a), mice (n = 10 per group) were inoculated subcutaneously with TC-1 cells on day 0 and then treated with phosphate-buffered saline (PBS) (◆), HPV-16 E7 (■), Fve (×) or HPV-16 E7 plus Fve (▲) at days 3, 10 and 17. For the therapeutic metastasis assay (b), mice (n = 5 per group) were intravenously injected with TC-1 cells. Their survival was monitored and analysed by log-rank test.

experiments, TC-1 cells were inoculated into the left flanks of mice 3 days prior to regular treatments with PBS, HPV-16 E7, Fve, and HPV-16 E7 plus Fve, respectively. Results showed that mice treated with HPV-16 E7 plus Fve had the highest tumour survival rate (Fig. 4a).

Further investigations were then carried out in a tumour metastasis model established by injecting TC-1 cells intraveneously into the tail vein of each mouse at day 0. Mice were then treated with same regimens. As shown in Fig. 4b, none of the mice in the groups immunized with PBS, Fve or HPV-16 E7 alone survived beyond 55 days, while mice treated with HPV-16 E7 plus Fve showed significantly prolonged survival for up to 120 days. These results indicate that Fve could significantly enhance HPV-16 E7-specific antitumour activity therapeutically.

In summary, the results of these series of proof-of-principle experiments strongly support the notion that Fve could enhance an antigen-specific immune response that not only confers long-term protection against tumour growth but also retards tumour growth at the early and advanced stages of tumour development.

Both $CD4^+$ and $CD8^+$ T-cell subsets and IFN-γ were essential for protection against tumours

As Fve significantly increased IFN-γ-secreting T cells and enhanced HPV-16 E7-specific antitumour immunity, we next performed *in vivo* antibody depletion assays to determine the roles of the T-cell subsets and IFN-γ in the antitumour effects induced by combined vaccination. As

expected, 60% of the HPV-16 E7 plus Fve co-immunized mice without any depletions remained tumour-free throughout the duration of this part of the study (Fig. 5a), while the tumours that developed in the remaining 40% of these mice were dramatically reduced in size (Fig. 5b). In contrast, all the mice depleted of $CD4^+$ T cells, $CD8^+$ T cells or IFN-γ developed tumours within 27 days. Interestingly, mice depleted of $CD8^+$ T cells had similar tumour sizes to the PBS control, whereas tumour growth was retarded in the mice depleted of $CD4^+$ T cells and of IFN-γ (Fig. 5b). These data suggested that, while $CD8^+$ T cells, $CD4^+$ T cells and IFN-γ are essential for the antitumour protection generated in mice co-immunized with HPV-16 E7 plus Fve, the $CD8^+$ T subset plays a more dominant role in these antitumour effects.

To further investigate the roles of T cells in the antitumour effects seen in the HPV-16 E7 plus Fve co-immunized mice, a T-cell adoptive transfer experiment was set up. The immunization and tumour challenge regimen was as detailed in Fig. 6a. Mice that received T cells from co-immunized mice showed a significant reduction in tumour growth (Fig. 6b) compared with those receiving T cells adoptively transferred from donor mice immunized with HPV-16 E7, Fve and PBS, respectively. These data support the notion that T cells play a pivotal role in therapeutic antitumour effects and such effects are probably directly correlated to the efficacy and magnitude of the HPV-16 E7-specific T-cell responses, as demonstrated in Fig. 2c,d.

Fve stimulated phenotypic maturation of splenic DCs *in vivo* and enhanced $CD8^+$ T-cell activation

To elucidate the mechanism of the adjuvant action of Fve, we further analysed the effect of Fve on DCs which

