國立臺灣大學獸醫專業學院獸醫學研究所

博士論文

Graduate Institute of Veterinary Medicine

School of Veterinary Medicine

National Taiwan University

Dissertation

檳榔子萃取物抑制T細胞功能及促進骨髓衍生抑制性細胞生成之免疫調節作用

The immunomodulatory effect of areca nut extract on the functionality of T cells and the induction of myeloid-derived suppressor cells

王家琪

Chia-Chi Wang

指導教授：詹東榮 博士

Advisor: Tong-Rong Jan, Ph.D.

中華民國 99 年 7 月

July 2010

中文摘要

流行病學研究顯示嚼食檳榔會提高罹患口腔黏膜下纖維化症及口腔癌的機率，且實驗證據指出病人免疫系統的惡化與嚼食檳榔所導致的口腔疾病病程的發展有重要的相關性。研究發現檳榔子萃取物能直接引起嗜中性球細胞及多種口腔細胞的死亡且增加細胞的氧化性傷害。由於 T 淋巴球細胞已被證實為主要浸潤於口腔癌前病變組織或口腔腫瘤的重要免疫細胞，因此推測此大量浸潤的 T 淋巴球可能會受到檳榔成分的直接作用而導致其媒介的免疫功能受到影響。本論文主要研究檳榔子萃取物對於 T 淋巴球活性及功能的影響，並進一步探討活性氧化物（ROS）於檳榔子萃取物的免疫調節作用所扮演的角色。此外更進一步研究檳榔子萃取物在活體動物中的免疫調節作用。實驗結果顯示檳榔子萃取物對於脾臟細胞具有明顯的毒性且會抑制介白素-2（interleukin-2）及干擾素-γ（interferon-γ）的分泌，而對於介白素-4 的抑制作用則不明顯。上述檳榔子萃取物的抑制作用可以被抗氧化劑 *N*-乙醯半胱胺酸（*N*-acetyl-$_{L}$-cysteine 以下簡稱 NAC）部分且有效的回復。檳榔子萃取物會增加 T 淋巴球 ROS 的增加及降低細胞內穀胱甘肽 （glutathione 以下簡稱 GSH）的含量，顯示檳榔子萃取物可藉由增加細胞內產生 ROS 來抑制 T 淋巴球的活化及第一型 T 幫手淋巴球細胞激素的製造。

檳榔子萃取物明顯地增加脾臟細胞的凋亡、促進粒線體膜電位的去極化、釋放粒線體內 cytochrome *c*、活化細胞內 caspase-9 及增加凋亡細胞的 ROS。除了

粒線體膜電位的去極化完全無法被 NAC 所預防，NAC 對於檳榔子萃取物所引起的上述作用都具有部分但顯著的回復效果。此研究結果指出檳榔子萃取物對於脾臟細胞的毒性作用與活化粒線體凋亡途徑有關，同時會導致細胞內 ROS 的增加。由於檳榔子萃取物含有豐富的多酚類，進一步研究檳榔子萃取物中的多酚類是否可以引起脾臟細胞的凋亡，結果發現多酚類含量較多的檳榔子萃取物（PANE）同樣具有導致脾臟細胞凋亡的效果，且進一步比較萃取物中不同聚合程度的前花青素（procyanidins），發現聚合程度大於五的 pentamers 即開始會引起脾臟細胞的凋亡，隨著前花青素聚合程度愈高引起細胞凋亡的程度也愈明顯。此外 PANE 也會導致細胞內 GSH 的下降，顯示細胞內氧化性傷害的增加，且細胞內 GSH 下降的程度也與前花青素聚合程度成正相關。此部分研究顯示檳榔子萃取物中高聚合程度的前花青素可能會導致細胞抗氧化物 GSH 的下降而引起細胞的凋亡。

活體動物的試驗中發現，腹腔注射檳榔子萃取物及 PANE 會導致脾臟的腫脹及增加一群 $CD11b^+$的骨髓衍生性細胞，此群細胞具有小鼠骨髓衍生性抑制性細胞（MDSC）的重要細胞表面標誌: CD11b 及 Gr-1。$CD11b^+Gr\text{-}1^+$ 的細胞族群在給予檳榔子萃取物的小鼠脾臟及血液中的比率有顯著的增加。進一步分析該群細胞的功能發現其抑制性細胞激素介白素-10（IL-10）的分泌量、第一型精胺酸酶（arginase-I）酵素的活性及表現第一型精胺酸酶及誘導型一氧化氮合成酶（inducible nitric oxide synthase；iNOS）基因的能力都有顯著地增加，顯示此群

細胞具有骨髓衍生抑制性細胞（myeloid-derived suppressor cell；MDSC）典型的功能特性。

綜合本論文的研究結果，發現檳榔子萃取物具有直接調節細胞媒介免疫反應的作用，包含降低 T 淋巴球細胞激素的分泌、導致脾臟細胞的凋亡及增加骨髓衍生抑制性細胞的數量。而檳榔子萃取物中的前花青素於上述的免疫調節作用中扮演重要的角色。本論文結果有助於了解檳榔子的成分如何調節免疫細胞及導致細胞媒介免疫反應惡化的可能機制。

關鍵字：檳榔子萃取物、前花青素、免疫抑制、細胞激素、凋亡、骨髓衍生抑制性細胞、氧化性傷害

Abstract

Areca quid chewing is a major risk factor associated with oral submucous fibrosis and oral cancer. Experimental evidence indicates that immune deterioration is closely associated with the pathophysiology of areca-associated oral diseases. In addition, the induction of oxidative stress and cell death has been shown to play a role in the cytotoxic and genotoxic effects induced by areca nut extracts (ANE) in oral cells and neutrophils. As T lymphocytes are one of the major immunocompetent cells present in the lesions of both OSF and oral cancer patients, it is hypothesized that T cell–mediated immune responses may be altered by ANE. The present studies investigated the immunomodulatory effect of ANE on T cell reactivity and the role of reactive oxygen species (ROS) in ANE-mediated effects *in vitro*. In addition, the immunomodulatory effect of ANE and polyphenol-enriched ANE (PANE) was examined *in vivo*. ANE induced a marked cytotoxic effect, and suppressed the production of IL-2 and IFN-γ by splenocytes, whereas the production of IL-4 was unaffected. The thiol antioxidant *N*-acetyl-$_{L}$-cysteine (NAC) partially but significantly attenuated ANE-mediated cytotoxicity and suppression of IL-2 and IFN-γ production. In splenic T cells, ANE increased the cellular ROS levels, which was also attenuated by the presence of NAC. Concordantly, the cellular level of glutathione was diminished by ANE in splenic T cells pretreated with NAC. These results

demonstrated that ANE markedly suppressed T-cell activation and Th1 cytokine production, which was mediated, at least in part, by the induction of oxidative stress.

ANE significantly enhanced splenocyte apoptosis. The depolarization of mitochondrial membrane potential ($\Delta\psi_m$), the release of cytochrome *c* and the activation of caspase-9 were induced by ANE, indicating the activation of the mitochondrion-dependent apoptotic pathway. Moreover, an increased level in the intracellular ROS was detected in ANE-treated splenocytes undergoing apoptosis. NAC significantly attenuated ANE-mediated apoptosis, caspase-9 activation and ROS production but not $\Delta\psi_m$ depolarization. These results demonstrated the pro-apoptotic effect of ANE in primary splenocytes, which was mediated by the activation of the mitochondrion-dependent pathway and oxidative stress. In addition, PANE and its fractionated oligomeric procyanidins from pentamers to decamers were active in inducing apoptosis. A marked diminishment in the level of intracellular thiols was revealed in splenocytes treated with pentamers to decamers. Pretreatment with NAC resulted in significant attenuation of both apoptosis and thiol diminishment induced by areca procyanidins. These results indicated that highly oligomeric procyanidins derived from areca nut induced a chain length-dependent pro-apoptotic effect in primary lymphocytes possibly via the diminishment of intracellular thiols.

Intraperitoneal administration of antigen-sensitized BALB/c mice with ANE or

PANE significantly increased the spleen index and the splenic cellularity of immature myeloid $CD11b^+$ cells. The population of $CD11b^+Gr\text{-}1^+$ cells in the spleen and peripheral blood was markedly enhanced by ANE and PANE. In addition, ANE administration significantly augmented the production of IL-10, and the mRNA expression of iNOS and arginase I by splenocytes and splenic $CD11b^+$ cells stimulated with lipopolysaccharide. These results suggested that ANE administration to antigen-sensitized mice enhanced the development of $CD11b^+Gr\text{-}1^+$ cells that exhibited a functional profile of myeloid-derived suppressor cells (MDSC).

Taken together, this study demonstrated the direct immunomodulatory effect of ANE on the down-regulation of Th1 cytokines *in vitro,* induction of lymphocyte apoptosis *in vitro* and generation of MDSC *in vivo.* In addition, areca-derived procyanidins may be the potential candidates responsible for the ANE-mediated immunomodulatory effects. These results provide evidence to show that areca constituents may directly compromise the cell-mediated immunity which was previously reported to be down-regulated in areca quid chewers with oral precancer and cancer.

Keywords: areca nut extract, procyanidins, immunosuppression, cytokines, apoptosis, myeloid-derived suppressor cells, oxidative stress

List of Abbreviations

ANE	areca nut extract
Apaf-1	apoptotic protease activating factor 1
AQ	areca quid
bFGF	basic fibroblast growth factor
CA	chromosome aberrations
CMF-DA	5-chloromethylfluorescein diacetate
ConA	concanavalin A
COX-2	cyclooxygenase-2
DC	dendritic cells
DCF-DA	2',7'-dichlorofluorescein diacetate
DD	death domain
ELISA	enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FSC	forward-angle light scatter
GSH	glutathione
HNSCC	head and neck squamous cell carcinomas
IFN	interferon
Ig	immunoglobulins
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IP-10	including IFN-inducible protein 10
JC-1	5,6,-tetrachloro-1,3-tetraethylbenzimidazolylcarbocyanine iodide
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MTT	3-(4,5-dimethyl- -thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NA	naïve
NAC	*N*-acetyl-$_{L}$-cysteine
NF-κB	nuclear factor-κB
NK	natural killer cells
NO	nitric oxide
OSCC	oral squamous cell carcinomas
OSF	oral submucous fibrosis
OVA	ovalbumin

PANE	polyphenol-enriched areca nut extract
PBMC	peripheral blood mononuclear cells
PBST	phosphate-buffered saline containing 0.02% Tween 20
PDGF	platelet-derived growth factor
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PI	phorbol 12-myristate 13-acetate plus ionomycin
ROS	reactive oxygen species
RT	room temperature
SCE	sister chromatid exchanges
STAT	signal transducer and activator of transcription
Tc	cytotoxic cells
TCR	T cell receptor
Th	T helper cells
TNF	tumor necrosis factor
T_{reg}	regulatory T cells
TUNEL	terminal dUTP nick-end labeling
UDS	unscheduled DNA synthesis
VCAM-1	vascular cell adhesion molecule-1
VH	vehicle
$\Delta\psi_m$	mitochondrial membrane potential
8-OH-dG	8-hydroxy-2-deoxyguanosine

Contents

List of Figures and Tables

Chapter 1. Background

1.1 Areca nut chewing related diseases

Areca quid (AQ) chewing is the fourth most addictive habit in the world. There are more than 6 hundred million AQ chewers in the world (Gupta and Warnakulasuriya, 2002), especially in Southeast Asia countries, India and Taiwan. The number of AQ chewers is estimated to be more than 2 million in Taiwan (Ko et al., 1992). Epidemiological studies indicated that AQ chewing is one of the major independent risk factors for leukoplakia, erythroplakia, oral submucous fibrosis and oral squamous cell carcinomas (OSCC) (Ko et al., 1995; Znaor et al., 2003). Oral submucous fibrosis (OSF), a chronic fibrotic disease, is characterized by mucosal rigidity due to fibro-elastic transformation of juxta-epithelial layer, burning sensation in the oral cavity and stiffening of the oral mucosa and oropharynx leading to the inability to open the mouth (Tilakaratne et al., 2006). OSCC of the head and neck cancers is the sixth most common human malignancy and the fourth leading cause of cancer death for males in Taiwan. Despite the advance in chemotherapy and surgery for cancer treatment, the overall five-year survival rate of oral cancer is not improved and remains as one of the lowest among common malignant tumors during the last two decades (Chang et al., 2005). It has been shown that AQ chewing is a risk factor of foregut cancers (Ahmed et al., 1997) and hepatocellular carcinoma (Tsai et al.,

2001), suggesting that AQ chewing-related injury is not restricted to the oral cavity. In addition, AQ chewing is associated with an increased risk of cardiovascular disease, chronic kidney diseases, metabolic syndrome and obesity (Chou et al., 2009; Lin et al., 2008; Lin et al., 2009). Daily consumption of AQ has been reported to correlate with the deregulation of humoral immunity in OSF patients (Chiang et al., 2002a). Likewise, immune deterioration is closely associated with the pathogenesis of oral cancer and precancers (Chang et al., 2005; Jeng et al., 2003; Verastegui et al., 2002). Collectively, AQ chewing is an important health issue which deserves further studies to elucidate the broad and complicated detrimental effect of AQ components.

1.2 Areca quid ingredients

AQ generally consists of areca nut (*Areca catechu* Linn), catechu (*Acacia catechu*), lime (calcium oxide and calcium hydroxide), with or without betel leaf (*Piper betle*). In India and Sri Lanka, most chewers of AQ add tobacco; in Taiwan, a piece of *Piper betle* inflorescence, instead of tobacco, is added to improve the flavor of AQ. Among different geographic locations, areca nuts are the main component in the AQ and the regular AQ chewers in Taiwan take a considerable number of areca nuts per day (Chang et al., 2005; Jeng et al., 2001; Nair et al., 2004). The areca nuts used in Taiwan are fresh, tender with husk, while the ripe without fibrous husk nuts are used in other countries (Chang et al., 2009). The major bioactive components of

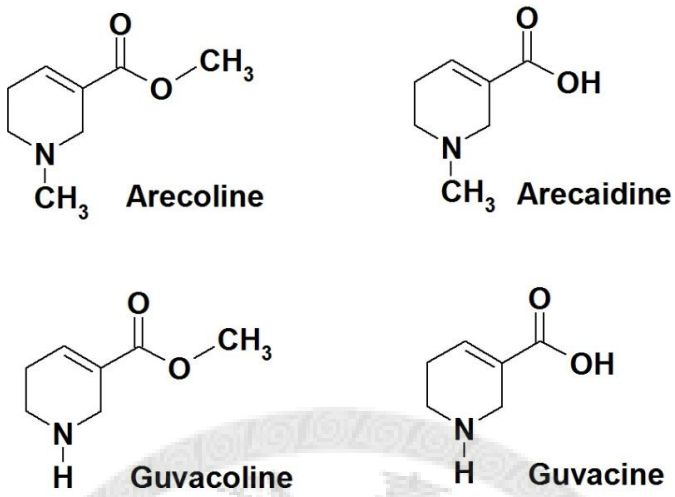
areca nuts are polyphenols (7-55%) and alkaloids (0.1-1%) (Mathew and Govindarajan, 1964; Ranadive et al., 1976). It has been shown that areca nut-derived polyphenols are mostly flavanols, including 10% of (+)-catechin, 2.5% of (-)-epicatechin, 12% of leucocyanidins and other flavonoids with various degrees of polymerization (Fig. 1B) (Mathew and Govindarajan, 1964). A series of procyanidins from dimers to tetramers and other oligomers have been isolated from areca nuts (Nonaka et al., 1981). It has been further reported that areca-derived flavan-3-ols and procyanidins are similar as those found in apple, grape seed and cocoa, except that grape procyanidins have gallic acid groups, whereas the others do not (Wu et al., 2007). The major alkaloids in areca nuts are arecoline, arecaidine, guvacine and guvacoline (Fig. 1A). Arecoline, a muscarinic and nicotinic agonist with parasympathomimetic properties, has been thought to be responsible for the warm sensation of the body and heightened alertness after AQ chewing (IARC, 2004).

1.3 Polyphenols and procyanidins

Polyphenols, a wide variety of molecules characteristic of several hydroxyl groups on aromatic rings, are ubiquitously distributed in daily foods, including vegetables, fruits, cereals, beans, nuts, beverages, spices, among many others (D'Archivio et al., 2007; Prior and Gu, 2005; Williamson and Manach, 2005). The main groups of polyphenols are categorized into hydrolysable tannins, flavonoids,

stilbenes and lignans (D'Archivio et al., 2007). Flavonoids possess a 15-carbon skeleton of diphenyl propanes, two benzene rings (ring A and B) joined by a linear 3-carbon chain. The central 3-carbon chain may form a closed pyran ring (ring C) with different oxidation state and thereby further classified into 6 subclasses, including flavonol, flavonoe, isoflavonoe, flavanone, anthocyanin and flavanol (catechins and procyanidins) (Fig. 2) (D'Archivio et al., 2007). Procyanidins are comprised of oligomeric and polymeric flavanols (flavan-3-ols) (D'Archivio et al., 2007; Prior and Gu, 2005). Flavan-3-ol units are mainly linked by type B linkage (C4-C8 and/or C4-C6 bonds) and can also be linked to an additional ether bond by C2-O-C7 or C2-O-C5 (type A linakage) (D'Archivio et al., 2007; Prior and Gu, 2005). (+)-Catechin and (-)-epicatechin are the two main monomeric flavanols in plants, whereas gallocatechin, epigallocatechin and epigallocatechin gallate are found in tea (D'Archivio et al., 2007). There are various isomeric forms of procyanidins according to the extent of polymerization, the nature of constituent units and their linkage. It has been suggested that the degree of polymerization might be an important factor affecting the bioactivities of procyanidins, such as the binding affinity of procyanidins to proteins and its antioxidant activity (Cheynier, 2005; Lotito et al., 2000; Verstraeten et al., 2003).

(A) Alkaloids in areca nut



(B) Catechin monomers and procyanidins

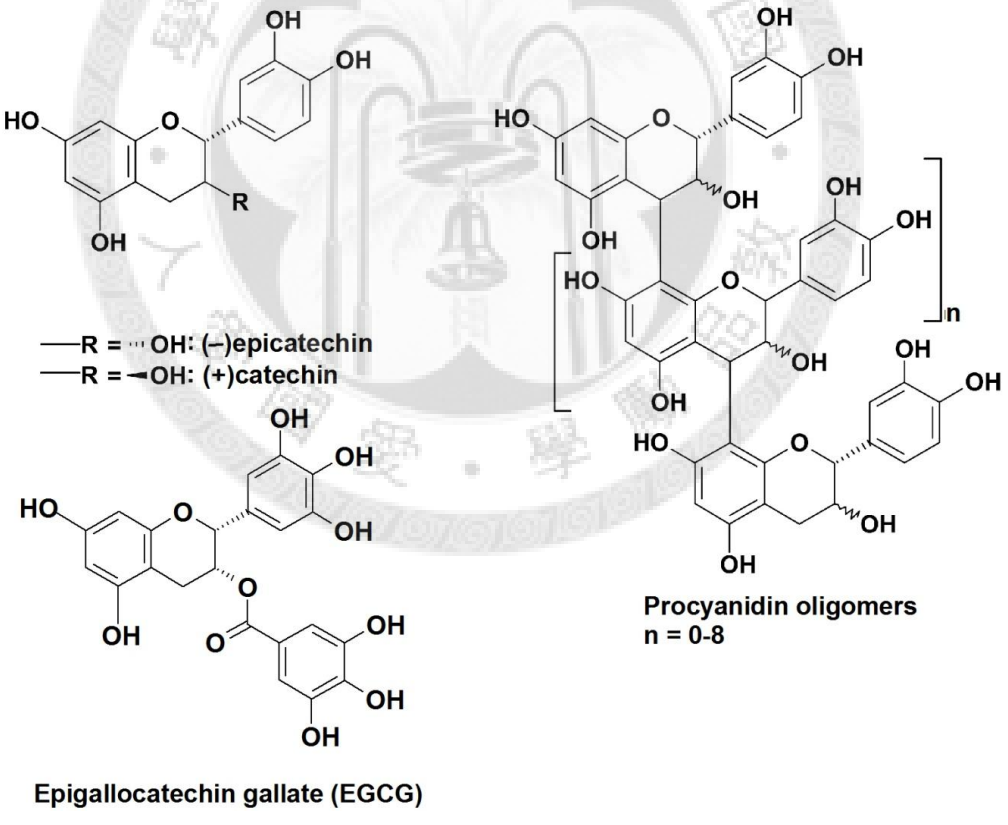


Figure 1. Structure of areca-derived alkaloids and polyphenols.

(A) There are four major alkaloids in areca nut, including arecoline, arecaidine, guvacine, guvacoline. (B) The chemical structures of catechin monomers, epigallocatechin gallate and areca-derived oligomeric procyanidins.

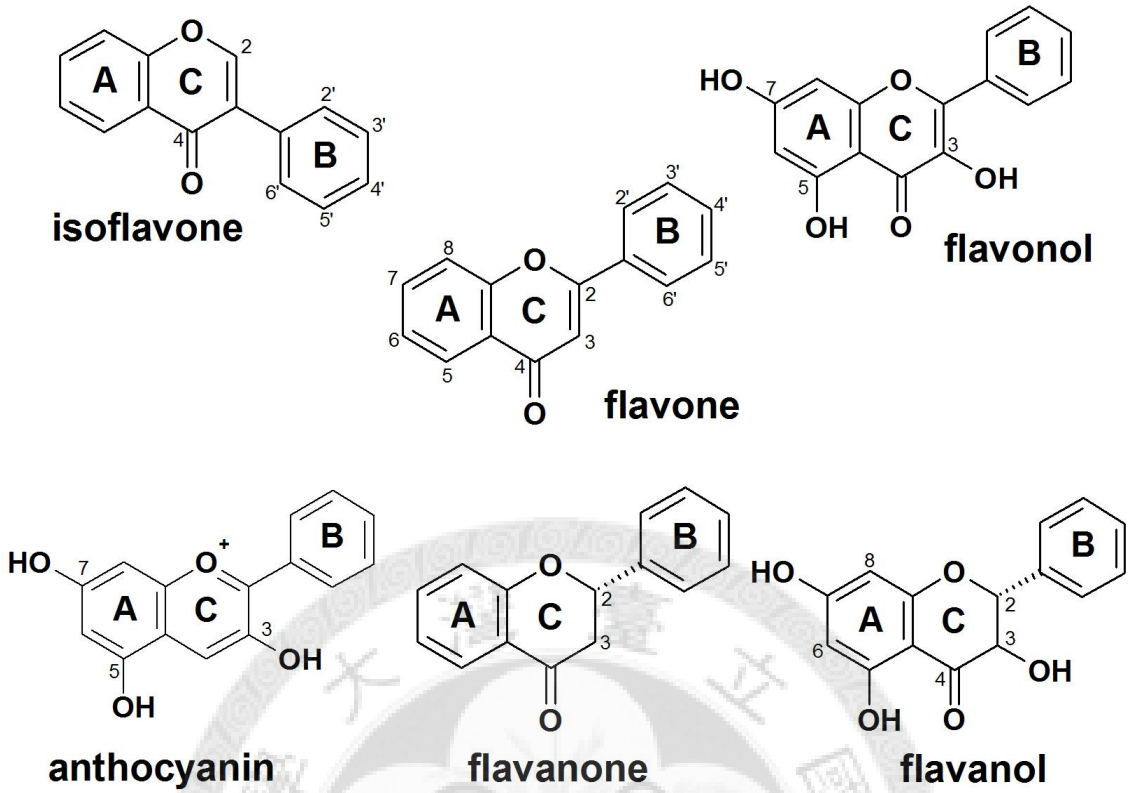


Figure 2. Structure of major flavonoids.

1.4 T cell-mediated immune responses

T cells play a pivotal role in the acquired immunity. They participate in a wide range of immune responses through a complicated cytokine network and via cell-cell interaction with other cells. T cells can be classified as $CD8^+$ cytotoxic (Tc) and $CD4^+$ helper (Th) T cells. Tc cells are effector cells involved in cell-mediated cytotoxic immune responses. Activated Tc cells recognize specific antigenic peptides bound to major histocompatibility complex (MHC) class I presented on infected self cells or transformed cells and kill these cells by releasing perforin and granzymes (Doherty, 1993). Th cells are involved in both cell-mediated and humoral immunity via expression of cytokines. Activation of Th cells by antigen presenting cells leads to the

expression of interleukin (IL)-2 which is a growth factor for various immune cells, including T cells, and is responsible for the clonal expansion of T cells (Bromley et al., 2001). Macrophages and dendritic cells (DC) are the professional antigen presenting cells that present the processed fragments of the antigen on MHC Class II and activate the antigen-specific Th cells (Heath and Carbone, 2001). Subsequently, Th cells undergo proliferation and differentiation, which is also dictated by the cytokine environment (Dong and Flavell, 2001).

According to the profile of cytokine expression, differentiated Th cells can be categorized into at least two subsets, Th1 and Th2 (Dong and Flavell, 2001). Th1 cells produce Th1 cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-β and IL-2, which mediate activation of Tc cells, macrophages and natural killer (NK) cells and thereby promote cell-mediated immune responses. IFN-γ, originally called macrophage-activating factor that was dominantly produced by NK, Th and Tc cells, can increase the lysosome activity, the synthesis of inducible nitric oxide and antigen presentation of macrophages. In addition, IFN-γ can promote NK cell activity and Th1 differentiation and suppress Th2 activity (Billiau and Matthys, 2009). On the other hand, Th2 cells express Th2 cytokines, including IL-4, IL-5, IL-10 and IL-13, which promote humoral immunity via stimulating B cell proliferation and differentiation. B cells may differentiate into long-lived plasma cells to produce

neutralizing immunoglobulins (Ig) in the presence of T cell help, thereby prolonging the humoral immune responses (O'Connor et al., 2003). Th2 cytokines trigger the isotype switching of antibody to IgG_1, IgG_{2a} and IgE and suppress differentiation of Th1 cells. Two pivotal cytokines that control the Th1 and Th2 differentiation are IL-12 and IL-4, respectively. The balance of Th1/Th2 plays a pivotal role to maintain immune homeostasis (Dong and Flavell, 2001). The suppression of Th1 lymphocytes and the over-activation of Th2-mediated humoral immunity have been reported in numerous cancers, including oral cancers (Chang et al., 2005). Moreover, the generation of regulatory T cells, including some suppressive $CD8^+$ T cells, $CD4^+CD25^+Foxp3^+$ regulatory T cells and other subset of suppressive T cells by tumor-secreted mediators may suppress the host immunity against tumor formation (Mougiakakos et al., 2010).

1.5 Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC), broadly defined as CD11b and Gr-1 double positive cells in mice, are an important element in the suppressive immune network reported in cancers and other pathological conditions, including chronic inflammation, infectious diseases, sepsis and trauma (Greifenberg et al., 2009; Marigo et al., 2008; Ostrand-Rosenberg and Sinha, 2009). MDSC consist of precursors of macrophages, granulocytes and DC, and accumulate in blood, spleen and lymphoid

tissues with rapid turnover and immunosuppressive capacity (Ostrand-Rosenberg and Sinha, 2009; Youn et al., 2008). An increased number of circulating MDSC in cancer patients has been reported with a good correlation with disease stages (Diaz-Montero et al., 2009). Tumor-induced MDSC have been described to mediate immune suppression in cancer, such as head and neck cancer and hepatocellular carcinomas (Korangy et al., 2010; Pak et al., 1995). Tumor-secreted and host-secreted inflammatory factors, such as IL-1β, IFN-γ, prostaglandin E_2 (PGE_2) and SA100 proteins, have been shown to induce generation of MDSC, a critical mechanisms contributing to inflammation-mediated tumor promotion (Dolcetti et al., 2008; Ostrand-Rosenberg and Sinha, 2009).

Several cytokines, including IFN-γ, IL-4 and IL-13, can activate the intracellular signaling pathways, including signal transducer and activator of transcription (STAT)-1, STAT6 and nuclear factor (NF)-κB in MDSC (Ostrand-Rosenberg and Sinha, 2009). The accumulation of MDSC may compromise the immune surveillance and antitumor immunity by down-regulating $CD4^+$ and $CD8^+$ T cell proliferation and skewing immunity toward tumor-promoting phenotype (Gabrilovich and Nagaraj, 2009; Marigo et al., 2008; Ostrand-Rosenberg and Sinha, 2009). It has been reported that MDSC suppress T cells by increasing metabolism of surrounding $_L$-arginine, an essential amino acid for T cell activation. Up-regulation of

enzymes, including arginase-I and inducible nitric oxide synthase (iNOS), to metabolize $_{L}$-arginine into $_{L}$-ornithine and nitric oxide (NO) has been shown to be a crucial mechanism contributing to the immunosuppressive effects of MDSC (Bronte and Zanovello, 2005). MDSC may release abundant amount of reactive oxygen species (ROS) and peroxynitrite to inhibit the function of Tc cells by nitration of tyrosine residues on T cell receptor (TCR) to interrupt T cell-peptide-MHC interactions (Nagaraj et al., 2007). MDSC also suppress the function of T cells by down-regulating the expression of TCR-associated ζ-chain to block further signaling transduction (Baniyash, 2004). Production of immunosuppressive cytokines, such as IL-10, by MDSC is responsible for the suppression of the functions of DC, macrophages and T cells and the generation of regulatory T cells (Sinha et al., 2007).

1.6 Apoptosis

Apoptosis, a process of programmed cell death, is involved in maintaining physiological homeostasis, such as the maintenance of balanced immunity. The morphologic changes of apoptotic cells include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation (Cohen et al., 1992; Fesus, 1992). The induction of apoptosis of immunocompetent cells may lead to the impairment of immune competency (Hildeman et al., 2003). Numerous reports have documented the complex molecular mechanisms that regulate apoptosis. There are

two major apoptotic pathways, extrinsic and intrinsic pathways that are activated in responses to various stimuli, such as death signals and oxidative stress. Caspases are cysteine proteases involved in the intracellular signaling of apoptosis. For example, caspase-3 is a downstream caspase of both intrinsic and extrinsic pathways and functions as the central executioners of apoptosis (Cohen et al., 1992). Mitochondria participate in many cellular processes, including the intrinsic apoptotic pathway. Disruption of the integrity of mitochondrial outer membrane by death signals may cause the release of apoptotic molecules, such as cytochrome *c*. The formation of apoptosome by apoptotic protease activating factor 1 (Apaf-1), cytochrome *c* may further cleave the pro-caspase-9 into activated form, which subsequently induces cell apoptosis (Knudson and Brown, 2008; Mohamad et al., 2005). Tumor necrosis factor family members, such as Fas and TNF receptors, are involved in the extrinsic apoptotic pathways. When specific ligands, such as FasL or TNF-α binds to the death receptors, the conformation change of these receptors leads to interact with adaptor proteins resulting in formation of death-inducing signaling complex, which initiates the activation of caspase-8 and downstream caspase cascade (Fig. 3) (Boise et al., 1995; Opferman and Korsmeyer, 2003).

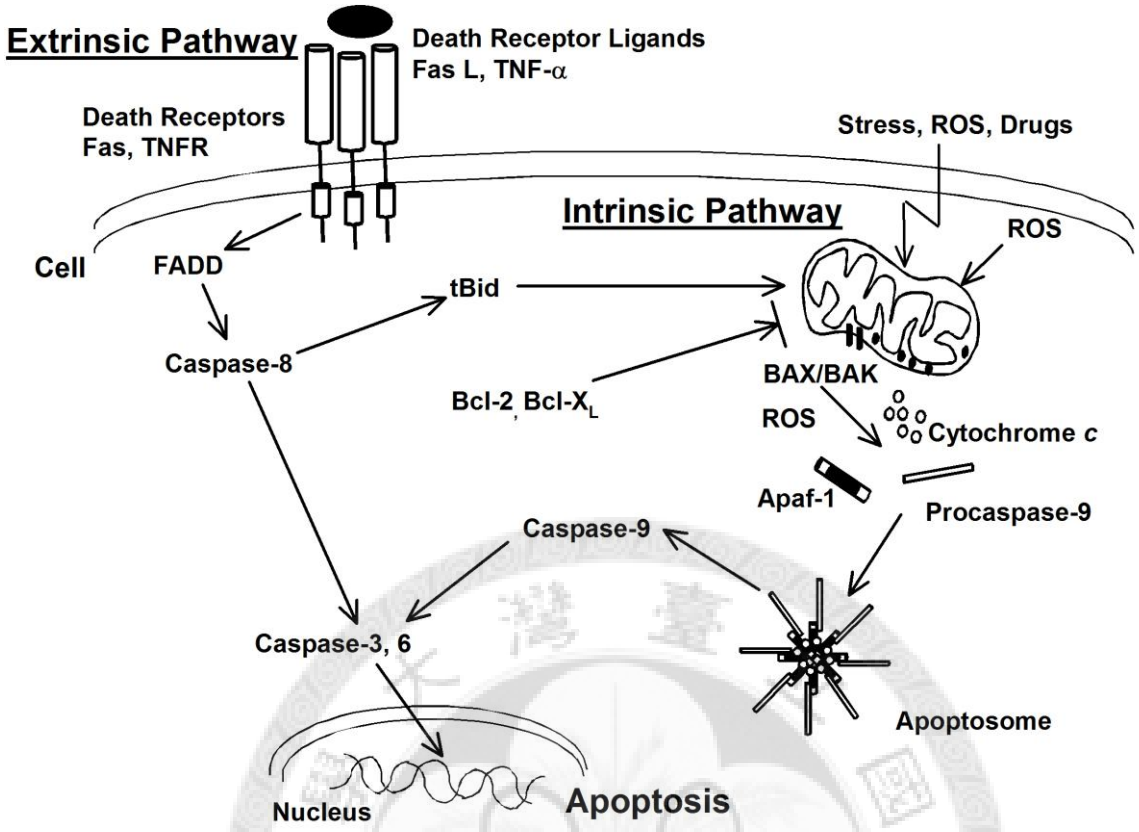


Figure 3. Caspase-dependent apoptosis pathways.

There are two major apoptosis pathways, the extrinsic and intrinsic pathways. The extrinsic pathway: ligation of death receptors, Fas or tumor necrosis factor receptors (TNFR), by their specific ligands induces the formation of a death-inducing signaling complex. Fas-associated death domain (FADD) then binds to the death domain (DD) of the receptors and further cleaves the procaspase-8 into activated caspase-8. The intrinsic pathway: apoptosis-inducing signals, ROS or stress, activate the pro-apoptotic members of the BCL_2 family, Bax or Bak, to permeabilize the mitochondrial outer membrane, leading to the release of cytochrome *c*. Apoptotic protease-activating factor-1 (Apaf-1) and cytochrome *c* then form an apoptosome that induces the cleavage and activation of procaspase-9. The activated caspase-8 and 9 further activate executioner caspases, such as caspase-3 and 6. The crosstalk between the extrinsic and intrinsic pathway mostly occurs through caspase-8 mediated cleavage of the Bid to truncated tBid, which activates Bax to trigger intrinsic pathway. Anti-apoptotic members such as Bcl-2 and Bcl-X_L bind and neutralize Bax and/or Bak.

1.7 Immune deterioration in AQ chewing-related oral diseases

1.7-1 Down-regulation of T cell responses

Immunohistochemical studies have shown that T lymphocytes were the major immunocompetent cells in both the subepithelial and epithelium of OSF tissues, while macrophages and B cells were the minor immunocompetent cells (Chiang et al., 2002b). The high number of infiltrated T cells and HLA-DR-positive cells in OSF tissues suggest a potential involvement of cellular immune responses in the OSF development (Haque et al., 1997). The induction of infiltrated T cells with high ratio of $CD4^+$ to $CD8^+$ cells implicates that T helper cells may play a role in the pathogenesis of OSF and oral cancer (Chiang et al., 2002b; Haque et al., 1997; Laad et al., 1996).

Nevertheless, it has been reported that the number of infiltrated T cells was significantly decreased during the development of advanced OSF and oral cancers (Chiang et al., 2002b; Pillai et al., 1987) and the frequencies of proliferating and cytotoxic lymphocytes from peripheral blood mononuclear cells (PBMC) and tumor tissue of oral cancer patients were markedly reduced (Laad et al., 1996). In addition, the proliferation and CD25 expression in mitogen-stimulated PBMC were depressed in leukoplakia and oral cancer patients (Murali et al., 1989). A diminished response to mitogenic stimuli was found in PBMC, tumor infiltrating lymphocytes and regional

lymph node cells of oral cancer and head and neck cancer patients (Heimdal et al., 1998; Murali et al., 1989; Verastegui et al., 2002). It has been reported a decrease in total number of $CD8^+$ T cells in regional and tumor-draining lymph nodes of oral cancer patients, indicating the deficiency of functional cytotoxic effector cells may deteriorate the antitumor immunity in oral cancers (Snyderman et al., 1991; Verastegui et al., 2002).

The functional disturbances were found in both Tc and Th cells of patients with oral cancer. A decrease number in total $CD4^+$ and activated $CD4^+$ cells in PBMC of oral cancer patients was observed (Laad et al., 1996). The production of IL-2 and IFN-γ by PBMC was diminished in patients with head and neck cancer and OSCC (Heimdal et al., 1998; Hsu et al., 2001; Murali et al., 1989). Collectively, ample evidence suggest that the alteration of lymphocyte subsets, the decline in the total number of T lymphocytes and the impairment of T cell functions may compromise the host T cell-mediated immunity. However, it is currently unclear whether AQ constituents affect the functionality of T lymphocytes.

1.7-2 Alteration of cytokine profile

Analysis of cytokine profile in OSF specimens showed an increased level of proinflammatory cytokines, such as IL-1, IL-6, TGF-β, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), and a decreased level of IFN-γ

(Haque et al., 1998). Furthermore, production of proinflammatory cytokines by PBMC of OSF patients was up-regulated, whereas the production of IFN-γ was down-regulated (Haque et al., 2000). Expression of IL-2 and IFN-γ by PBMC of patients with early-stage oral cancer was higher compared to normal control subjects, but high levels of IL-4 and IL-10 were expressed in PBMC of patients with advanced oral cancers. It seems that the development of oral cancer may skew the expression of cytokines from the Th1- toward the Th2-dominant direction (Agarwal et al., 2003). It has been reported that the production of IL-2 by phytohemagglutinin (PHA)-stimulated PBMC of patients with leukoplakia, OSF and oral cancer was decreased (Murali et al., 1989), whereas the secretion of IFN-γ by PBMC of advanced OSF and oral cancer patients was diminished (Hsu et al., 2001). Interestingly, the production of IFN-γ but not IL-2 by PBMC in AQ chewers without precancer lesions, was significantly lower than normal control subjects (Hsu et al., 2001). IFN-γ exhibits anti-fibrotic activity via the inhibition of collagen synthesis (Lee et al., 1991); intralesional injections with IFN-γ may improve the mouth opening in OSF patients (Haque et al., 2001). Together these data suggests that the down-regulation of IFN-γ may be involved in the pathophysiology of the oral diseases. However, little is known regarding the effect of AQ constituents on the production of IFN-γ by T cells.

1.7-3 Dysfunction of humoral immunity

Epidemiological evidence revealed the augmentation of circulating immune complexes and autoantibodies in OSF and oral cancer patients, indicating the deregulation of humoral immunity in AQ chewers (Balaram et al., 1987; Chiang et al., 2002a). The levels of serum IgA, IgD and IgE were elevated in OSF and oral cancer patients (Rajendran et al., 1986). The high incidence of antinuclear, anti-smooth muscle, anti-gastric parietal cell and anti-thyroid microsomal autoantibodies has been documented in patients with OSF (Canniff et al., 1986). The presence of these autoantibodies was significantly associated with daily consumption of AQ and might be induced by altered autoantigens released from AQ components-damaged cells (Chiang et al., 2002a). Although the total number of rosette-forming cells was unaltered, the number of high-affinity rosette-forming cells was significantly diminished in patients with OSF (Rajendran et al., 1986). In premalignant lesions, it has been reported that both the population and number of B cells were significantly increased (Pillai et al., 1987). On the contrary, a decrease in B cells was reported in oral precancerous patients (Dammer et al., 1997). Together, the above findings indicate a dysfunction and/or deregulation of B cell-mediated immunity in AQ-related oral diseases. However, it is currently unclear if AQ components affect the humoral immunity.