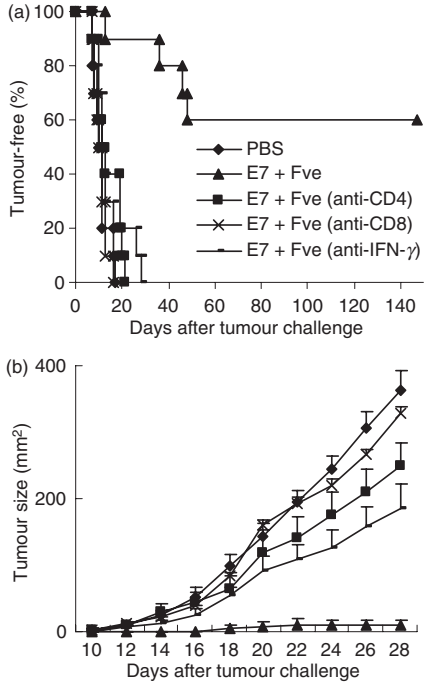

Figure 5. $CD4^+$ and $CD8^+$ T cells and interferon (IFN)-γ were essential for protection against tumours in mice immunized with human papillomavirus (HPV)-16 E7 plus Fve. Mice (n = 10 per group) were immunized with phosphate-buffered saline (PBS) or 20 μg of HPV-16 E7 plus 20 μg of Fve at days 0, 14 and 28. TC-1 cells (5×10^4) were inoculated subcutaneously into the right flanks of mice at day 30. To deplete $CD4^+$ T cells (■), $CD8^+$ T cells (×) or IFN-γ (-), mice immunized with HPV-16 E7 plus Fve were injected intraperitoneally with anti-CD4, anti-CD8, or anti-IFN-γ monoclonal antibodies (mAbs), respectively, at days −4, −1, 6, 13, 20, 27, 34, 41 and 48. Mice immunized with PBS (◆) and HPV-16 E7 plus Fve (▲) without depletion were included as the control groups. The mice were monitored daily for tumour growth by palpation (a) and the tumour size was measured every 2 days (b). Error bars represent the standard error of the mean.

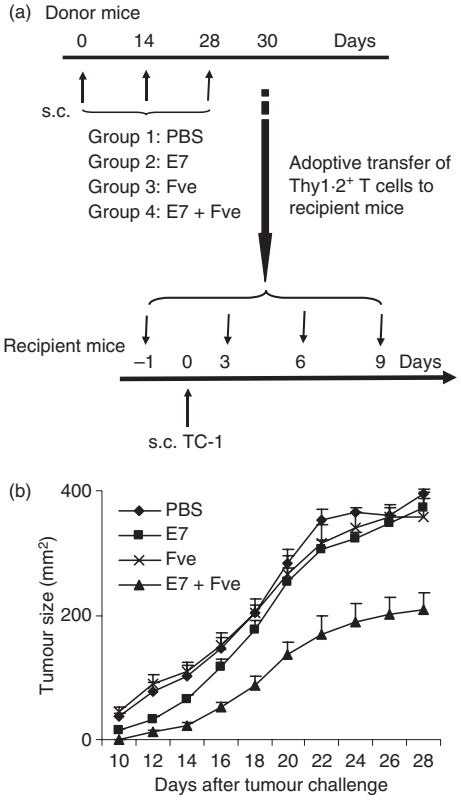

Figure 6. Adoptive transfer of T cells from mice immunized with human papillomavirus (HPV)-16 E7 plus Fve retarded tumour growth and prolonged survival. Eight million T cells purified from mice immunized with phosphate-buffered saline (PBS) (◆), HPV-16 E7 (■), Fve (×) or HPV-16 E7 plus Fve (▲) were adoptively transferred to recipient mice at days −1, 3, 6 and 9. Recipient mice (n = 10 per group) were inoculated subcutaneously with 5×10^4 TC-1 cells at day 0 (a). Ten days after the tumour challenge, the size of the tumour formed was measured on alternate days. Data are presented as mean ± standard error of the mean (b).