1.7-4 Immunosuppression associated with oral diseases

The activity of NK cells in oral precancer and cancer patients has been shown to be diminished with a good correlation with the progression of oral cancer (Balaram et al., 1992; Pillai et al., 1990). A reduced activity of lmyphokine-activated killer cells has been also reported (Tatake et al., 1989). The induction of $CD4^+CD25^+$ regulatory T cells and immunosuppressive factors, including PGE_2, TGF-β and IL-10, was found in head and neck squamous cell carcinomas (HNSCC) (Rice et al., 1992; Young et al., 1996). These immunosuppressive cytokines secreted by tumor-infiltrating lymphocytes or tumor cells may suppress the function of both $CD4^+$ and $CD8^+$ lymphocytes and modulate the progression of tumor cells (Rice et al., 1992; Wustrow and Mahnke, 1996).

Interestingly, generation of $CD34^+$ granulocyte-macrophage progenitor inhibitory cells has been shown to decrease the function of $CD4^+$ T cells in both primary and metastatic HNSCC (Young et al., 1996). The induction of $CD11b^+Gr\text{-}1^+$ cells, broadly defined as MDSC in mice, has been reported in mice bearing transplants of oral carcinoma cells (Tanaka et al., 2007). The high level of FasL expressed on the HNSCC was proposed to be associated with the induction of T cell apoptosis in the tumor microenvironment (Gastman et al., 1999; Reichert et al., 2002). In addition, the down-regulation of ζ-chain expression in peripheral blood

lymphocytes and tumor-infiltrated lymphocytes of patients with oral cancer was found to correlate with lymphocyte apoptosis (Reichert et al., 2002). The decrease in the number of mature DC and the presence of $CD123^+$ plasmacytoid DC in tumor sites were associated with a poor outcome in oral cancer patients (O'Donnell et al., 2007). Vascular endothelial growth factor secreted by oral cancer cell lines might inhibit the differentiation of $CD1a^+$ DC from progenitor cells and increase the number of dysfunctional $CD83^+$ DC (Kikuchi et al., 2006). Taken together, oral cancer cells have been shown to produce factors that suppress mitogenic capacity of NK, Th and Tc cells, attract regulatory T cells and MDSC, induce lymphocyte apoptosis and interfere with the function of antigen-presenting cells, to compromise host cell-mediated immunity. To date, little is known regarding the role of AQ constituents in the immunosuppression associated with oral diseases.

1.8 The direct effect of AQ components on oral and immune cells

1.8-1 Mitogenic and cytotoxic effect of AQ components on oral cells

Carcinogenesis, chronic inflammation and immune dysfunction are the three major potential mechanisms responsible for the generation of areca-related oral cancer (Chang et al., 2005; Jeng et al., 2001; Nair et al., 2004). The toxicity of AQ components on oral cells and the underlying mechanisms has been under extensively investigation. An ethanolic extract of areca nuts has been shown to stimulate the

synthesis of collagen by oral mucosal fibroblasts and this effect may result from the contents of arecoline and arecaidine in the extract (Canniff and Harvey, 1981). In addition, arecoline and arecaidine stimulated the proliferation of fibroblasts from normal or OSF buccal mucosa (Chen et al., 1995). The direct stimulation of fibroblast growth and collagen synthesis by areca nut extract (ANE; 10 μg/mL) or its alkaloids may be involved in the pathophysiology of OSF. On the contrary, high concentrations of ANE (50-150 μg/mL) and arecoline (100 μg/mL) have been reported to suppress the growth of fibroblasts (Jeng et al., 1994; Jeng et al., 1996). Furthermore, arecoline inhibited the attachment, spreading and migration of fibroblasts, which was associated with the depletion of cellular glutathione (GSH) (Jeng et al., 1996). In addition, arecoline induced cell death and cell cycle arrest by inhibiting mitochondrial activity and depleting intracellular thiols (Chang et al., 2001b). The cytotoxicity of ANE and arecoline has also been demonstrated in gingival keratinocytes and oral epithelial KB cells (Chang et al., 2004; Jeng et al., 1999; Jeng et al., 2000). The induction of unscheduled DNA synthesis (UDS) of keratinocytes by ANE was concomitant with the presence of intracellular vacuolization indicating the genotoxic effect of ANE (Jeng et al., 1999); glutathione, N-acetyl-$_L$-cysteine dramatically reversed ANE-induced UDS in keratinocytes (Chang et al., 2002). ANE and arecoline caused cell cycle arrest by disrupting $\Delta\psi_m$ and increasing H_2O_2 production in KB epithelial

cells (Chang et al., 2001a). Arecoline induced cell apoptosis and interfered with the regulation of S and/or G2/M cell cycle-related proteins in keratinocytes and KB cells (Lee et al., 2006). Moreover, areca nut ingredients caused oxidative damage in keratinocytes and CHO-K1 cells (Lai and Lee, 2006; Liu et al., 1996), as well as in the oral cavity of volunteers chewing areca quid and the buccal pouch of hamsters exposed to ANE (Chen et al., 2002; Chiang et al., 2004). The elevated expression of heme oxygenase-1, an oxidative stress responsive protein, in oral cancer specimens may provide a protective response for tumor cells against oxidative stress (Lee et al., 2008). Taken together, these results suggest a role for oxidative stress in ANE-induced cytotoxicity in oral cells. Since ANE contains a rich amount of polyphenols, the auto-oxidation of ANE-derived polyphenols to generate radicals has been speculated to be a contributing factor for ANE-mediated cytotoxicity and oxidative stress in oral cells (Liu et al., 1996).

1.8-2 Proinflammatory effect of AQ components

Abundant epidemiological and experimental data show a strong correlation between inflammation and cancer formation. The inflammation of keratinocytes has been documented to play a critical role in the progression of oral cancer (Barker et al., 1991). ANE induced the production of PGE_2 and 6-keto-$PGF_{1\alpha}$ in keratinocytes; Up-regulation of cyclooxygenase-2 (COX-2) expression leading to the production of

prostaglandins was associated with tissue inflammation (Jeng et al., 2000). The production of IL-6, TNF-α and PGE_2 was increased after exposure to ANE in keratinocytes (Jeng et al., 2003). The activation of NF-κB, c-Jun N-terminal kinase and extracellular signal-regulated kinase by ANE has been demonstrated in keratinocytes (Lin et al., 2005). Additionally, ANE enhanced the production of IL-6, TNF-α, PGE_2 and PGI_2 by oral epithelial cells (Jeng et al., 2000; Jeng et al., 2003). It has been reported that ANE augmented the production of IL-1β, TNF-α, IL-6 and IL-8 by human PBMC (Chang et al., 2009; Chang et al., 2006). Together, these reports demonstrated the direct stimulatory effect of AQ components on the production of proinflammatory cytokines by both oral cells and immune cells. The induced inflammatory mediators may attract more immune cells infiltrating into local lesions and prolong the inflammation that lead to further tissue damage and tumor promotion.

1.8-3 Immunomodulatory effect of AQ components

As areca chewing is the main etiological factor for oral cancer whose pathogenesis is associated closely with immune deterioration, it is therefore crucial to know whether areca ingredients inhibit the functions of immune cells. ANE has been reported to inhibit the DNA synthesis in the PHA-stimulated human lymphocytes (Yang et al., 1979). The activation of PHA-induced $CD4^+$ and $CD8^+$ lymphocytes was

inhibited by the conditioned medium of ANE and arecoline-treated keratinocyte culture (Jeng et al., 2003). Arecoline markedly suppressed proliferation and IL-2 production of splenocytes. Subcutaneously injection of arecoline in mice suppressed the delayed type hypersensitivity responses and anti-sheep red blood cell antibody production (Selvan et al., 1991). The production of IL-2, TNF-α and TGF-β by PBMC of healthy or OSF donors was attenuated by exposure to arecoline *in vitro* (Hsu et al., 2001). Moreover, pro-apoptotic effect of arecoline has been reported in splenocytes *in vivo* (Dasgupta et al., 2006). ANE, arecoline and arecaidine induced the sister chromatid exchanges and chromosomal breaks in murine bone marrow cells (Panigrahi and Rao, 1982, 1984, 1986). The chromosomal aberrations in PBMC of ANE and arecoline-treated mice were associated with ROS production and cellular GSH level (Kumpawat et al., 2003). It has been shown that the phagocytic and bactericidal activities of neutrophils were suppressed by ANE (Hung et al., 2006). ANE induced the apoptosis and necrosis of neutrophils, in which glycogen synthase kinase-3α/β may be involved (Ho et al., 2010). Production of inflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α, by PBMC was elevated by ANE (Chang et al., 2009; Chang et al., 2006). Together these results demonstrated that neutrophils and lymphocytes were sensitive targets for ANE. However, little is known regarding the direct effect of ANE on T cell function and T cell-mediated immune responses.

Chapter 2. Rationale

AQ chewing is one of the most popular addictive habits in the world. In Taiwan, the number of AQ chewers has been estimated to be more than 2 million among the 23 million inhabitants. Epidemiological data suggest that AQ chewing is one of the major risk factors for the development of OSF and OSCC. A line of evidence suggests that the pathogenesis of AQ-related oral diseases is closely associated with immune deterioration in the patients. Deficiencies in T cell-mediated immunity have been reported in patients with OSF and OSCC. The impairment of T cell activation and functions, the deregulation of T cell cytokine production, and the generation of immunosuppressive cells have been found in oral diseases. As AQ chewing is the main etiological factor for oral cancer whose pathogenesis is closely linked to immune dysfunction, it is vital to study whether areca ingredients suppress the functions of T cells and induce immunosuppressive cells.

T cells play a pivotal role in the regulation of cell-mediated immune responses against infection and tumor formation. Clinical reports indicated that the T cells are the major immunocompetent cells in the oral tissues of OSF patients. Moreover, the decreased production of IFN-γ was reported to be involved in the pathogenesis of OSF. However, little is known regarding the direct effect of AQ ingredients on T cell functions and its underlying mechanisms. Among different geographic locations,

areca nuts are the major component of areca quid. It has been shown that the phagocytic and bactericidal activities of neutrophils were suppressed by ANE. ANE have been documented to be toxic to various immune cells, including bone marrow cells, thymocytes and splenocytes. Cytotoxicity, carcinogenicity and genotoxicity of ANE have been well documented in many oral cells. ANE is cytotoxic to epithelial cells, fibroblasts and keratinocytes by inducing cell cycle arrest or apoptosis. In addition, the activity of areca ingredients to induce reactive oxygen species and diminishment of cellular thiols in oral cells and bone marrow cells has been documented. An induction of oxidative damage in the hamster's buccal pouch exposed to ANE and the increased ROS level detected in the oral cavity of AQ chewers indicate a crucial role of ROS in areca-induced cytotoxic effect. On the base of these lines of evidence, it is hypothesized that ANE may possess direct deteriorating effects on the activity and cytokine production by T cells. To test this hypothesis, the direct influence of ANE on T cell viability and cytokine production was studied *in vitro*. The enhancement of cell apoptosis, impairment of mitochondrial function, induction of ROS and depletion of cellular thiols were investigated to study the underlying mechanisms of ANE-mediated cytotoxic effects on T cells. In addition, the *in vivo* effect of ANE and areca-derived procyanidins on generation of myeloid-derived suppressor cells was investigated.

Chapter 3. Materials and Methods

3.1 Reagents and areca nut extract

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Areca nut extract (ANE) was prepared as previously described (Liu et al., 1996). Briefly, nuts were sliced and extracted 3 times with water and filtered. The filtrate was freeze-dried as ANE. A polyphenol-enriched areca nut extract (PANE) was prepared as previously described (Wang and Lee, 1996). Briefly, nuts were extracted 3 times with 80% aqueous acetone (1:10, w/v) and filtrated. The filtrate was evaporated to remove acetone, partitioned with n-hexane and ethyl-ether to remove lipids and then freeze-dried that consisted of 90% condensed tannins (unpublished data). The fractionation of PANE was performed by normal-phase HPLC as previously described (Gu et al., 2006). In brief, PANE was dissolved in 30% aqueous methanol for application to a 250 × 4.6 mm Phenomenex Luna Silica column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) dichloromethane: methanol: 50% acetic acid = 82: 14: 4, and (B) methanol: 50% acetic acid = 96: 4. The gradient was programmed at: 0-20 min, 14-23.6% B; 20-50 min, 23.6-35% B; 50-55 min, 35-86% B; 55-65 min, 86% B; 65-70 min, 86-14% B with 1 mL/min flow rate and monitored at 280 nm with UV detector. The chromatographic distribution of monomeric fraction was confirmed with authentic

standards, catechin and epicatechin. To measure the degree of polymerization, each fraction was thiolyzed and analyzed by reverse phase-HPLC using a LichroCART C_{18} 250 × 4.6 mm column (Merck & Co., Germany). The mobile phase consisted of (A) 0.1% acetic acid and (B) 0.1% acetic acid in acetonitrile. The gradient was as follows: 0-8 min, 8% B; 8-40 min, 8-24% B; 40-50 min, 24-80% B; 50-55 min, 80-8% B and the flow rate was 1 mL/min (Monagas et al., 2003). All signals were detected at 280 nm with UV detector (Gu et al., 2006). The PANE used in this study contained dimers (6.5%), trimers (5.2%), tetramers (5.8%), pentamers (3.8%), hexamers (3.4%), heptamers (5.0%), octomers (3.6%), nonamers (2.9%) and decamers (3.7%). The chromatography was repeated three times with similar results. The ANE and PANE have been confirmed to be endotoxin free using a commercial Limulus amebocyte lysate assay kit (Kinetic-QCL® ; Lonza Walkersville, Walkersville, MD, USA).

3.2 Animals

Male BALB/c mice, 5–6 weeks of age were purchased from the Animal Breeding Center of the National Taiwan University Hospital (Taipei, Taiwan). On arrival, the mice were randomized, transferred to plastic cages containing a saw-dust bedding (five mice per cage) and quarantined at least for 1 week. The animal was housed in a temperature (25±2℃), humidity (50±20%), and light-controlled environment (12 h light/dark cycle) with free access to standard laboratory food and

water.

3.3 Protocol of ANE treatment and OVA sensitization

Male BALB/c mice (6-8 weeks old; 4–5 animals/group) were either left untreated (naïve; NA), or daily administered by intraperitoneal (IP) injection with ANE (5, 25, 50 mg/kg; 0.1 mL/mouse), PANE (25 mg/kg; 0.1 mL/mouse) arecoline (5 mg/kg) and/or vehicle (VH; saline) on day 1–5 and day 8–12. Except for the NA group, mice were sensitized with OVA 6 h after the ANE administration on day 3 as described above (Fig. 26). The mice were then sacrificed, and their blood samples and spleens were harvested for further experimentation. The animal experiments were approved by the Institutional Animal Care and Use Committee of the National Taiwan University.

3.4 Cell isolation and culture

Spleens were isolated aseptically and made into single cell suspensions as described previously (Kaminski et al., 1994). Briefly, the spleens were washed three times with RPMI-1640 medium and made into single cell suspension by pressing spleens with microtubes in 5 cm petri dishes in RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 5% heat inactivated FBS (referred to as complete RPMI). Cells were transferred into a 15 mL microtube and the tube was put on ice for 3 min to remove the debris. Splenocytes were centrifuged

at 1200 x rpm for 7 min and the pellet was resuspended with medium after decanting the suspension. After repeating the washing steps for three times, the erythrocytes were lyzed by ACK buffer (0.15M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) and washed 2 more times to get single cell suspensions. Peripheral blood mononuclear cells (PBMC) were separated from the peripheral blood by standard gradient centrifugation with Ficoll-Hypaque. The blood samples was centrifuged at 3000 x g for 15 min, and buffy coat were collected and the residuary erythrocytes were lyzed by ACK buffer and wash 3 times to remove the residue erythrocytes. Splenocytes and PBMC were cultured in complete RPMI at 37℃ in 5% CO_2.

3.5 Spleen index

The degree of splenomegaly was expressed as the spleen index which was calculated as the spleen weight (mg) per body weight (g). Animals were sacrificed and their spleens were dissected out and weighed immediately. Body weight was measured before sacrifice.

3.6 MTT assay

The metabolic activity of the cells was determined by the 3-(4,5-dimethyl--thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay previously described (Mosmann, 1983). Briefly, cells were cultured in triplicate (100 μL/well) in 96-well culture plates and received ANE treatments with various concentrations (*in vitro*

studies) or left untreated (*ex vivo* studies), followed by stimulation with phorbol 12-myristate 13-acetate plus ionomycin (PI; 80 nM/1μM) for 24 h, concanavalin A (ConA; 5 μg/mL) for 48 h, lipopolysaccharide (LPS; 5 μg/mL) for 48 h or ovalbumin (OVA; 50 μg/mL) for 72 h . After stimulation, a MTT stock solution (5 mg/mL in PBS) was added to each well (10 μg/well) and incubated for 4 h. The formed formazan was then dissolved by adding 100 μL lysis buffer (10% SDS in *N*,*N*-demethylformamine) per well overnight in the dark. The plate was read using a microplate reader (Dynatech Laboratories, Chantilly, VA, USA) at 570 nm using 630 nm as background reference.

3.7 Enzyme-linked immunosorbent assay (ELISA) for cytokine quantification

Mouse recombinant IL-2, IL-4, IFN-γ and IL-10 standards, purified rat anti-mouse IL-2, IL-4, IFN-γ and IL-10 antibodies and biotinylated anti-mouse IL-2, IL-4, IFN-γ and IL-10 antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Splenocytes (5×10^6 cells/mL) were cultured in triplicate in 48-well plates (0.25 mL/well) and treated with ANE or left untreated (*in vitro* studies) or left untreated (*ex vivo* studies), followed by stimulation with PI (80 nM/1 μM) for 24 h, ConA (5 μg/mL) for 48 h, LPS (5 μg/mL) for 48 h or OVA (50 μg/mL) for 72 h. The supernatants from each well were collected and quantified for IL-2, IL-4, IFN-γ and

IL-10 by standard sandwich ELISA as previously described (Wang et al., 2007). Briefly, ELISA plates were coated overnight at 4℃ with 50 μL/well of 0.1% purified antibody or 0.05% OVA in 0.1 M $NaHCO_3$ buffer (pH 8.2). After washing with phosphate-buffered saline containing 0.02% Tween 20 (PBST), wells were blocked with 200 μL/well of 1% bovine serum albumin in PBST (blocking buffer) for 1 h at room temperature (RT). After washing with PBST, samples at appropriate dilutions were added into wells (50 μL/well) and incubated for 1 h at RT. Wells were again washed three times and 50 μL/well of the paired biotinylated antibody (0.05% in blocking buffer) was added followed by incubation for 1 h at RT. After washing, 50 μL/well of streptavidin peroxidase was added and incubated for 1 h at RT. Lastly, the plates were washed six times and the bound peroxidase conjugate was detected by addition of substrate solution (50 μL/well) for appropriate time. 6N H_2SO_4 (150 μL/well) was added to terminate the reaction and optical density was measured at 450 nm using a microplate reader (Dynatech Laboratories, Chantilly, VA, USA).

3.8 Cell cycle analysis of splenic lymphocytes

Cell cycle distribution of splenocytes was determined by flow cytometry using propidium iodide staining. Splenocytes were washed twice with PBS (pH 7.4) and fixed with 70% ethanol at -20°C overnight, followed by incubation with RNase (0.1 mg/mL) and propidium iodide (50 μg/mL) for 30 min at 37°C in the dark. After

staining, the cell suspensions were filtrated with mesh to remove aggregated cells and transferred to flow tubes. The single cell fluorescence of 5,000 cells for each sample was measured by excitation at 488 nm and detected in the FL2 channel (575 nm emission filter) using a BD FACS Calibur flow cytometer (San Jose, CA). The list mode data were analyzed using the software Flowjo 8.0. DNA histograms of splenocytes display the characteristic cell-cycle distribution with the first peak corresponding to the diploid peak (G_0/G_1-phase cells) and the second peak to cells with doubled DNA content (G_2/M-phase cells). S-phase cells are with an intermediate DNA content. The apoptotic cells were defined as sub- G_0/G_1-phase cells with hypodiploid DNA content (Nicoletti *et al*., 1991).

3.9 Terminal dUTP nick-end labeling (TUNEL) assay

Splenocytes were washed twice with PBS (pH 7.4) and fixed with 70% ethanol for 1 h. The DNA single strand breaks of apoptotic cells were determined by a commercial TUNEL assay kit (Roche Diagnostics GmbH, Penzgerg, Germany). Briefly, fixed cells were stained with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP for 1 h at 37°C. After washing with PBS, the single cell fluorescence of 5,000 cells for each sample was measured using a flow cytometer at emission of 525 nm (FL1). The data were analyzed by the software Flowjo 8.0.

3.10 Hoechst staining

The cells (1.2 x 10^5 cells in 100 μL) were spun onto a glass slide by a cytospin centrifuge at 500 x rpm for 10 min at RT. The slides were then fixed in 100% acetone at -20°C for 10 min. The fixed cells were stained with Hoechst 33258 (1 μg/mL) for 5 min at RT in the dark, and then washed with PBS. The slides were examined and photographed using a fluorescence microscope (Optiphoto II, Nikon, Tokyo, Japan) with an excitation wavelength of 330-380 nm.

3.11 Determination of mitochondria membrane potential by JC-1 staining

The mitochondria membrane potential ($\Delta\psi_m$) of splenic lymphocytes was measured using 5,6,-tetrachloro-1,3,-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) as described previously (Cossarizza et al., 1993). JC-1 is a cationic dye that is accumulated in mitochondria to forms JC-1 aggregates (590 nm emission; orange color) in proportion to $\Delta\psi_m$. When $\Delta\psi_m$ decreases, JC-1 aggregates depart from mitochondria and change to JC-1 monomers (530 nm emission; green color). Therefore, JC-1 has been used to detect the occurrence of $\Delta\psi_m$ depolarization in the early stages of apoptosis (Salvioli et al., 1997). Briefly, the cells were washed and then incubated with 500 μL JC-1 (2.5 μg/mL dissolved in RPMI-1640) for 20 min at RT. After washing, the cells were resuspended in 200 μL PBS and transferred to flow

tubes. The single cell fluorescence of 10,000 cells was measure using a flow cytometer at emission of 525 and 590 nm. The data were analyzed using the software Flowjo 8.0.

3.12 Western blotting analysis for cytochrome *c*

Splenocytes (1×10^7) were cultured in a 10 cm petri-dish (10 mL/dish) for treatment of ANE (10–60 μg/mL) for 3 h. At the end of incubation, cells were immediately harvested on ice and washed twice with cold PBS (pH 7.4) and then resuspended in 50 μl lysis buffer (250 mM sucrose, 10 mM HEPES-KOH, pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and protease inhibitors) on ice for 1 h. Mitochondrial and cytosolic fractions were prepared by following the subcellular fractionation procedure previously described (Yu, 2002; Fukumori, 2003). The lysates were then passed through a 26-gauge syringe 15 times and centrifuged at 1,200 x g to completely remove nuclei and cell debris. Next, the supernatant was subjected to centrifugation at 10,000 x g to isolate mitochondria. The pellet (mitochondrial fraction) was resuspended in hypotonic RIPA buffer (containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 10 μg/mL aprotinin, DTT and leupetin) and the supernatant was collected as cytosol fraction and was further centrifuged at 13,000 x g to eliminate DNA fragment. Fractions were boiled for 5 min at 95℃ in loading buffer (62.5 μM Tris-HCl, pH 6.8, 2% SDS, 5%

0.01% -bromophenol blue), subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PDVF membrane (Pierce) for western blot analysis. The membrane was incubated for cytochrome *c* monoclonal antibody (Biosource, Camarillo, USA). Positive bands were identified using the enhanced chemiluminescence system (Pierce, Rockford, USA) and quantified by software (Image quantity).

3.13 Detection of caspase activation

The activation of caspase-3, -8 and -9 was measured using cell-permeable specific substrate of caspase-3, -8 and -9 (FAM-DEVD-FMK, FAM-IETD-FMK and FAM-LEHD-FMK, respectively) (Chemicon, Temecula, CA). In brief, splenic lymphocytes were treated with ANE (20 μg/mL) for 1-12 h. After washing, the cells were incubated with different substrates of caspases for 1 h at 37℃ in the dark, and then washed again. The single cell fluorescence of 10,000 cells for each sample was measured by a flow cytometer at emission of 525 nm. The data were analyzed using the software Flowjo 8.0.

3.14 Measurement of ROS level

2',7'-Dichlorofluorescein diacetate (DCF-DA), a cell-permeable probe that emits fluorescence upon oxidization by hydrogen peroxide, was used to monitor intracellular generation of ROS. Splenic lymphocytes were either left untreated (NA)

or treated with ANE. After washing, the cells were resuspended in 0.5 mL PBS, and stained with DCF-DA (20 μM) for 15 min at 37℃ in the dark. After incubation, cells was washed twice and then transferred to flow tubes. The single cell fluorescence of 10,000 cells for each sample was measured using a flow cytometer at emission of 525 nm. The data were analyzed using the software Flowjo 8.0.

3.15 Detection of intracellular thiols using flow cytometry

5-Chloromethylfluorescein diacetate (CMF-DA), a cell-permeable probe that emits green fluorescence by reacting with cellular thiols, was used to measure intracellular levels of thiols. After treatment, cells were stained with 25 μM of CMF-DA for 25 min at 37℃ in the dark. The single cell fluorescence of 10,000 cells for each sample was measured using a flow cytometer at emission of 525 nm. The data were analyzed using Flowjo 8.0.

3.16 Cellularity of PBMC and splenocytes

The expression of $CD4^+$, $CD8^+$, $B220^+$, $Gr\text{-}1^+$ and $CD11b^+$ by splenocytes and PBMC was measured by flow cytometry. Briefly, splenocytes and PBMC (10^6 cells in 20 μL) were stained with 1 μL of rat anti-mouse CD4 and Gr-1 conjugated with FITC and rat anti-mouse CD8, CD11b, B220 conjugated with PE-Cy5 antibodies (BioLegend; San Diego, CA) in staining buffer (PBS containing 2% FBS and 0.09% sodium azide) in the dark on ice for 30 min. Appropriate rat anti-mouse antibodies

were employed as the isotype control for evaluating non-specific binding. After washing, the single cell fluorescence of 10,000 cells for each sample was measured by a flow cytometer (BD FACSCalibur, San Jose, CA, USA). Date was analyzed using Flowjo 8.0 software.

3.17 Measurement of Gr-1 expression and morphology of splenic CD11b$^+$ cells

Splenic CD11b$^+$ cells were isolated using a commercial kit (BD IMag Cell Separation System; BD Biosciences, San Jose, CA) according to the supplier's instructions. The purity of CD11b$^+$ cells was determined to be ≥ 90% by flow cytometry. For morphologic examination, CD11b$^+$ cells (2.5 x 10^5 cells) were centrifuged onto slides by a cytospin centrifuge and fixed in 100% pre-cold acetone at –20℃ for 10 min. The cells were stained with Diff-Quick dye according to standard protocols, examined and photographed under a microscope (Optiphoto II, Nikon, Tokyo, Japan). For measurement of Gr-1 expression, CD11b$^+$ cells were examined by flow cytometry using a rat anti-mouse Gr-1 antibody conjugated with FITC.

3.18 Detection of arginase-I activity

The activity of arginase-I was measured as described previously with minor modification (Corraliza et al., 1994). Splenic CD11b$^+$ cells (2 x 10^6 cells) were stimulated with LPS for 18 h. After washing, the cells were lyzed with 50 μL 0.4%

Triton X-100 containing proteinase inhibitor cocktails for 30 min at 37℃. Lysates were centrifuge at 1,200 x rpm to move cell debris. 45 μL of lysates were added to 45 μL of 25 mM Tris-HCl (pH 7.4) and 10 μL of 10 mM $MnCl_2$ and heated for 10 min at 56℃ to activate arginase. Arginine hydrolysis was conducted by incubating the lysates with 100 μL of 0.5 M $_L$-arginine (pH 9.7) at 37℃ for 2 h. The reaction was stopped with 400 μL of H_2SO_4-H_3PO_4-H_2O at a ratio of 1:3:7. For colorimetric determination of urea, 100 μL of 9% α-isonitrosopropiophenone (dissolved in ethanol) was added to tube and heated at 100℃ for 60 min. The urea concentration was measured at 540 nm. The standard curve of urea was performed in parallel (1-100 μM of urea).

3.19 Reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA expression of iNOS and arginase-I was analyzed by RT-PCR. 100 ng total RNA of each sample was reversely transcribed into cDNA by 40 units of MMLV reverse transcriptase using oligo-dT as the primer. The reverse transcription proceeded as the following: 42℃ for 15 min and 95℃ for 5 min. The PCR mixture containing PCR buffer, 4 mM $MgCl_2$, 6 pmole each of forward and reverse primers, and 2.5 units of *Taq* DNA polymerase was added to each cDNA sample. Samples were heated to 94℃ for 3 min and cycled 24–34 times at 94℃ for 15 sec, 60℃ for 30 sec, and 72℃ for 30 sec followed by an additional step at 72℃ for 5 min. The PCR products were

electrophoresed in 2% agarose gels and visualized with ethidium bromide staining. The DNA products were quantified using the alpha imager 1200 digital imaging system (Alpha Innotech Crop, San Leandro, CA). The expression level of the housekeeping gene β-actin was employed as the control for semi-quantification of target genes. The sequence of primers for RT-PCR is shown below:

Primer		Sequence	Product Size (bp)	Cycle
iNOS	Forward	CATGGCTTGCCCCTGGAAGTTTCTCTTCAAAG	828	32
	Reverse	GCAGCATCCCCTCTGATGGTGCCATCG		
Arginase-I	Forward	CAGAGTATGACGTGAGAGACCAC	491	34
	Reverse	CAGCTTGTCTACTTCAGTCATGGAG		
β-actin	Forward	AGGGAAATCGTGCGTGACATAAAA	478	24
	Reverse	ACTCATCGTACTCCTGCTTGCTGA		

Chapter 4. Experimental Results

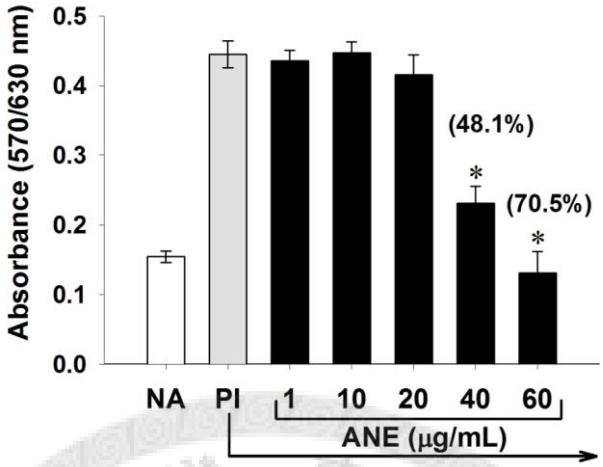
4.1 ANE suppresses T-cell activation and cytokine production via the induction of oxidative stress

4.1-1 Cytotoxicity of ANE on splenocytes

ANE are known to be cytotoxic to a number of oral cells. To study the cytotoxicity of ANE on immune cells, murine splenocytes were treated with ANE (1–60 μg/mL) for 24 h in the presence of phorbol-12-myristate-13-acetate plus ionomycin (PI; 80 nM/1 μM). The cytotoxicity of ANE was determined by the MTT assay that measures the metabolic activity of viable cells. The range of concentrations between 1 and 60 μg/mL of ANE was selected according to previous reports demonstrating that these concentrations inhibited the phagocytic activity of neutrophils (Hung et al., 2005; Hung et al., 2006). As expected, ANE inhibited the metabolic activity of splenocytes in a concentration-dependent manner (Fig. 4A). The magnitude of inhibition induced by ANE 40 and 60 μg/mL was 48.1% and 70.5%, respectively. Since ANE has been shown to induce oxidative stress in oral cells, the thiol-antioxidant NAC was employed to further study the involvement of oxidative stress in ANE-mediated cytotoxicity. NAC markedly attenuated ANE-mediated cytotoxicity. In the presence of NAC the magnitude of inhibition on splenocyte

metabolic activity by ANE 40 and 60 μg/mL was 15.6% and 34.7%, respectively (Fig. 4B).

(A) Without NAC



(B) With NAC

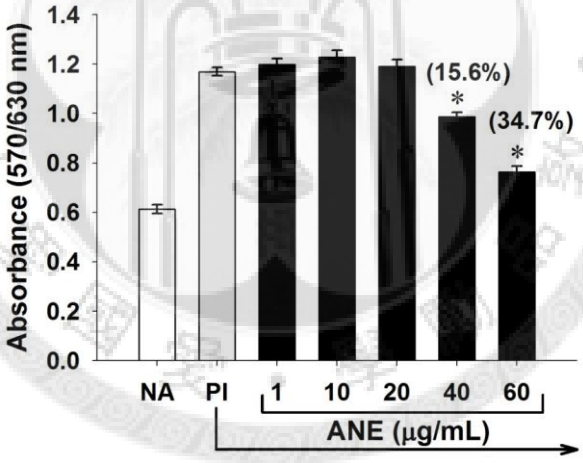


Figure 4. The cytotoxic effects of ANE on murine splenocytes.

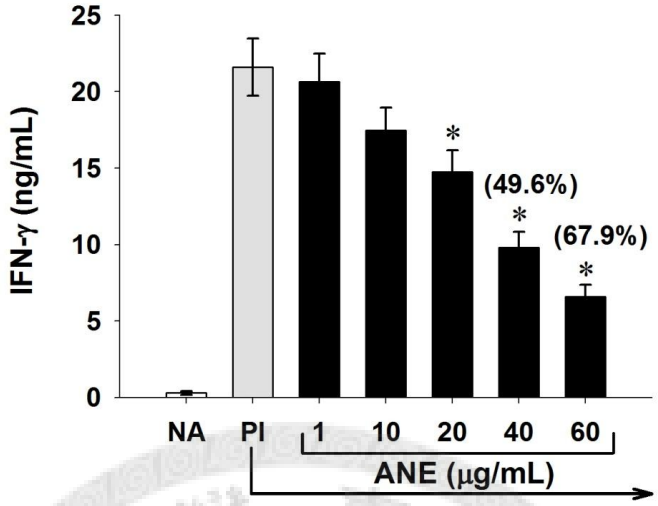
Splenocytes (5×10^6 cells/mL) were either untreated (NA), or treated with ANE (1-60 μg/mL) in the absence (A) or presence (B) of pretreatment of *N*-acetyl-$_L$-cysteine (NAC; 4 mM) for 30 min. Except for the NA group, cells were stimulated with phorbol 12-myristate 13-acetate plus ionomycin (PI; 80 nM/1 μM) for 24 h. The metabolic activity of viable cells was determined using the MTT assay. Data are expressed as the mean ± SE of triplicate cultures. Results are representative of six independent experiments. The percentage of inhibition induced by ANE (40-60 μg/mL) was indicated in the parentheses. *$p < 0.05$ as compared with the PI group.

4.1-2 Differential effects of ANE on IFN-γ, IL-2 and IL-4 production by splenocytes

Cytokines expressed by T-cells plays an essential role in various immune reactions. To investigate the influence of ANE on functional activity of T-cells, IL-2, IL-4 and IFN-γ were selected to examine the effect of ANE on cytokine production. Splenocytes were treated with ANE (1–60 μg/mL) for 30 min followed by stimulation with PI for 24 h for 48 h. The concentration of cytokines in supernatants was quantified by ELISA. ANE markedly suppressed the production of IFN-γ and IL-2, whereas the production of IL-4 was unaffected (Fig. 5-7). The above finding showing a partial reversal of ANE-mediated cytotoxicity by NAC, NAC was employed to study whether ANE-mediated inhibition of IL-2 and IFN-γ production was associated with the induction of oxidative stress. In parallel to the reversal of cytotoxicity, the presence of NAC (4 mM) dramatically reversed ANE-mediated inhibition of IFN-γ production. In the absence of NAC the magnitude of inhibition by 40 and 60 μg/mL of ANE on IFN-γ production was 49.6% and 67.9%, respectively (Fig. 5A), which was markedly attenuated in the presence of NAC (4 mM) to 12.8% and 19.1%, respectively (Fig. 5B). Likewise, a partial reversal of ANE-mediated inhibition of IL-2 was observed. The magnitude of inhibition by 40 and 60 μg/mL of ANE on IL-2

production was 50.0% and 76.2% (Fig. 6A), respectively, which was attenuated in the presence of NAC (4 mM) to 22.4% and 51.3% (Fig. 6B), respectively.

(A) Without NAC



(B) With NAC

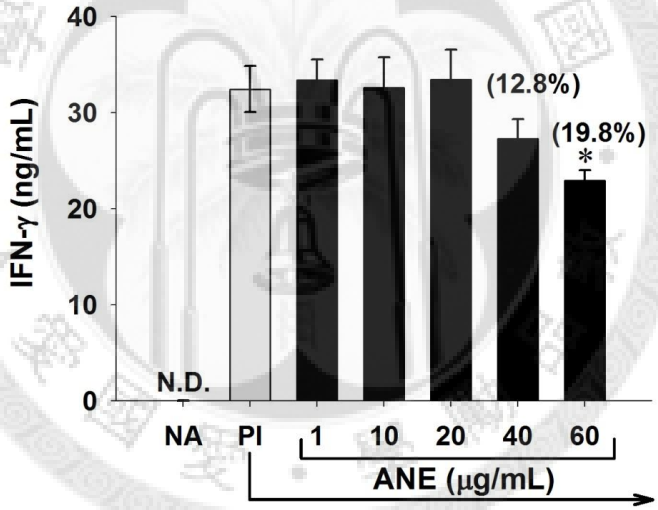
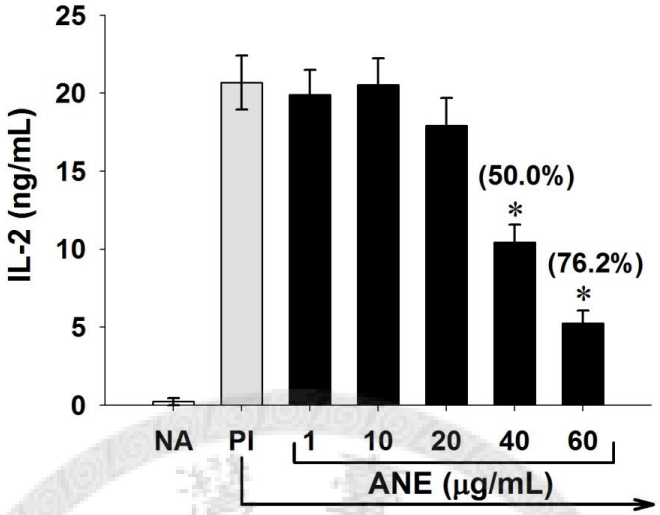


Figure 5. The effect of ANE on IFN-γ production by PI-stimulated splenocytes. Splenocytes (5×10^6 cells/mL) were either untreated (NA), or treated with ANE (1-60 μg/mL) in the absence (A) or presence (B) of pretreatment of *N*-acetyl-$_L$-cysteine (NAC; 4 mM) for 30 min. Except for the NA group, cells were stimulated with PI (80 nM/1 μM) for 24 h. IFN-γ in the supernatants were measured by ELISA. Data are expressed as the mean ± SE of triplicate cultures. Results are representative of six independent experiments. The percentage of inhibition induced by ANE (40-60 μg/mL) was indicated in the parentheses. *$p < 0.05$ as compared with the PI group. ND: not detectable.