are responsible for the priming of the specific immune response.[25,26] To this end, splenic CD11c$^+$ DCs were enriched from spleens of mice which were intravenously injected with Fve prior to harvest and analysed for surface expression of major histocompatibility complex (MHC) class II and CD86. As shown in Fig. 7c, DCs isolated from Fve-treated mice up-regulated MHC-II and CD86 molecules in both CD11c^{hi} and CD11c^{int} subsets as compared with those from control mice. CD11c^{hi} DCs can be further subdivided into CD4$^+$, CD8α^+ and CD4$^-$ CD8α^- subtypes (Fig. 7b). Up-regulation of MHC-II and CD86 was seen in all three subtypes of CD11c^{hi} DCs (Fig. 7d), especially in the CD8α^+ DCs. This indicates that Fve stimulates phenotypic maturation of splenic DCs *in vivo*. Subsequently, the *in vitro* functional assays for these Fve-stimulated DCs were performed using OVA-specific CD8$^+$ and CD4$^+$ T cells from OT-I and OT-II mice, respectively. Analysis of culture supernatants of OVA-specific CD8$^+$ T cells co-cultured with OVA-laden DCs isolated from OVA plus Fve co-injected mice showed that production of IFN-γ and IL-2 was greatly increased compared with those from all the other experimental control groups (Fig. 8a). Such a marked enhancement of IFN-γ and IL-2 production by OVA-specific CD4$^+$ T cells was not observed in similar parallel functional assays performed using CD4$^+$ T cells from OT-II mice (Fig. 8b). There was no increased induction of IL-4, the signature cytokine for Th2 responses, by either CD4$^+$ or CD8$^+$ OVA-specific T cells. These data suggest that Fve can efficiently enhance the OVA-specific CD8$^+$ T-cell immune response, probably by modifying the ability of DCs to present antigen.

Discussion

Antigen-specific immunotherapy is a promising strategy to eradicate systemic tumours at multiple sites while conferring the advantage of specific discrimination between neoplastic and non-neoplastic cells. However, a major hurdle for the development of such vaccines for treatment and prevention of cancer is the poor immunogenicity of tumour-associated antigens. An attractive strategy to overcome this problem is the use of an immune modulator as an adjuvant to boost antigen-specific immunity and enhance the efficacy of tumour vaccines.[27]

Our *in vivo* tumour protection results showed that 60% of the mice co-immunized with HPV-16 E7 plus Fve remained tumour-free after tumour challenge as compared with only 20% of mice immunized with HPV-16 E7 alone (Fig. 3). More importantly, the enhanced antitumour effects induced by HPV-16 E7 plus Fve co-immunization were also observed in the therapeutic tumour model (Fig. 4). These data indicate that HPV-16 E7 plus Fve co-immunization is more efficacious for the protection of mice against tumour challenge and the eradication of established tumours, and that Fve enhances antitumour effects. It is conceivable that the enhanced HPV-16 E7-specific T-cell immunity and increased IFN-γ production by these T cells induced by Fve may contribute, at least