(A) Without NAC



(B) With NAC

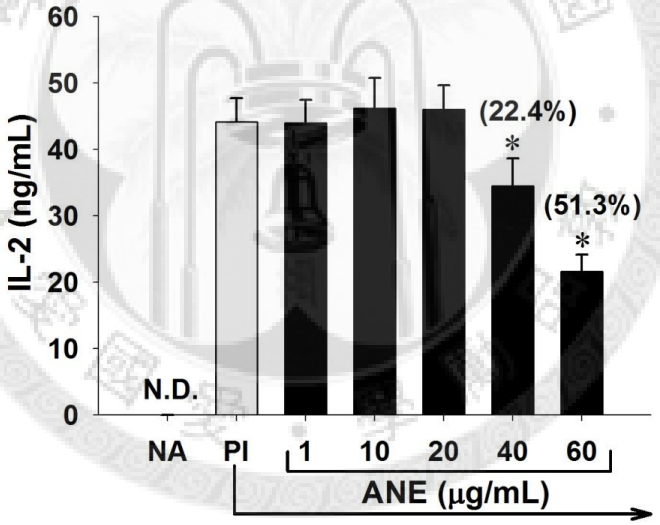


Figure 6. The effect of ANE on IL-2 production by PI-stimulated splenocytes.
Splenocytes (5×10^6 cells/mL) were either untreated (NA), or treated with ANE (1-60 μg/mL) in the absence (A) or presence (B) of pretreatment of *N*-acetyl-$_L$-cysteine (NAC; 4 mM) for 30 min. Except for the NA group, cells were then stimulated with PI (80 nM/1 μM) for 24 h. IL-2 in the supernatants were measured by ELISA. Data are expressed as the mean ± SE of triplicate cultures. Results are representative of six independent experiments. The percentage of inhibition induced by ANE (40-60 μg/mL) was indicated in the parentheses. *$p < 0.05$ as compared with the PI group. ND: not detectable.

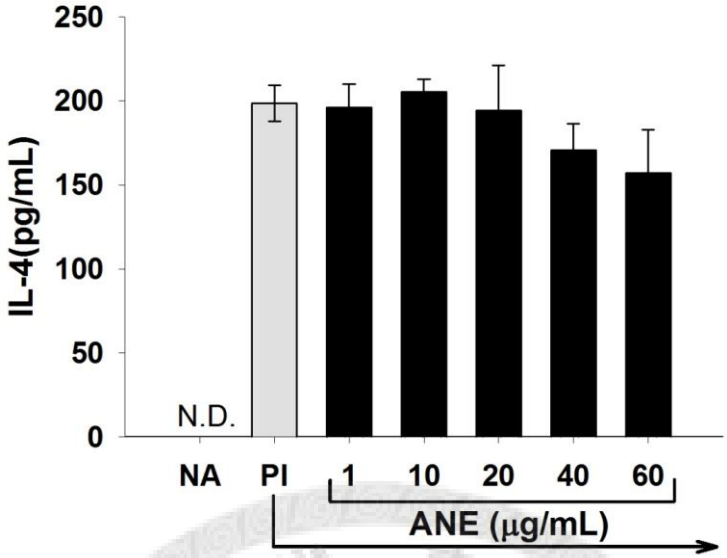


Figure 7. The effect of ANE on IL-4 production by PI-stimulated splenocytes. Splenocytes (5×10^6 cells/mL) were either untreated (NA), or treated with ANE (1-60 μg/mL). Except for the NA group, cells were then stimulated with PI (80 nM/1 μM) for 24 h. IL-4 in the supernatants were measured by ELISA. Data are expressed as the mean ± SE of triplicate cultures. Results are representative of six independent experiments. ND: not detectable.

4.1-3 Induction of ROS and diminishment of cellular thiols by ANE in splenic T cells

The above results suggest that the induction of cytotoxicity and suppression of cytokine production by ANE are associated with oxidative stress. Murine splenocytes are primarily composed of T and B cells, and macrophages. The splenic T-cells are likely the major cell type to produce IFN-γ and IL-2 in splenocytes. Hence, the influence of ANE on the cellular levels of ROS in splenic T-cells was examined. Proliferation of naïve splenic T-cells was induced by concanavalin A (ConA; 5 μg/mL) for 48 h. The splenic T-cells were then treated with ANE (10-40 μg/mL) for 12 h. The cellular ROS levels were quantified by flow cytometry using DCF-DA as a fluorescent indicator for intracellular ROS. ANE (10-40 μg/mL) markedly increased the DCF fluorescence in a concentration-dependent manner (Fig. 8) in splenic T cells. Consistent with the reversal of ANE-mediated cytotoxicity, and inhibition of IL-2 and IFN-γ production, pretreatment of the splenic T-cells with NAC (4 mM) attenuated ANE-induced elevated levels of the cellular ROS (Fig. 8B). In addition, the influence of ANE on the cellular thiols level was examined. The level of cellular thiols was measured by flow cytometry using CMF-DA as a fluorescent indicator for thiols. As expected, the presence of NAC elevated the level of intracellular thiols. In accordance

with the induction of cellular ROS, treatment of NAC-incubated splenic T-cells with ANE markedly diminished the cellular level of thiols (Fig. 9).

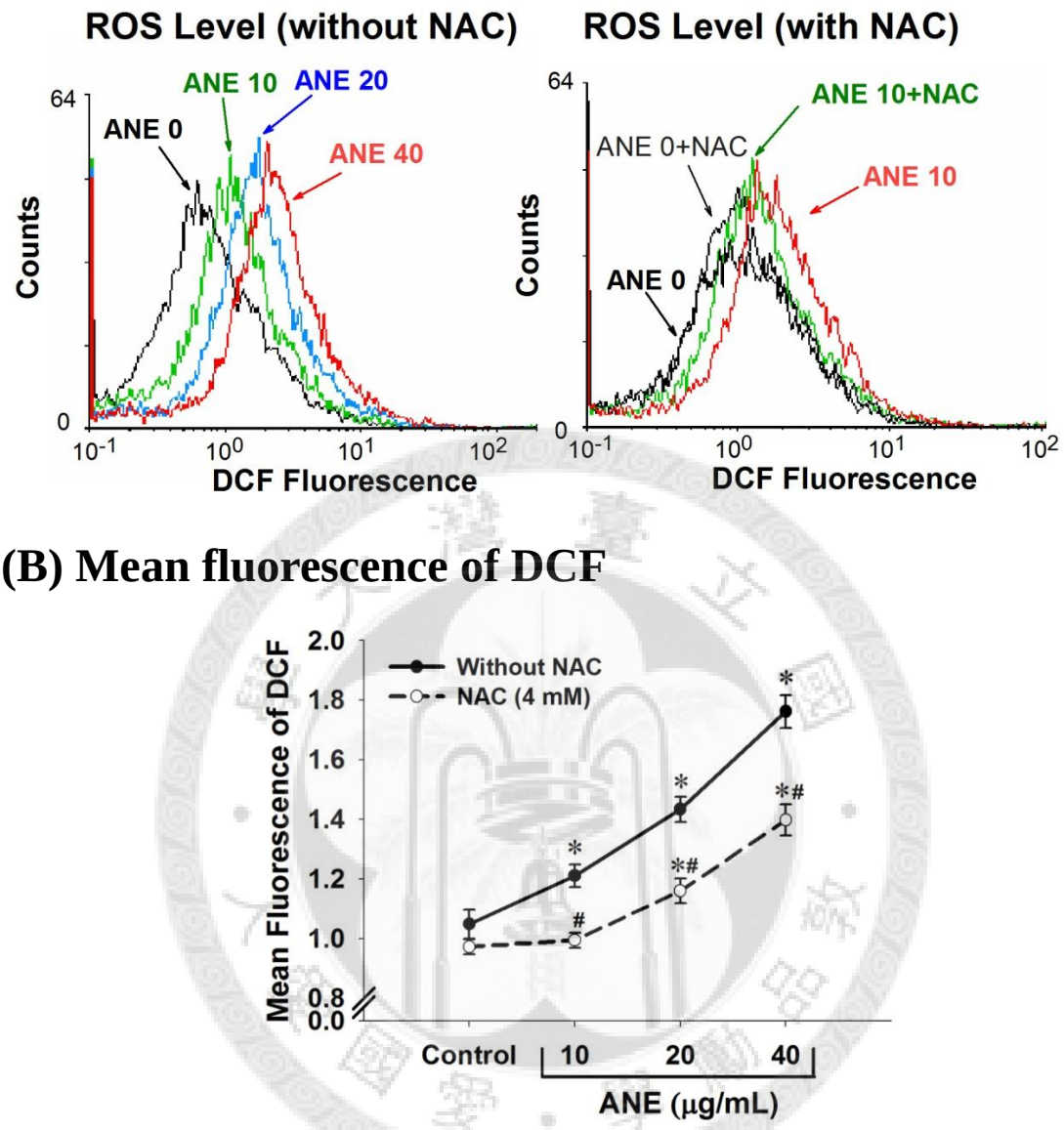
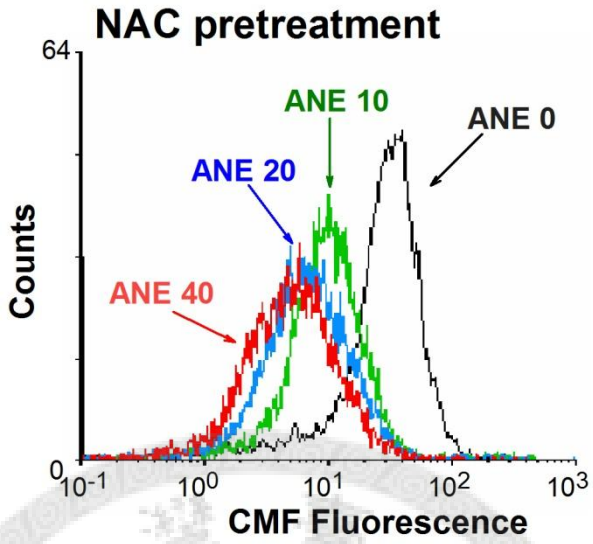


Figure 8. Attenuation by NAC of ANE-mediated induction of cellular ROS in ConA-stimulated splenocytes.

Splenocytes were cultured in complete RPMI with ConA (5 μg/mL) for 48 h to stimulated T-cell proliferation. The harvested splenic T-cells (5×10^6 cells/mL) were untreated or treated with ANE (10-40 μg/mL) for 12 h in the absence or presence of NAC (4 mM). After washing, the cells were incubated with DCF-DA (10 μM) for 15 min and the single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. The data are expressed as the histograms of DCF fluorescence of cells (A). The mean fluorescence of DCF was expressed as the mean ± SE of triplicate cultures (B). Results are representative of three independent experiments. $^{*}p < 0.05$ as compared with the control group. $^{\#}p < 0.05$ as compared with the matched group without NAC pretreatment.

(A) Histogram of CMF



(B) Mean fluorescence of CMF

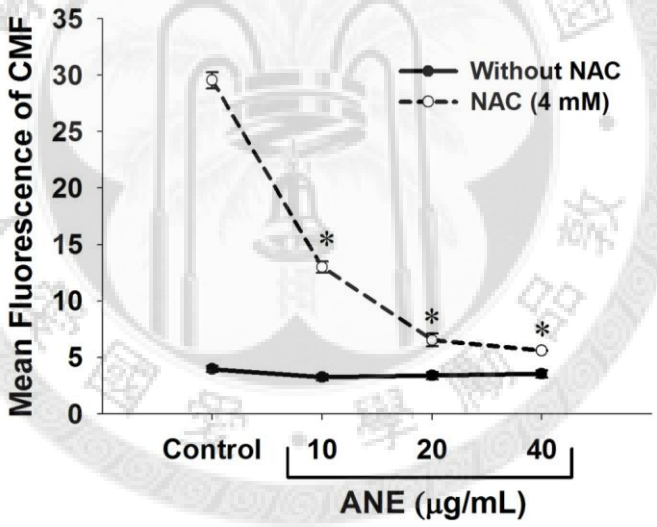


Figure 9. Diminishment of the level of cellular thiols in ConA-stimulated splenocytes by ANE.

Splenic T-cells (5×10^6 cells/mL) were untreated or treated with ANE (10-40 μg/mL) for 12 h in the absence or presence of NAC (4 mM). After washing, the cells were incubated with CMF-DA (25 μM) for 15 min and the single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. The data are expressed as the histograms of CMF fluorescence of cells (B). The mean fluorescence of CMF (B) was expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. *$p < 0.05$ as compared with the control group. $^{\#}p < 0.05$ as compared with the matched group without NAC pretreatment.

4.2 Involvement of the mitochondrion-dependent pathway and oxidative stress in ANE-induced splenocyte apoptosis

4.2-1 Induction of apoptosis of splenocytes by ANE

The effect of ANE on the apoptosis of splenocytes was estimated. Splenocytes were left untreated (NA) or treated with ANE for 1-12 h and the cell cycle distribution was analyzed by flow cytometry. The freshly isolated NA splenocytes underwent spontaneous apoptosis; the apoptotic rates were approximately 3 and 19% at 1 and 12 h in culture, respectively (Fig. 10A). Treatment of splenocytes with ANE (20 μg/mL) for 3-12 h gradually enhanced apoptosis in a time-dependent manner (Fig. 10B). The pro-apoptotic effect by ANE (20-60 μg/mL) was concentration-dependent; the apoptotic rate of splenocytes exposed to 60 μg/mL of ANE for 12 h reached 62% that was 3 times greater than that of the NA group (19%; Fig. 10A). In addition, the results of TUNEL staining confirmed that ANE (20 μg/mL) treatment markedly increased the proportion of splenocytes with DNA single strand breaks (Fig. 11). ANE-mediated apoptosis was further examined by analyzing the nuclear morphology of cells using Hoechst staining. In the control (NA) group, the nuclei of splenocytes were round and large with regular shape and size. In contrast, ANE-treated cells showed small nuclei with condensed chromatin that displayed brighter staining than that in NA cells (Fig. 12). The apoptotic effect of ANE on different subset of T cells

was further studied. Splenocytes were stained with annexin V and CD4 and CD8 antibodies. Annexin V protein has a strong and specific affinity for phosphatidylserine, which is translocated form the inner membrane to the outer membrane in apoptotic cells. As illustrated in Fig.13, ANE induced $CD4^+$ and $CD8^+$ lymphocyte apoptosis. $CD4^+$ cells were more sensitive to ANE-mediated apoptosis than $CD8^+$ cells. Caspases are a group of enzymes participated in the signaling and execution of apoptosis. Therefore, caspase inhibitors were employed to investigate the involvement of caspases in ANE-mediated apoptosis. All of the tested caspase inhibitors showed a remarkable reversal of ANE-induced apoptosis, indicating the activation of caspase-dependent apoptosis pathway by ANE (Fig. 14A). Moreover, ANE enhanced the activation of caspase-3, -8 and -9 (Fig. 14B).

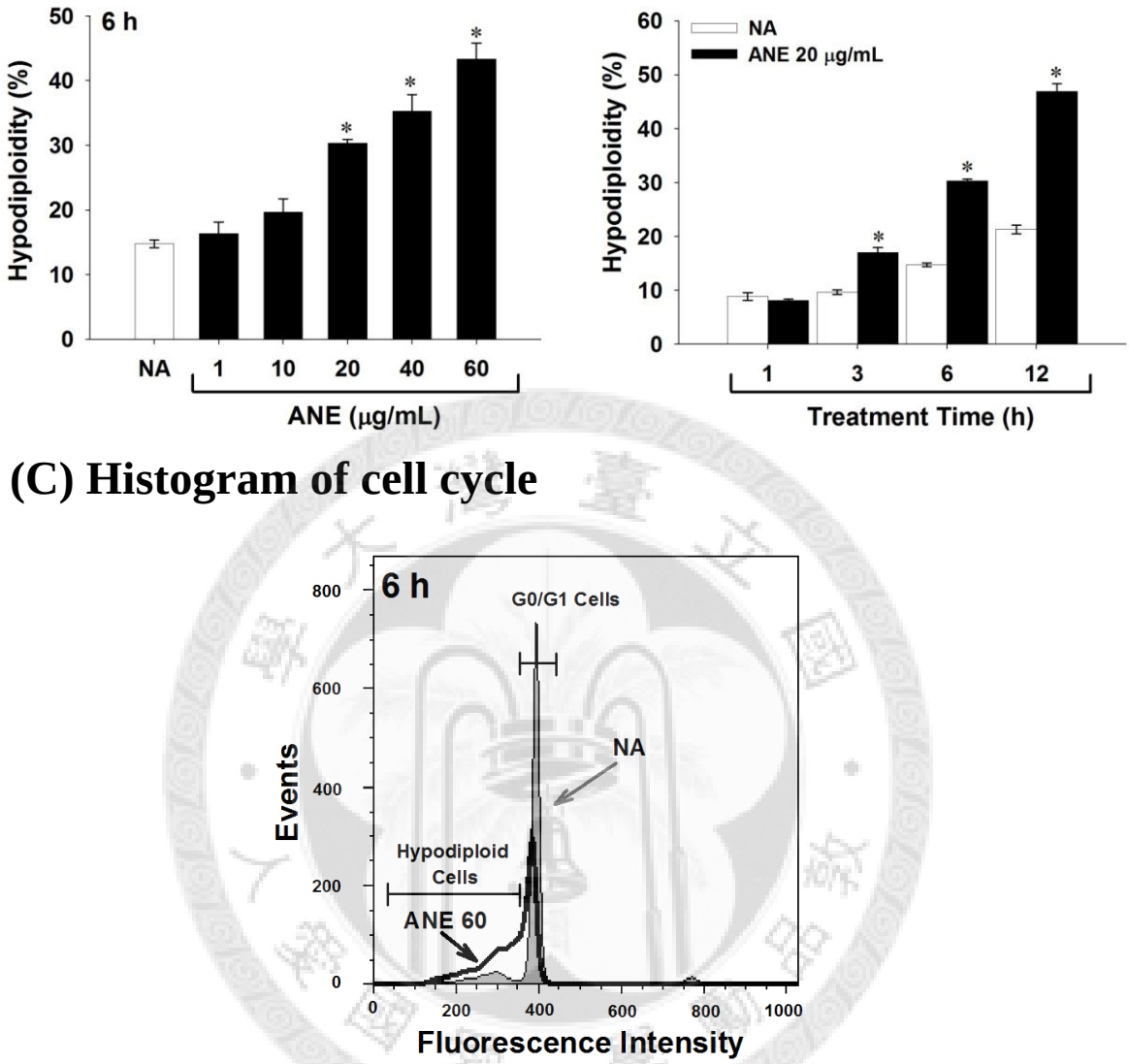


Figure 10. Enhancement of DNA hypodiploidity in splenocytes by ANE.

Splenocytes (5×10^6 cells/mL) were left untreated (naïve; NA) or treated with ANE (1-60 μg/mL) for 1-12 h. The cells were washed and fixed with 70% ethanol at -20°C for at least 1 h. The cells was washed with PBS and resuspended in 400 μL propidium iodide staining buffer (50 μg/mL propidium iodide and 20 μg/mL ribonuclease in PBS) at 37℃ for 30 min. The single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. The percentage of hypodiploid cells at sub-G_0/G_1 stage was expressed as the mean ± SE of triplicate cultures. Results are representative of six independent experiments. *$p < 0.05$ as compared with the NA group. Representative histograms of cell cycle analysis on NA and ANE (60 μg/mL)-treated cells were shown in panel C.

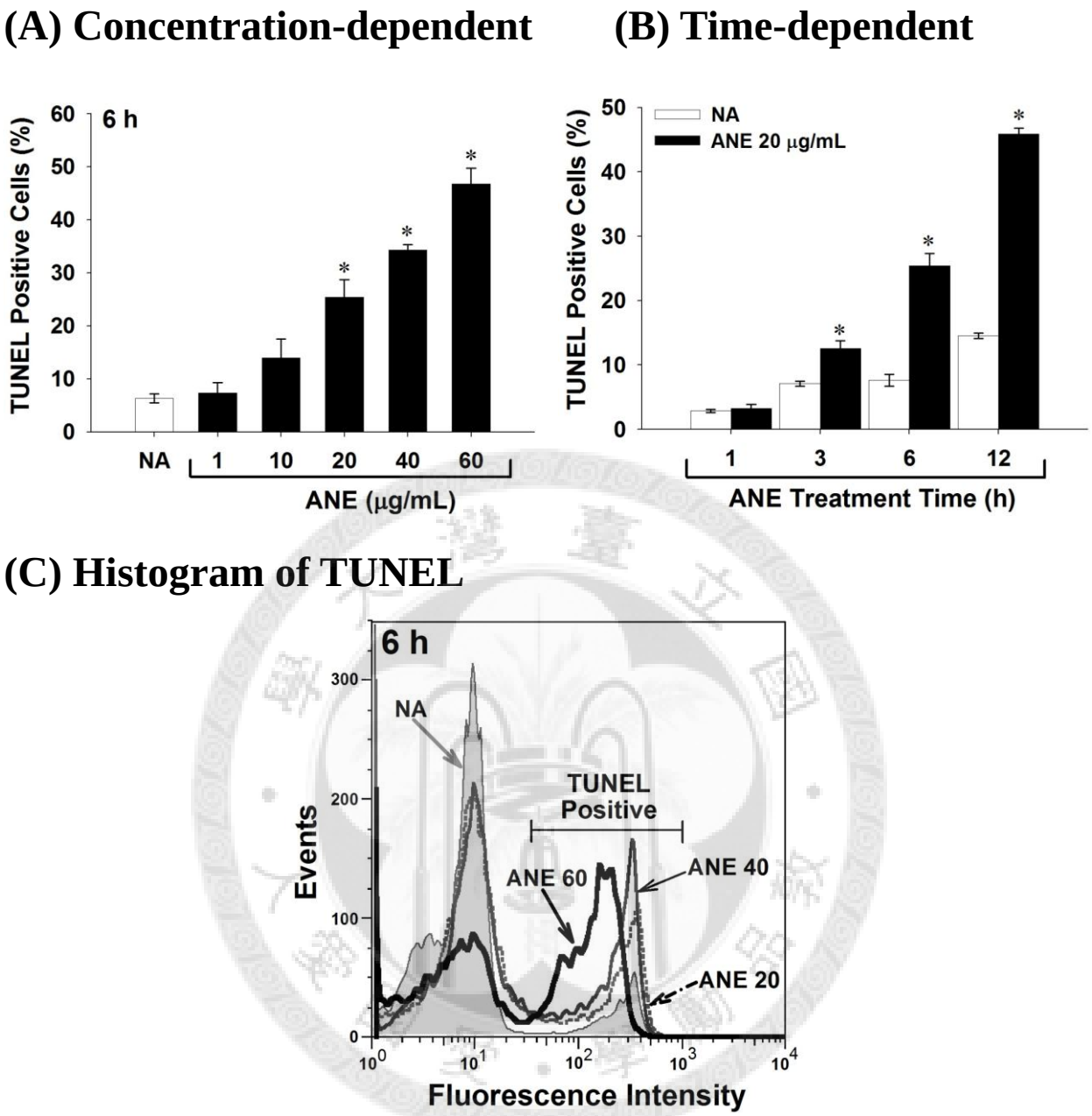


Figure 11. Induction of splenocyte apoptosis by ANE.

Splenocytes (5×10^6 cells/mL) were left untreated (naïve; NA) or treated with ANE (1-60 μg/mL) for 1-12 h. After washing, the cells were fixed with 70% ethanol at -20℃ for at least 1 h. Fixed cells were stained with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP for 1 h at 37℃. After washing with PBS, the single cell fluorescence of 5,000 cells for each sample was measured by flow cytometry. The percentage of $TUNEL^+$ cells was expressed as the mean ± SE of triplicate cultures (Fig. 12A and 12B). Results are representative of three independent experiments. *$p < 0.05$ as compared with the NA group. Representative histograms of TUNEL analysis on NA and ANE (20-60 μg/mL)-treated cells were shown in panel C.

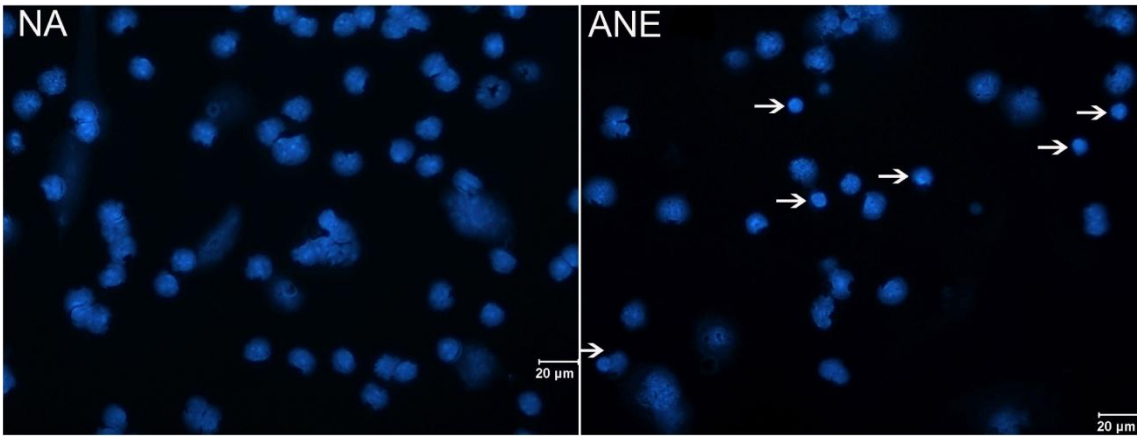
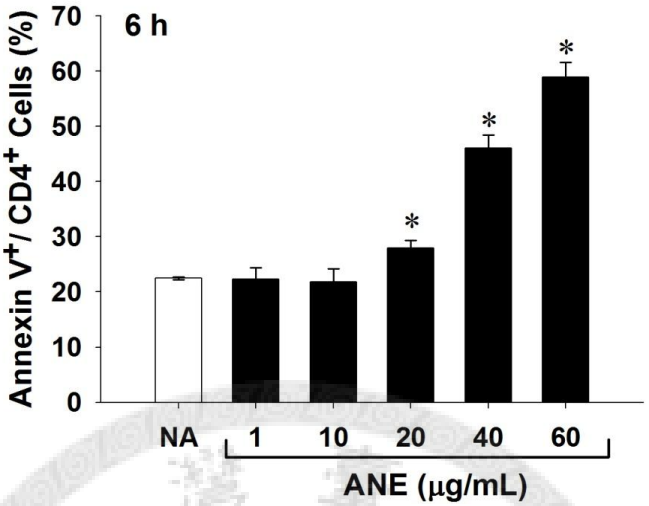


Figure 12. Nuclear condensation in ANE-treated splenocytes.
Splenocytes (5×10^6 cells/mL) were left untreated (naïve; NA) or treated with ANE (40 μg/mL) for 6 h. The morphological features of splenocytes were determined by Hoechst staining. Splenocytes were stained with Hoechst 33258 (1 μg/mL) and photographed. White arrows indicate apoptotic splenocytes showing nuclear condensation (original magnification, × 400).

(A) CD4+ cells



(B) CD8+ cells

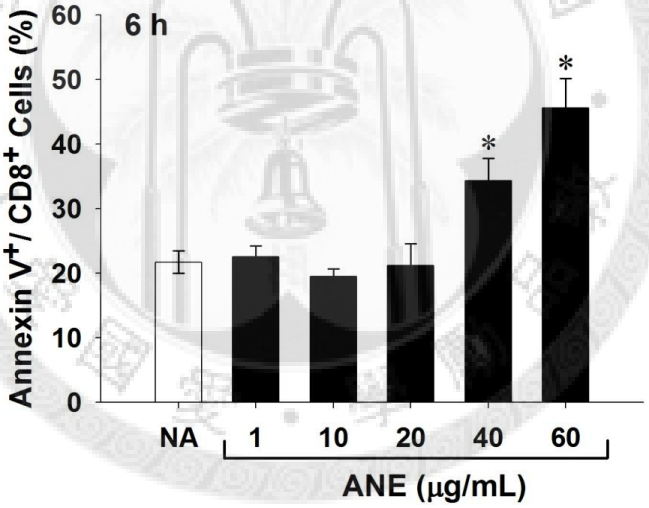
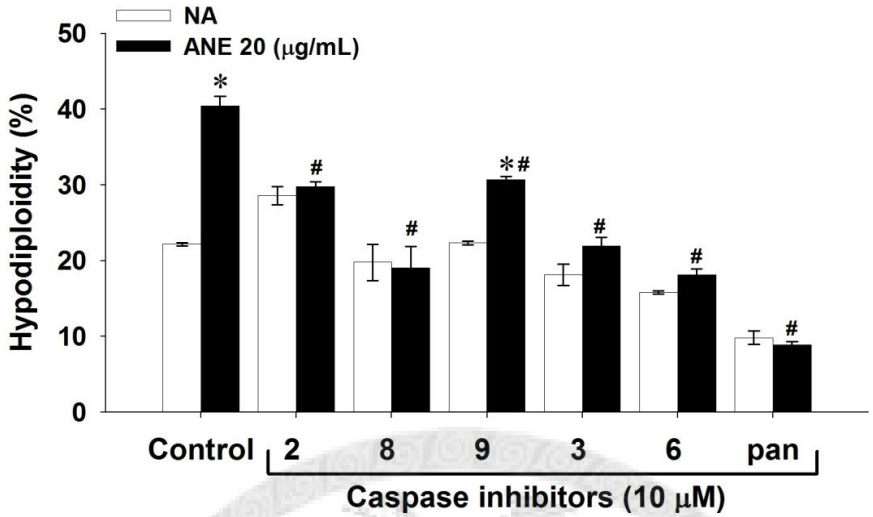


Figure 13. Enhancement of $CD4^+$ and $CD8^+$ lymphocyte apoptosis by ANE.
Splenocytes (5×10^6 cells/mL) were left untreated (naïve; NA) or treated with ANE (1-60 μg/mL) for 6 h. After washing, the cells were stained with FITC-annexin V and PE/Cy5 conjugated anti-mouse CD4 or CD8 antibodies at RT for 20 min. The single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. The percentage of annexin $V^+/CD4^+$ and annexin $V^+/ CD8^+$ cells was expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. *$p < 0.05$ as compared with the NA group.

(A) Caspase inhibitors



(B) Caspase activity

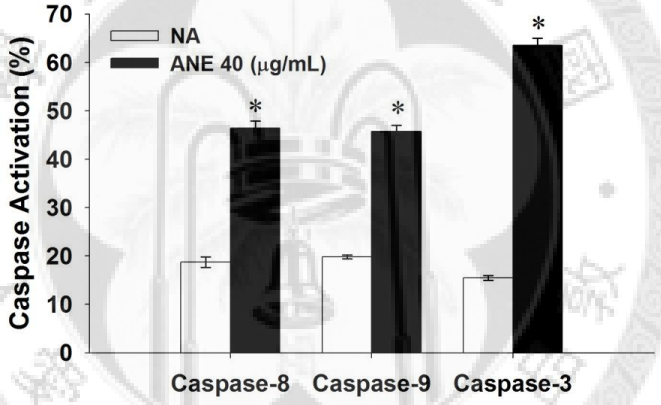


Figure 14. Induction of caspase-dependent apoptosis in splenocytes by ANE.
Splenocytes were left untreated (naïve; NA) or treated with ANE (20 μg/mL) for 12 h in the presence of Z-VDVAD-FMK, Z-IETD-FMK, Z-LEHD-FMK, Z-DEVD-FMK, Z-VEID-FMK (caspase inhibitors for caspse-2, -8, -9, -3 and -6, respectively) and pan caspase inhibitor, Z-VAD-FMK. (A) The percentage of hypodiploid cells was quantified by cell cycle analysis and expressed as the mean ± SE of triplicate cultures. $*p < 0.05$ as compared with the NA group. $^{\#}p < 0.05$ as compared with the matched group without caspase inhibitors. (B) The activation of caspase-8, -9 and -3 in ANE-treated splenocytes was measured using specific cell-permeable substrate of caspase-8, -9 and -3 (FAM-IETD-FMK, FAM-LEHD-FMK and FAM-DEVD-FMK, respectively). The single cell fluorescence of 10,000 cells was measured by a flow cytometer at emission of 525nm. The percentage of activated-caspase positive cells was expressed as the mean ± SE of triplicate cultures. $*p < 0.05$ as compared with the NA group. Results are representative of three independent experiments.

4.2-2 Involvement of the mitochondrion-dependent pathway in ANE-mediated splenocyte apoptosis

In response to a variety of apoptotic stimuli, mitochondria may trigger the intrinsic pathway signaling accompanied by mitochondrial membrane potential ($\Delta\psi_m$) depolarization, ROS generation and the release of mitochondrion-associated pro-apoptotic proteins, such as cytochrome *c* (Mohamad et al., 2005). As previous studies showed the disruption of $\Delta\psi_m$ by ANE in oral cells (Chang et al., 2001), the role of mitochondria in ANE-mediated apoptosis in splenocytes was explored. As shown in Fig. 15C, a marked increase in the proportion of $\Delta\psi_m$ depolarized cells (42.5%) was detected in splenocytes exposed to ANE (20 μg/mL) for 12 h, as compared to the NA group (27.8%). ANE-induced $\Delta\psi_m$ depolarization was time- and concentration-dependent (Fig. 15A and 15B). The induction of $\Delta\psi_m$ depolarization began at 0.5 h post ANE (20 μg/mL) treatment (Fig. 15B). According to the above results, it is speculated that ANE might activate the mitochondrion-mediated apoptotic signaling. The influence of ANE on the release of cytochrome *c* was therefore examined. Exposure of splenocytes to ANE (10-60 μg/mL) for 3 h significantly enhanced the release of cytochrome *c* in a concentration-dependent manner, as compared to the NA group (Fig. 16). Caspase-9 is the key initiator caspase in the intrinsic pathway. Upon activation, caspase-9 induces the activation of downstream

caspases, such as caspase-3, to execute the apoptotic program (Orrenius et al., 2007). The effect of ANE on the activation of caspase-9 in splenocytes was examined, and the results showed that ANE (20 μg/mL) treatment gradually induced the activation of caspase-9 in a time- and concentration-dependent manner (Fig. 17A and 17B).

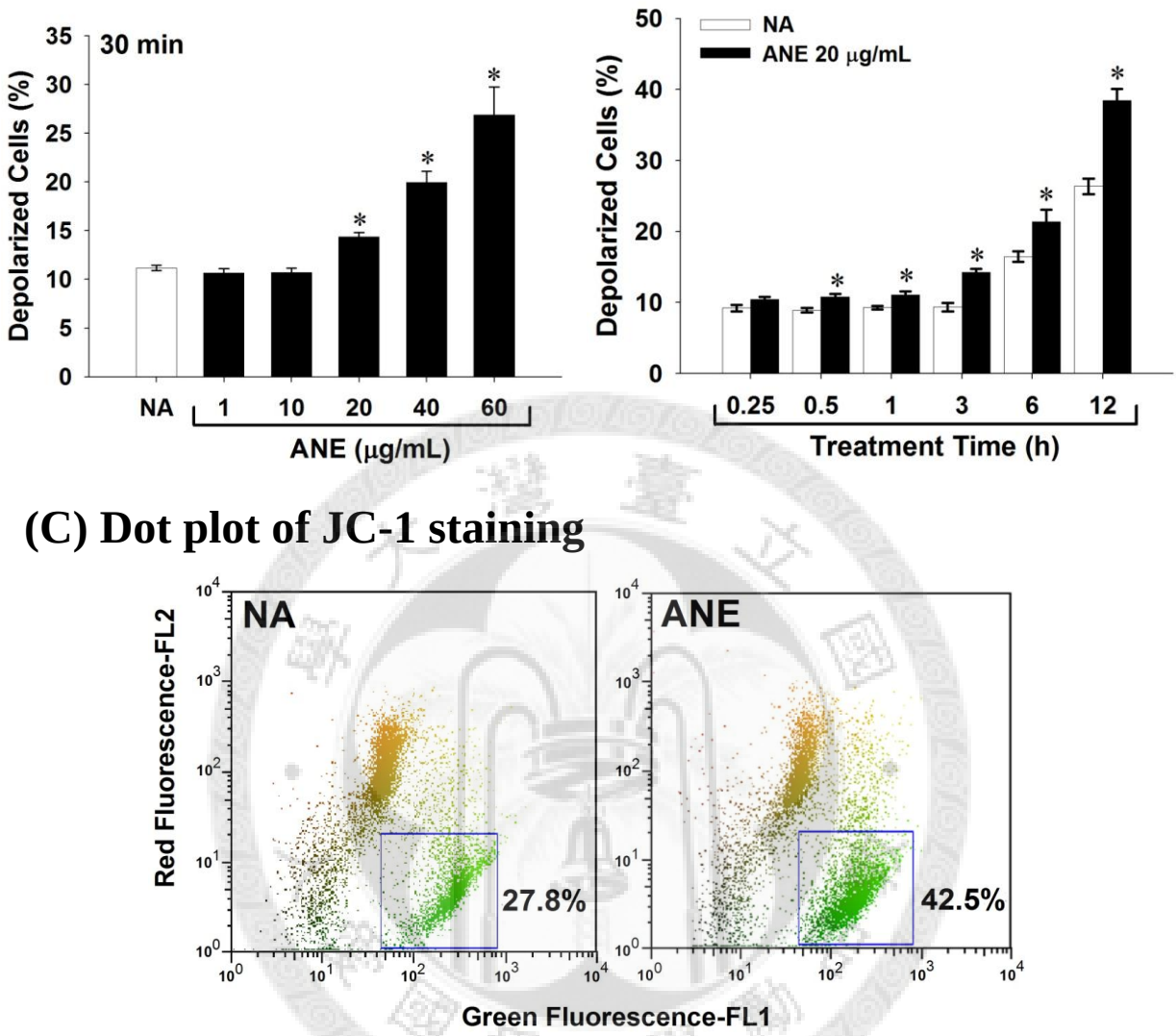


Figure 15. Induction of the depolarization of mitochondria membrane potential ($\Delta\psi_m$) in splenocytes by ANE.

Splenocytes were left untreated (naïve; NA) or treated with ANE (1-60 μg/mL) from 0.25-12 h. $\Delta\psi_m$ was measured by flow cytometry using JC-1 staining. The single fluorescence of 10,000 cells was measured by flow cytometry. In proportion to $\Delta\psi_m$, JC-1 accumulated in mitochondria to form JC-1 aggregates (590 nm emission; orange color) whereas JC-1 aggregates depart from mitochondria and change to JC-1 monomer (530 nm emission; green color) in response to loss of $\Delta\psi_m$. (A and B) The data were expressed as mean ± SE of triplicate cultures. Results are representative of six independent experiments. *p < 0.05 as compared with the NA group. (C) Representative dot plots of JC-1 analysis on NA and ANE (20 μg/mL)-treated cells were shown in panel C. The percentage of $\Delta\psi_m$ depolarized cells was shown in open box area.