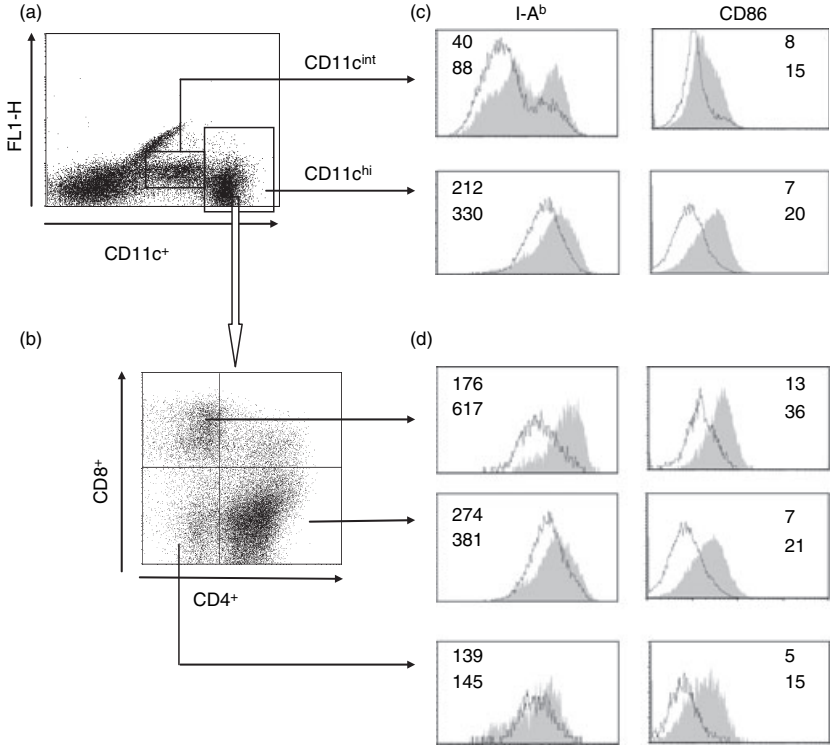

Figure 7. Fve induced splenic dendritic cell (DC) phenotypic maturation *in vivo*. Mice (n = 8 mice/group) were intraveneously injected with phosphate-buffered saline (PBS) or Fve 12 hr prior to cell harvest. DCs were enriched from spleens for staining of major histocompatibility complex (MHC) class II and CD86. Splenic DCs were separated into CD11c^{int} and CD11c^{hi} populations (a) and CD11c^{hi} DCs were further divided into CD4$^+$, CD8α^+ and CD4$^-$ CD8α^- subpopulations (b). MHC class II and CD86 up-regulation could be seen in both CD11c^{int} and CD11c^{hi} populations (c) and the three CD11c^{hi} subpopulations of DCs (d) after stimulation with Fve *in vivo*. Open histograms delineated with a dark line represent PBS-stimulated DCs, and filled grey histograms represent Fve-stimulated DCs. The numbers in the histograms indicate the mean fluorescent intensities (MFIs) of DCs stimulated with PBS (upper number) and Fve (lower number), respectively.

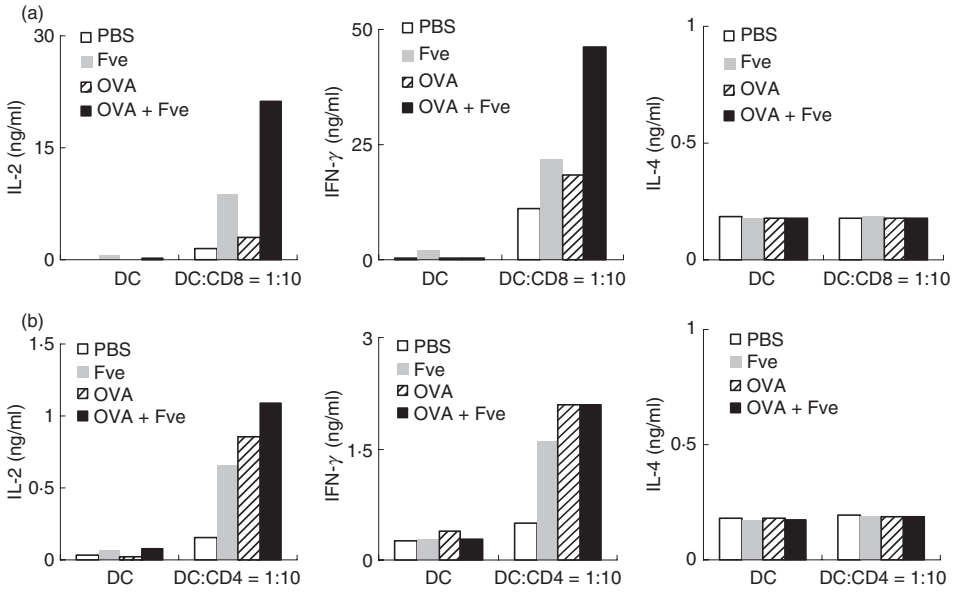

Figure 8. Fve greatly enhanced antigen-specific responses of $CD8^+$ T cells. Mice (n = 8 mice/group) were intraveneously injected with phosphate-buffered saline (PBS), ovalbumin (OVA), Fve or OVA plus Fve and the dendritic cells (DCs) were isolated from the pool of eight spleens harvested from each group of mice 24 hr later. Purified DCs were pulsed with 1 μM $OVA_{257–264}$ peptide or 1 μM $OVA_{323–367}$ peptide for 2 hr at 37° and washed three times before co-culturing with $CD8^+$ T cells from OT-I mice or $CD4^+$ T cells from OT-II mice, respectively, for 72 hr. Supernatants were collected for cytokine assays by enzyme-linked immunosorbent assay (ELISA). The experiments were performed three times and representative data from one of these experiments are presented. DC, dendritic cells alone; DC:CD8 = 1 : 10, the ratio of DCs to $CD8^+$ T cells from OT-I mice is 1 : 10; DC:CD4 = 1 : 10, the ratio of DCs to $CD4^+$ T cells from OT-II mice is 1 : 10.

in part, to the enhanced antitumour effects observed in this study.