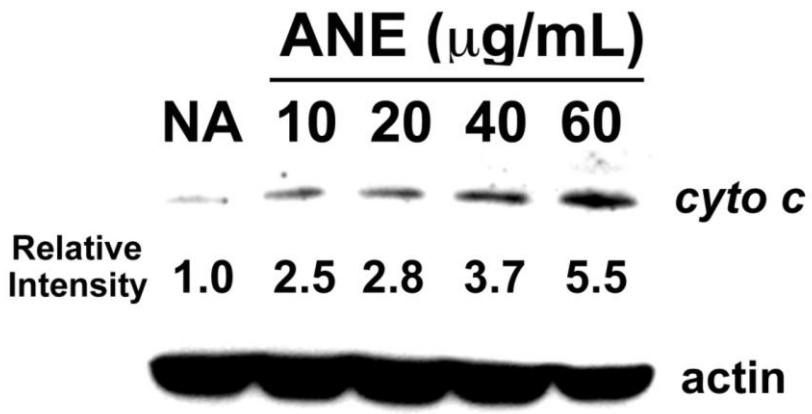
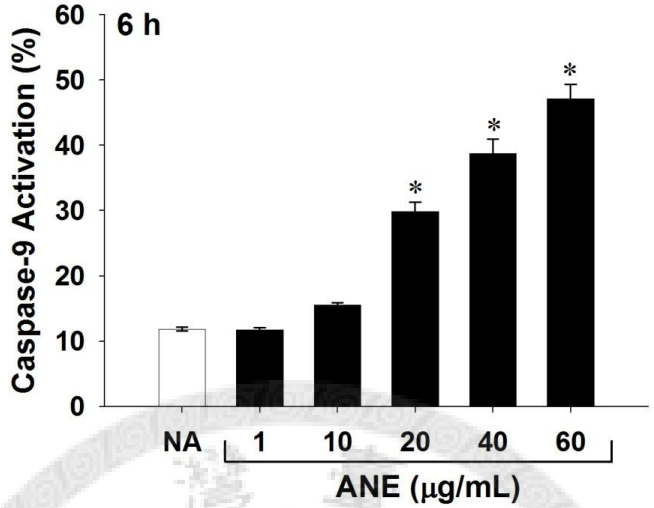


Figure 16. Induction of cytochrome *c* release in splenocytes by ANE.

Splenocytes were left untreated (NA) or treated with ANE (1-60 μg/mL) for 3 h. Cells were lyzed in lysis buffer and centrifuged at 1,200 x rpm to remove nuclei. The supernatant was centrifuged again at 10,000 x g and the cytosolic extracts were collected and electrophoresed by SDS-PAGE and transferred to a PDVF membrane. The blots were probed with anti-mouse cytochrome *c* antibody. The density of actin was determined as loading control. The density of cytochrome *c* (cyto c) was expressed as relative intensity to the NA group. The data were representative of three independent experiments.

(A) Concentration-dependent



(B) Time-dependent

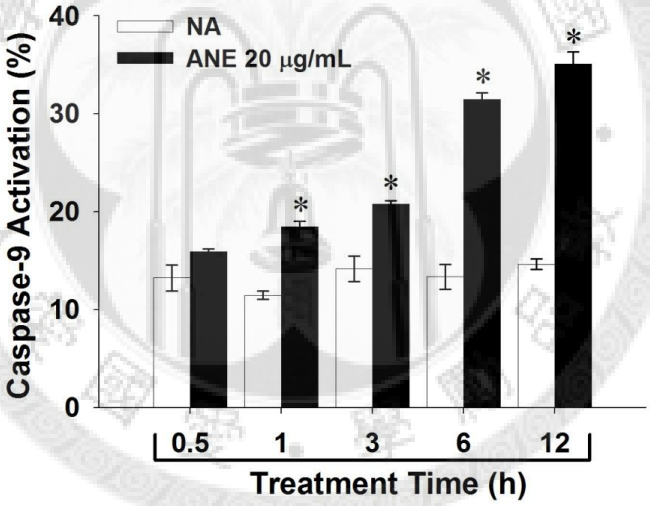


Figure 17. Activation of capsase-9 in splenocytes by ANE.

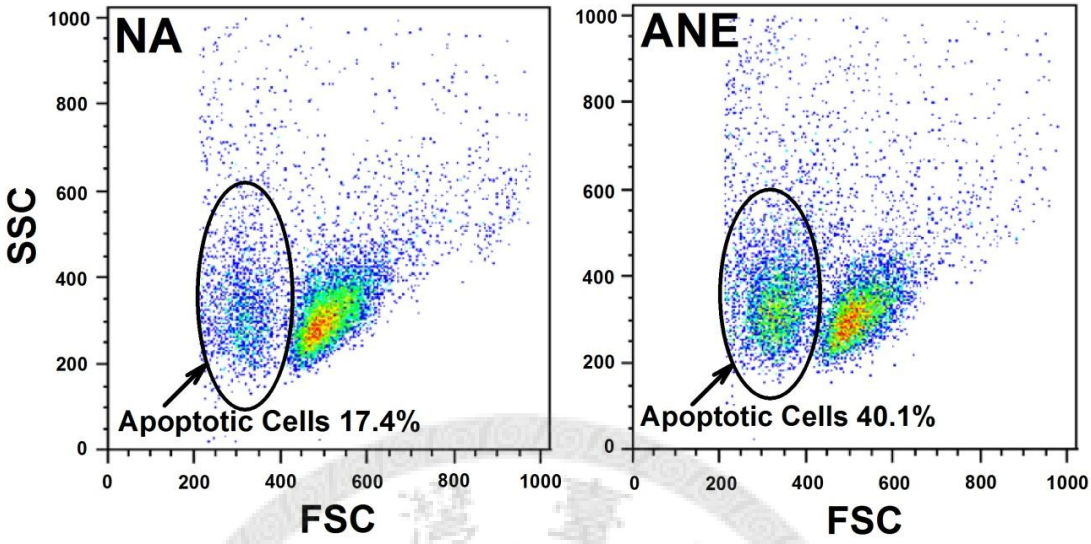
Splenocytes were either left untreated (NA) or treated with ANE (1-60 μg/mL) for 0.5-12 h. The activation of caspase-9 in ANE-treated splenocytes was measured using specific cell-permeable substrate of caspase-9 (FAM-LEHD-FMK). After washing, splenocytes were incubated with FAM-LEHD-FMK for 1 h at 37℃ in the dark. The single cell fluorescence of 10,000 cells was measured by a flow cytometer at emission of 525nm. The percentage of active capase-9 positive cells was expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. *$p < 0.05$ as compared with the NA group.

4.2-3 The role of ROS in ANE-mediated splenocyte apoptosis

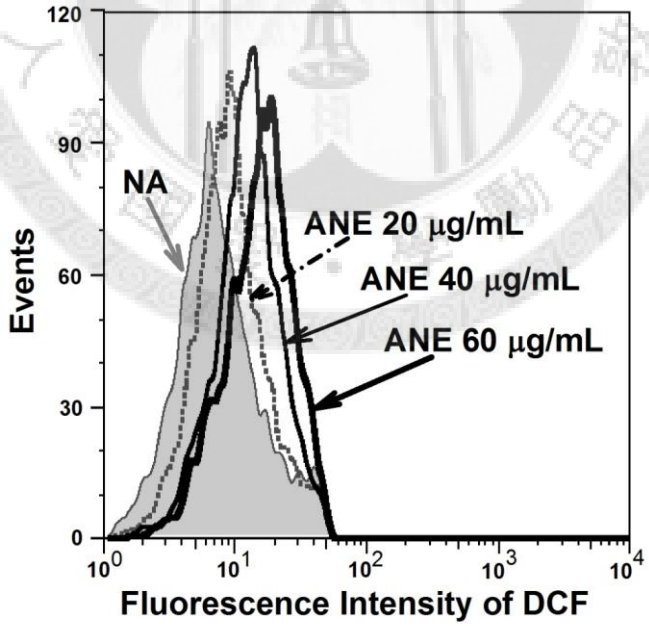
To study the role of ROS in ANE-mediated apoptosis, the generation of cellular levels of ROS was determined by DCF-DA staining. It has been well established that a reduction of forward-angle light scatter (FSC) is a characteristics of apoptotic cells measured by flow cytometry (Banfalvi et al., 2007; Darzynkiewicz et al., 1992; Swat et al., 1991; Vermes et al., 2000). In flow cytometric experiments, both NA and ANE-treated splenocytes showed the presence of 2 main cell populations based on their FSC signal. As shown in Fig. 18A, the low FSC population of NA and ANE (40 μg/mL)-treated splenocytes was 17.4% and 40.1%, respectively. Interestingly, the DCF fluorescence of apoptotic cells was significantly increased by ANE treatment for 3-6 h in a concentration-dependent manner (Fig. 18C). In contrast, the DCF fluorescence in non-apoptotic splenocytes with the high FSC signal was not altered by ANE (Fig. 18D). These data demonstrated the increased production of ROS in apoptotic splenocytes treated with ANE. To further investigate the involvement of ROS in ANE-mediated apoptosis, the thiol antioxidant NAC was employed. Splenocytes were treated with ANE (20-60 μg/mL) in the absence or presence of NAC (4 mM), and the levels of cellular ROS, hypodiploidity, caspase-9 activation and $\Delta\psi_m$ were measured. As shown in Figs. 19A-C, the presence of NAC partially but significantly attenuated the ROS generation, apoptosis enhancement and caspase-9

activation induced by ANE (20-60 μg/mL). In contrast, NAC did not affect ANE-mediated alteration in $\Delta\psi_m$ (Fig. 19D).

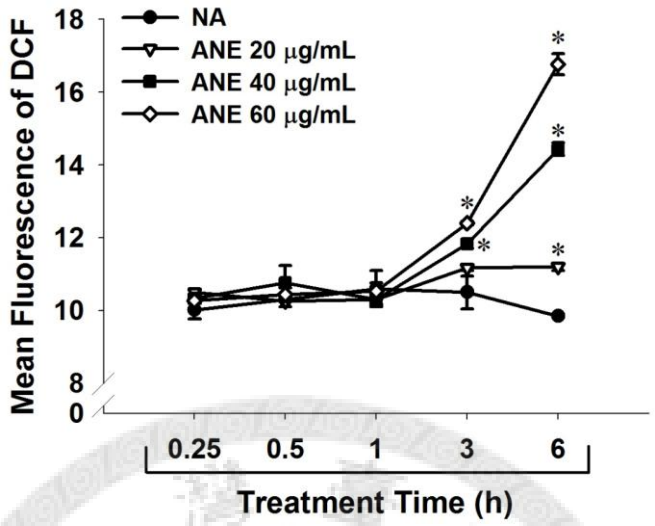
(A) FSC-SSC dot plots



(B) Histogram of DCF



(C) ROS level in apoptotic cells



(D) ROS level in non-apoptotic cells

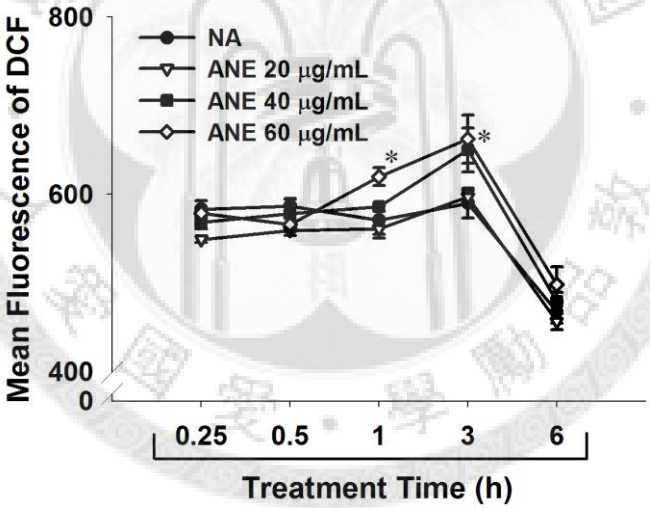


Figure 18. The effect of ANE on the reactive oxygen species (ROS) production in splenocytes.

Splenocytes were either left untreated or treated with ANE (20-60 μg/mL) for 15 min to 12 h. After washing, cells were stained with DCF-DA (10 μM) for 15 min and the single cell fluorescence of 10,000 cells was measured by a flow cytometer at emission of 525nm. (A) Representative FSC-SSC dot plots were shown. The low FSC population was referred to apoptotic cells. (B) Representative histograms of the DCF fluorescence in apoptotic cells were shown. (C, D) The mean fluorescence of DCF was expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. *$p < 0.05$ as compared with the NA group.

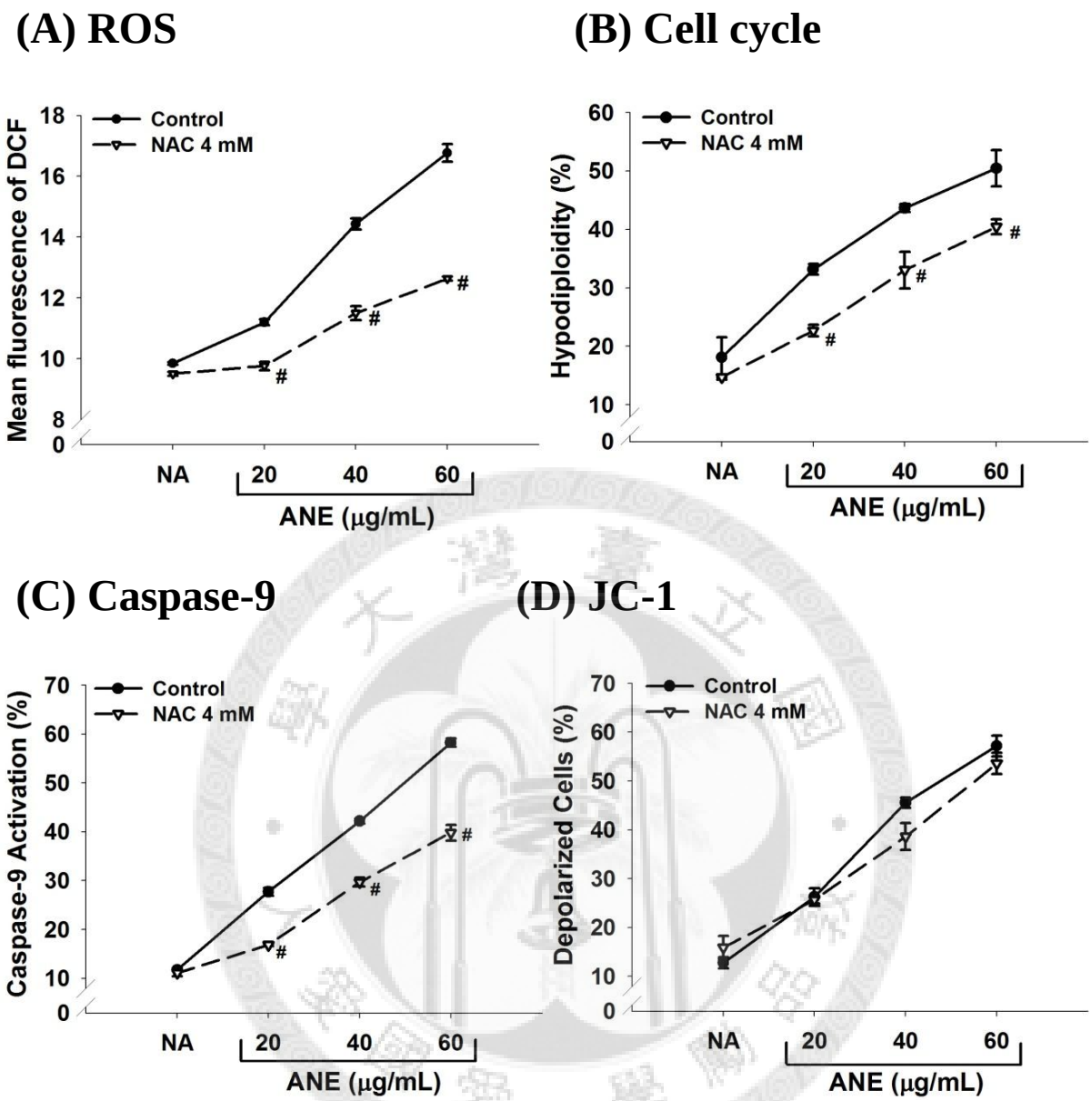


Figure 19. The effect of NAC on ANE-mediated ROS production, apoptosis, caspase-9 activation and $\Delta\psi_m$ depolarization.

Splenocytes were either untreated (NA), or treated with ANE (20-60 μg/mL) in the absent (Control) or present of NAC (4 mM). (A) The production of ROS in NA and ANE-treated cells were measured by DCF-DA. (B) The apoptosis of cells was evaluated by cell cycle analysis. (C) The activation of caspase-9 was determined by FAM-LEHD-FMK staining. (D) The depolarized cells were measured by flow cytometry using JC-1 probe. Data are expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. $^{\#}p < 0.05$ as compared with the matched group without NAC.

4.3 Areca-derived highly oligomeric procyanidins induce splenocyte apoptosis via the depletion of intracellular thiols

4.3-1 Polyphenol-enriched ANE (PANE) and areca-derived highly oligomeric procyanidins induced splenocyte apoptosis

Because ANE contain a rich amount of polyphenols, it is speculated that areca derived polyphenols may be the potential constituents responsible for the pro-apoptotic effect on splenocytes. It has been shown that areca nut-derived polyphenols are mostly flavanols, including catechin and epicatechin monomers and procyanidins with various degrees of polymerization. At first, catechin, epicatechin, epigallocatechin gallate and polyphenol-enriched ANE were tested for the induction of apoptosis in splenocytes. Catechin and epicatechin slightly induced splenocyte apoptosis (Fig. 20A and 20B). Exposure of splenocytes to catechin and epicatechin (100-1000 μM) increased approximately 15% of apoptotic cells. In accordance with the induction of splenocyte apoptosis by ANE, PANE induced splenocyte apoptosis in a concentration- and time-dependent manner (Fig. 20D, 21A). The apoptotic rate in cells treated with 100 μg/mL of PANE for 12 h reached 66% that was three times greater than that in the VH group (Fig. 20D). The apoptotic effect of PANE was confirmed using Hoechst staining, in which splenic lymphocytes exposed to PANE (40 μg/mL) for 6 h demonstrated small and condensed nuclei that showed bright

staining (Fig. 21B). To further confirm the induction of apoptotic death by PANE treatment, cell-permeable inhibitors for caspase-6 and -3 (Z-VEID-FMK and Z-DEVD-FMK, respectively) were applied. PANE-mediated apoptosis of splenocytes was almost completely reversed by the presence of either inhibitor (Fig. 22). The pro-apoptotic activity of fractionated areca procyanidins from dimers to decamers was compared. As demonstrated in Fig. 23A, oligomers up to tetramers did not affect the apoptosis of splenic lymphocytes, as compared to the VH group. In contrast, pentamers to decamers significantly enhanced apoptosis in a concentration- and time-dependent manner (Fig. 23A & 23B). The lowest effective concentrations for pentamers, heptamers, and octamers were 40, 20, and 10 μg/mL, respectively (Fig. 23A). As 40 μg/mL is the lowest effective concentration for pentamers, and is also an effective concentration for the other higher oligomers, this concentration was used for time-course analysis. Cells were treated with oligomers from tetramers to decamers (40 μg/mL) for 0.5-12 h. No alteration in apoptotic rates was found at all time points in tetramers-treated cells, whereas the other oligomers gradually enhanced apoptosis in a time-dependent manner (Fig. 23B). The effect of pentamers was statistically significant beginning at 4 h post treatment, and the other higher oligomers were effective at all time points examined. Table 1 summarized the 50% effective concentration (EC_{50}) of oligomeric procyanidins-induced apoptosis, which was

calculated using the apoptotic rate of cells treated with the highest concentration (100 μg/mL) as the maximal response. The EC_{50} for pentamers to decamers were from 56.27-41.80 μg/mL. Together these results demonstrated that pentamers were the minimal degree of polymerization for the pro-apoptotic activity, and that the active oligomers with longer chain length induced a slightly more potent and rapid pro-apoptotic effect than shorter ones.