It is well known that antigen-specific T-cell immunity plays a critical role in tumour immunotherapy.[28] Previous studies using other adjuvants such as heat shock protein 65,[9] bacteria exotoxin,[11] IL-12,[7] CpG[12] and 3-*O*-deacylated monophosphoryl lipid A (MPL) mixed with a purified *Quillala saponaria* saponin immunologic adjuvant (QS21)[29] found that $CD4^+$ and/or $CD8^+$ cells play major roles in protecting animals from challenge with HPV-16 E7-expressing TC-1 cells. In this study, we performed *in vivo* T-cell subset depletion assays to elucidate the contribution of $CD4^+$ and $CD8^+$ T cells to antitumour activity. We found that both T-cell subsets are essential for the inhibition of tumour growth and $CD8^+$ T cells appear to play a more dominant role in protection against tumours. These results concur well with the conventional dogma that $CD8^+$ T cells are pivotal and highly specialized for cytolytic function and thus have been the main focus of cancer immunotherapy, whereas $CD4^+$ T cells confer helper functions in the antitumour effect by providing activation signals to $CD8^+$ T cells[30,31] and contributing to the survival maintenance of $CD8^+$ T memory cells.[32–36] Recent studies, however, found that tumour-specific $CD4^+$ T cell were able to eliminate a wide variety of tumours that were resistant to CD8-mediated rejection,[37,38] providing new supporting evidence for the hypothesis that $CD4^+$ T cells may play a broader role in antitumour responses. Moreover, we found that tumour-free mice protected by immunization with HPV-16 E7 plus Fve had higher levels of IFN-γ production at 167 days after tumour inoculation (Fig. 3c), indicating that Fve enhances HPV-16 E7-specific memory immunity to protect mice against tumour growth. These findings are important because the capacity to elicit an effective long-term memory immune response is essential for the success of a vaccination strategy.[39–41]

We also adoptively transferred total T cells from HPV-16 E7 plus Fve immunized mice into tumour-bearing recipient mice to address the importance of T cells in mediating the antitumour effects seen in our study. Results indicated that T cells from mice immunized with HPV-16 E7 plus Fve were more efficacious in suppressing tumour growth than those from mice immunized with HPV-16 E7 alone (Fig. 6b). This enhanced efficacy was probably correlated to the increased number of HPV-16 E7-specific effector T cells induced by co-immunization. However, other possibilities, such as increased HPV-16 E7-specific T-cell avidity, cannot be excluded as antigen-specific $CD8^+$ T cells with high avidity are known to produce stronger antitumour effects in vaccinated mice than low-avidity $CD8^+$ T cells.[42,43] In recent years, adoptive transfer of antigen-specific T cells into patients has emerged as a promising new approach to cancer treatment.[44–46] Our T-cell transfer data suggest that the Fve protein may be a good immunotherapeutic vaccine adjuvant to enhance the antitumour immunity mediated by

tumour antigen-specific T cells, thereby providing a promising new strategy to improve the efficacy of the adoptive cell therapy approach.

In addition, we found that HPV-16 E7 plus Fve co-immunization significantly up-regulated HPV-16 E7-specific IgG1 and IgG2c. The enhanced production of IgG2c in C57BL/6 (Fig. 2b) and IgG2a in BALB/cJ mice (Fig. S2b) was consistent with the previous finding that Fve can increase the Th1-skewed humoral OVA-specific immune response.[20] Previous studies using the NY-ESO-1 tumour antigen as a model antigen have shown that antigen-specific IgG2a antibodies contribute to DC maturation and cross-priming of $CD8^+$ T cells, probably through a mechanism mediated by antigen–antibody immune complexes.[47,48] Although there is no direct evidence that antibody-mediated responses play an important role in controlling HPV-associated malignancies, a possible role of increased production of HPV-16 E7-specific antibody, which may improve antitumour activity, cannot be ruled out and warrants further study.

IFN-γ has been shown to inhibit tumour growth *in vivo* by up-regulation of MHC class I molecules, inducing inflammation at tumour sites as well as eliciting an angiostatic effect.[49–54] Our study also demonstrated that IFN-γ was critical for generating potent antitumour effects against TC-1 tumour challenge. We found that the tumour protection effect of the HPV-16 E7 plus Fve vaccine was significantly attenuated in IFN-γ-depleted mice (Fig. 5). These results are consistent with previous studies demonstrating the important role of IFN-γ in the antitumour effect.[51,53,55,56] Interestingly, we found that the mean tumour size of HPV-16 E7 plus Fve-immunized mice with IFN-γ depletion was smaller than that of PBS-immunized mice (Fig. 5b), suggesting that additional IFN-γ-independent mechanisms may also contribute to the suppression of tumour growth found.

It is worth noting that the Fve protein alone conferred some antitumour effects compared with the effects of PBS (Fig. 3b). This interesting observation may be explained by the fact that the Fve protein mitogenically expands the T-cell pool and induces high levels of IFN-γ production (Fig. 1), creating a microenvironment to confer partial antitumour effects. This may also represent an additional beneficial effect of using Fve as an adjuvant for antitumour immunotherapeutic vaccines.

It is well known that activation of innate immunity is a prerequisite for an adjuvant function.[57] The roles of DCs in the priming and differentiation of naïve T cells are well recognized.[26] The majority of adjuvants are microbial products that activate innate responses through pattern recognition receptors, such as Toll-like receptors (TLRs) on DCs. For example, monophosphoryl lipid A, imiquimod and CpG motifs, which are agonists for TLR4, TLR7 and TLR9, respectively, have been developed as vaccine adjuvants for the treatment of cancer.[58,59] In this study, we found that Fve induced maturation of DCs *in vivo* and, notably, it appears that Fve preferentially, although not exclusively, drives maturation of the $CD8^+$ DC subset. Moreover, *in vitro* functional assays using OVA-specific $CD8^+$ T cells from OT-I mice clearly showed that DCs from mice co-immunized with Fve and OVA greatly enhanced the activation of antigen-specific $CD8^+$ T cells (Fig. 8a). It is well known that there are at least three distinct subsets ($CD4^+$ $CD8^-$, $CD4^-$ $CD8^+$ and $CD4^-$ $CD8^-$) of DCs found in the mouse spleen. Previous studies have shown that these subsets exhibit intrinsic differential capacities to present soluble antigen and activate antigen-specific $CD4^+$ and $CD8^+$ T subsets. The $CD8^-$ subsets show the greatest ability to stimulate antigen-specific MHC class II-restricted $CD4^+$ T cells, whereas the $CD8^+$ DCs are much more efficient at cross-presenting antigen and stimulating MHC class I-restricted $CD8^+$ T cells.[60,61] Taking these findings together, it is reasonable to propose that Fve modifies the ability of DCs (for example, by up-regulating the $CD8^+$ DC subset to enhance $CD8^+$ T cells via the cross-presentation pathway) to generate robust and long-lasting HPV-16 E7-specific $CD8^+$ T-cell immune responses for antitumour effects. This may be a unique feature of the adjuvant effects of Fve and therefore further work is warranted to validate this notion. Thus, our future research will focus on elucidation of the effects of Fve on the various DC subsets and the subsequent priming and polarization of antigen-specific T-cell subsets.

In summary, our study has demonstrated for the first time that an immunomodulatory protein, Fve, exhibited effective adjuvant effects to enhance robust and long-lasting adaptive antigen-specific immune responses that conferred strong prophylactic and therapeutic antitumour effects. Notably, it appears that, by targeting $CD8^+$ DCs, Fve can effectively enhance antigen-specific $CD8^+$ T-cell immune responses. For HPV-associated malignancies, prophylactic vaccines which aim to induce neutralizing antibodies have been successfully used in clinical applications.[62,63] However, the challenge for the future is to combine the prophylactic approach with therapeutic immunization, for example, combining E7 with the HPV capsid protein L1 or L2 to develop chimeric vaccines.[64–66] We envisage that Fve could potentially be an effective adjuvant not only for such HPV chimeric vaccines; it could also be generally exploited to develop other efficacious anticancer or antiviral vaccines.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary materials and methods

Figure S1. Fve stimulates mouse splenic T-cell proliferation in an accessory-cell-dependent manner.

Figure S2. Fve enhanced human papillomavirus (HPV)-16 E7-specific antibody production in BALB/cJ mice.

Figure S3. Fve enhanced interferon (IFN)-γ production in C57BL/6 mice co-immunized with human papillomavirus (HPV)-16 E7 plus Fve.

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