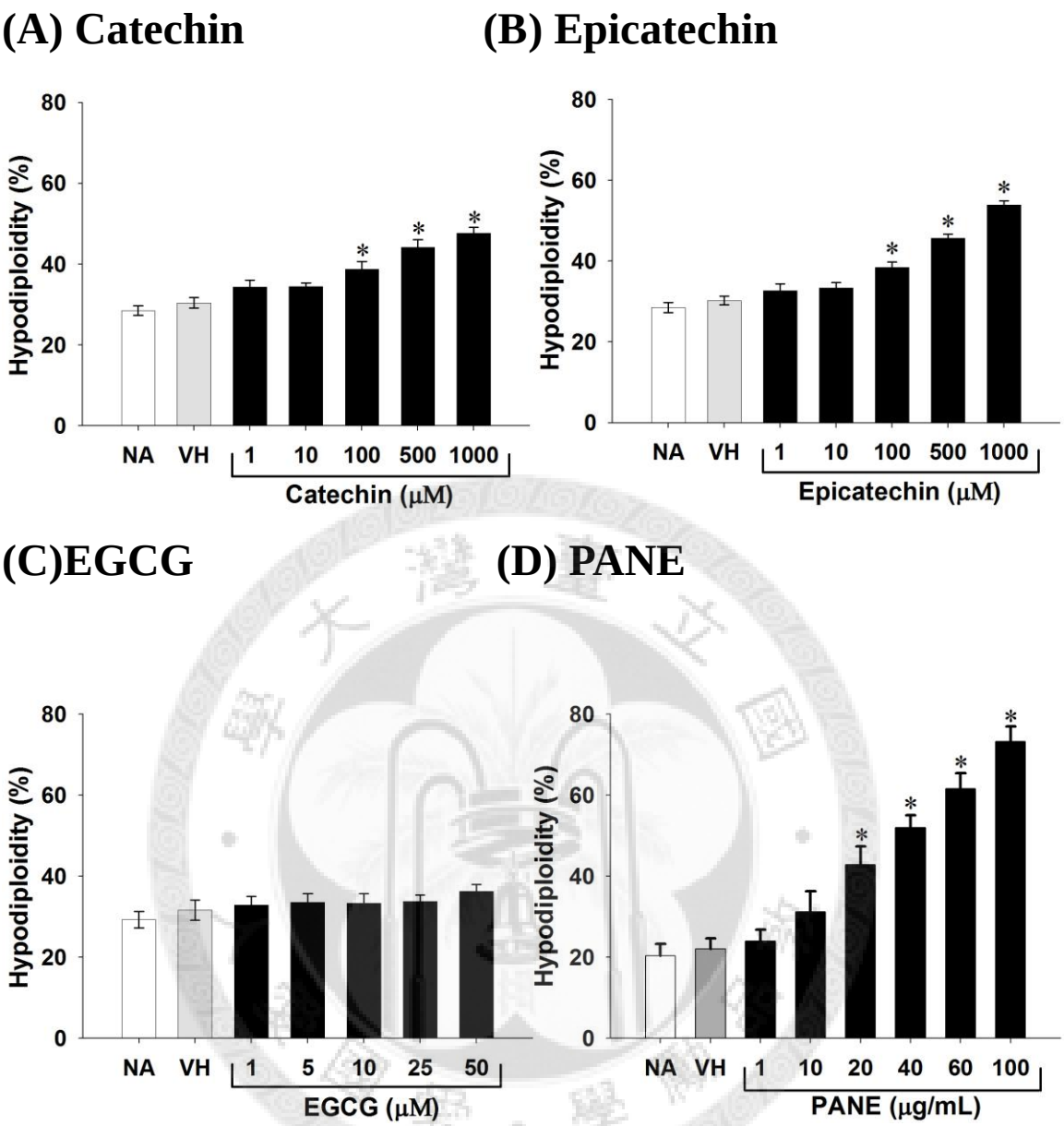
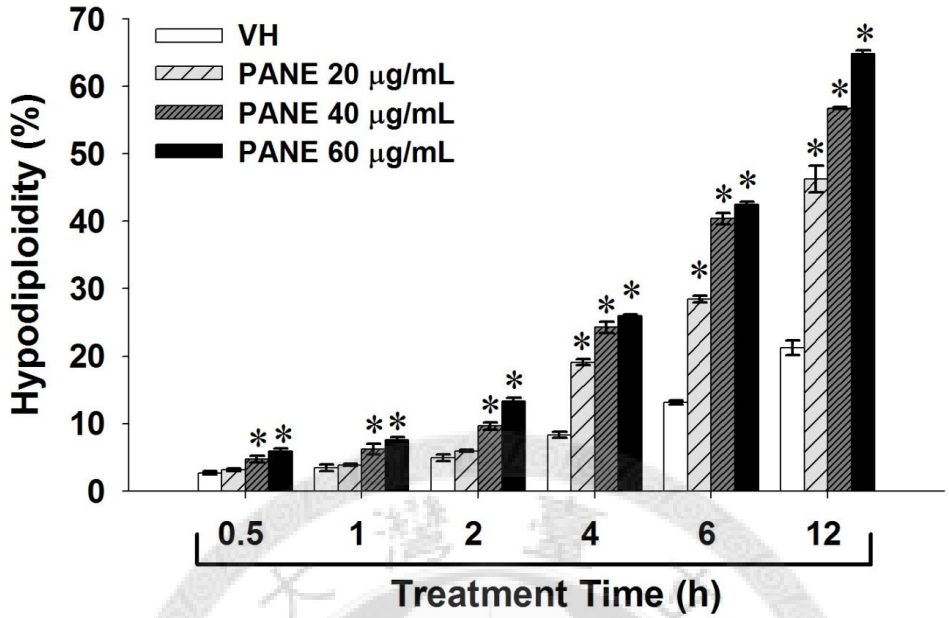


Figure 20. Induction of splenocyte apoptosis by catechin, epicatchin and polyphenol-enriched ANE (PANE).

Splenocytes were left untreated (naïve; NA) or treated with catechin (1-1000 μM), epicatechin (1-1000 μM), epigallocatechin gallate (EGCG; 1-50 μM) and PANE (1-100 μg/mL) and /or vehicle (VH; 0.1% DMSO) for 12 h. After washing, cells were fixed with 70% ethanol at -20℃ for at least 1 h. Cells were washed with PBS and resuspended in 400 μL propidium iodide staining buffer (50 μg/mL propidium iodide and 20 μg/mL ribonuclease in PBS) at 37° C for 30 min. The single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. The percentage of hypodiploid cells at sub-G_0/G_1 stage was expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. *$p < 0.05$ as compared with the VH group.

(A)



(B)

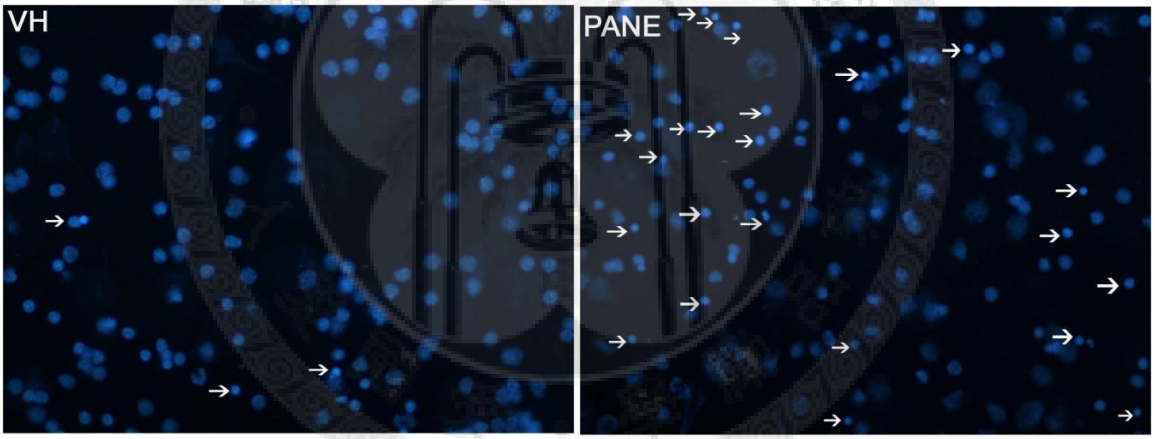


Figure 21. Induction of splenocyte apoptosis by PANE.

Splenocytes (5×10^6 cells/mL) were treated with PANE (2-60 μg/mL) and/or vehicle (VH; 0.1% DMSO) for 0.5-12 h. Cells were washed and fixed with 70% ethanol at -20° C for at least 1 h. Cells were stained with 400 μL propidium iodide staining buffer (50 μg/mL propidium iodide and 20 μg/mL ribonuclease in PBS) at 37℃ for 30 min. The single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. (A) The percentage of hypodiploid cells at sub-G_0/G_1 stage was expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. *p < 0.05 as compared with the VH group. (B) The morphological features of splenocytes were determined by Hoechst staining. White arrows indicate apoptotic splenocytes showing nuclear condensation (original magnification, × 200).

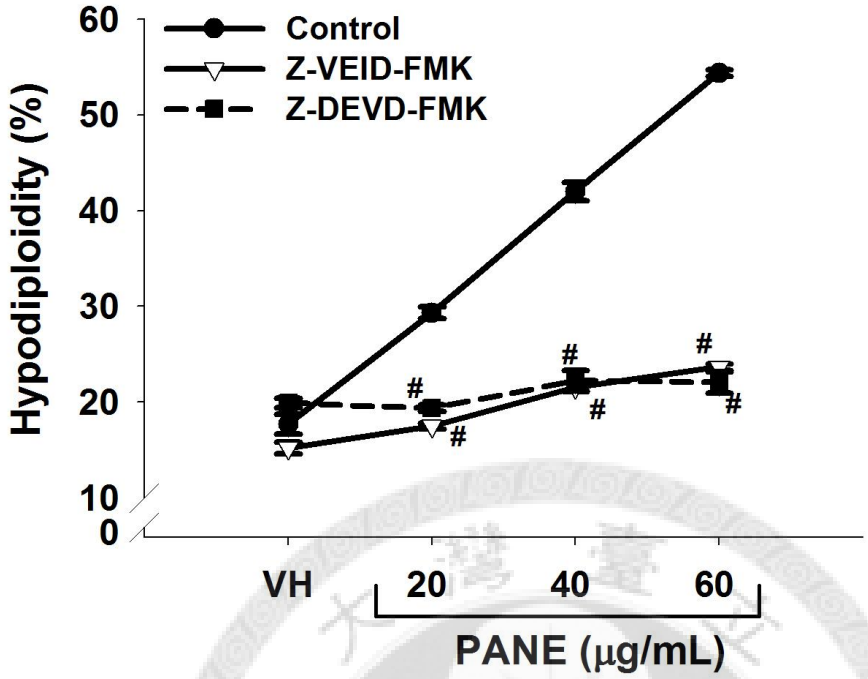
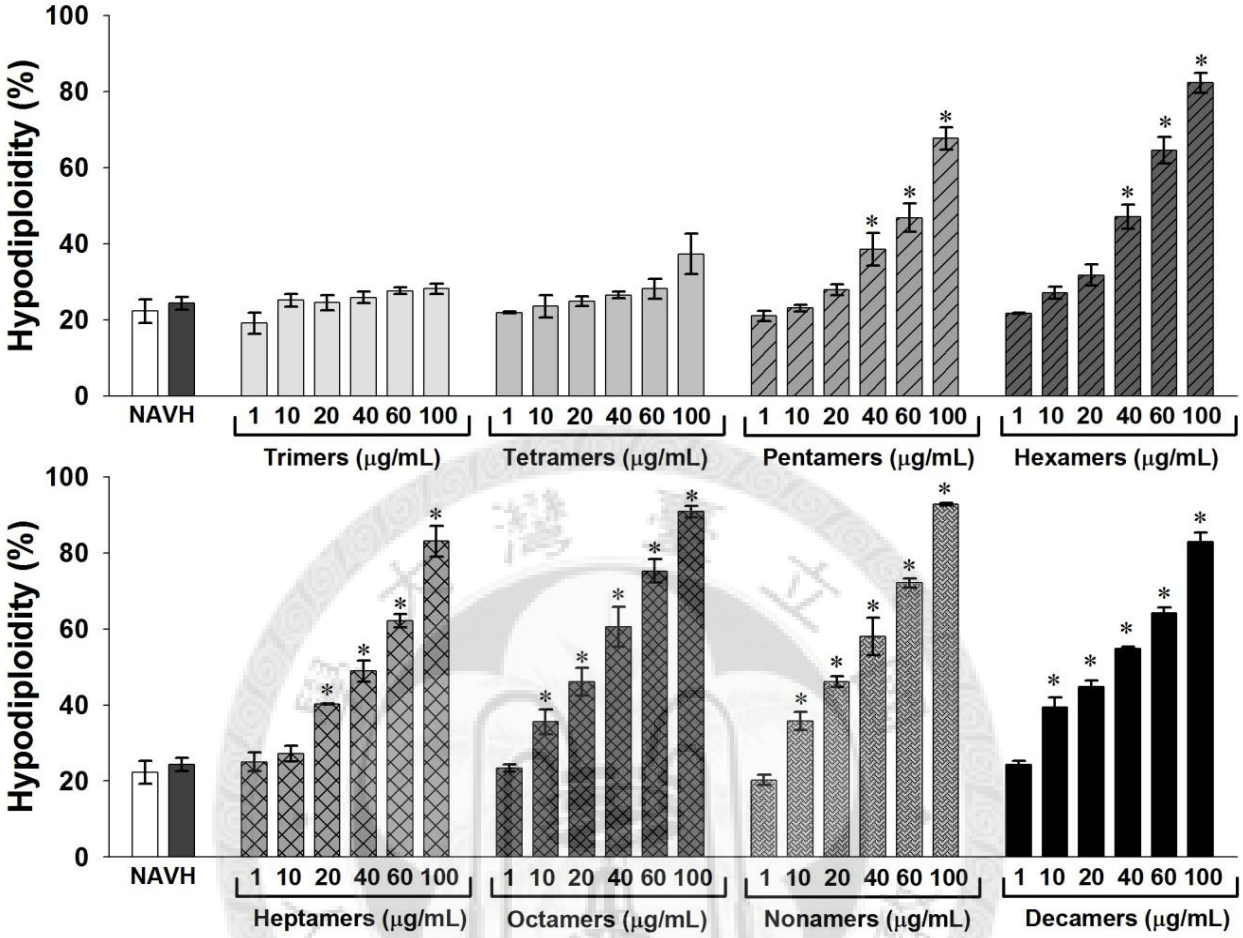


Figure 22. Induction of caspase-dependent apoptosis in splenocytes by PANE. Splenocytes were treated with PANE (20-60 μg/mL) and/or VH for 12 h in the presence of Z-DEVD-FMK and Z-VEID-FMK (caspase inhibitors for caspse-3 -6, respectively). The percentage of hypodiploid cells was quantified by cell cycle analysis and expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. $^{\#}p < 0.05$ as compared with the matched group without caspase inhibitors.

(A) Concentration-dependent



(B) Time-dependent

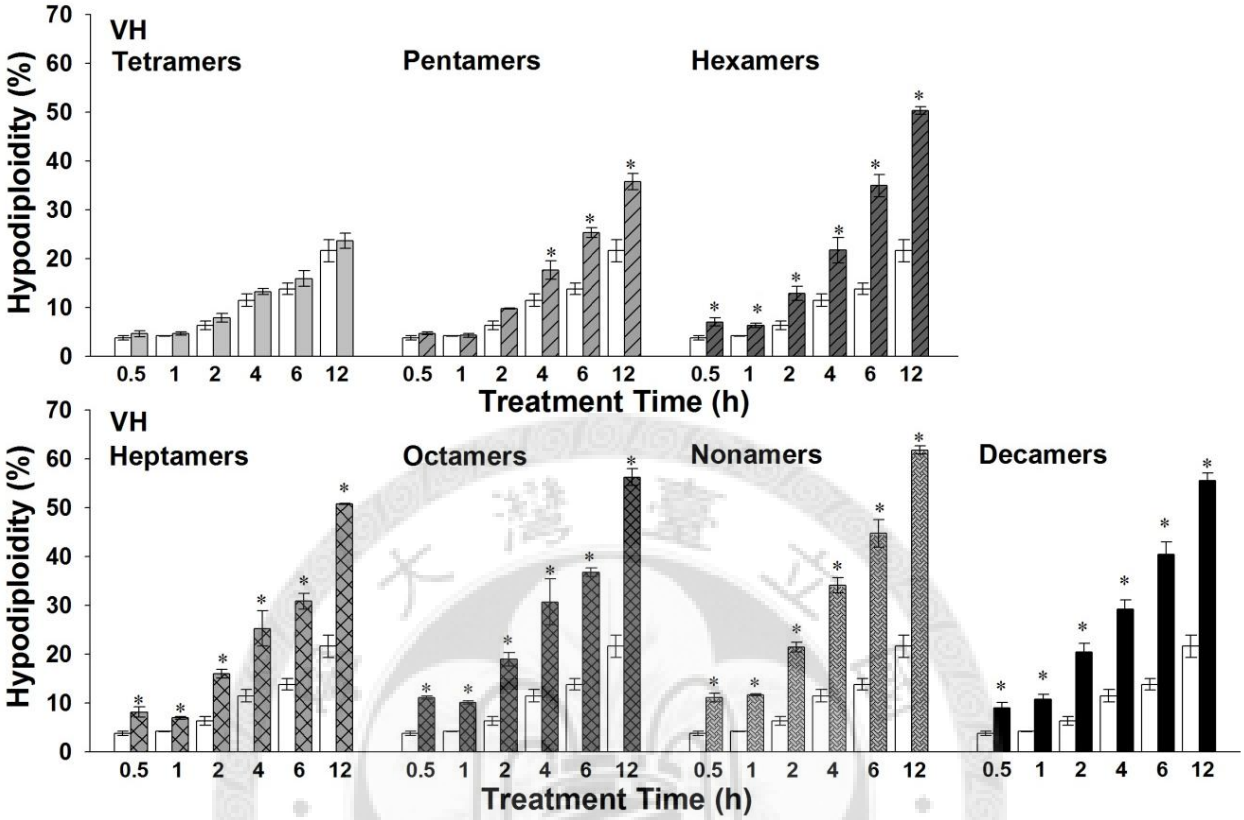


Figure 23. The effect of areca-derived oligomeric procyanidins on the apoptosis of splenocytes.

(A) Splenocytes (5×10^6 cells/mL) were either left untreated (NA) or treated with oligomeric procyanidins from trimers to dacamers (1-100 μg/mL) and/or VH (0.1% DMSO) for 12 h. (B) Splenocytes (5×10^6 cells/mL) were either left untreated (NA) or treated with oligomeric procyanidins from tetramers to dacamers (1-100 μg/mL) and/or VH (0.1% DMSO) for 0.5-12 h. Cells were washed and fixed with 70% ethanol at -20° C for at least 1 h. Cells were stained with 400 μL propidium iodide staining buffer (50 μg/mL propidium iodide and 20 μg/mL ribonuclease in PBS) at 37 ° C for 30 min. The single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. (A) The percentage of hypodiploid cells was expressed as the mean ± SE of pooled data from three independent experiments. *$p < 0.05$ as compared with the VH group.

Table 1. The 50% effective concentration (EC_{50}) of procyanidins-induced apoptosis.

Procyanidins	[EC50] (μg/mL)	Apoptotic rate (%) at 100 μg/mL at 12 h
Dimer	N.D.	2.66 ± 1.94
Trimer	N.D.	3.74 ± 1.68
Tetramer	N.D.	12.91 ± 3.70
Pentamer	56.27 ± 1.15	43.37 ± 1.91
Hexamer	50.03 ± 3.06	57.97 ± 3.20
Heptamer	48.27 ± 2.80	58.73 ± 4.40
Octamer	40.83 ± 2.03	66.23 ± 1.63
Nonamer	44.67 ± 1.81	68.41 ± 1.99
Decamer	41.80 ± 2.25	58.64 ± 2.74

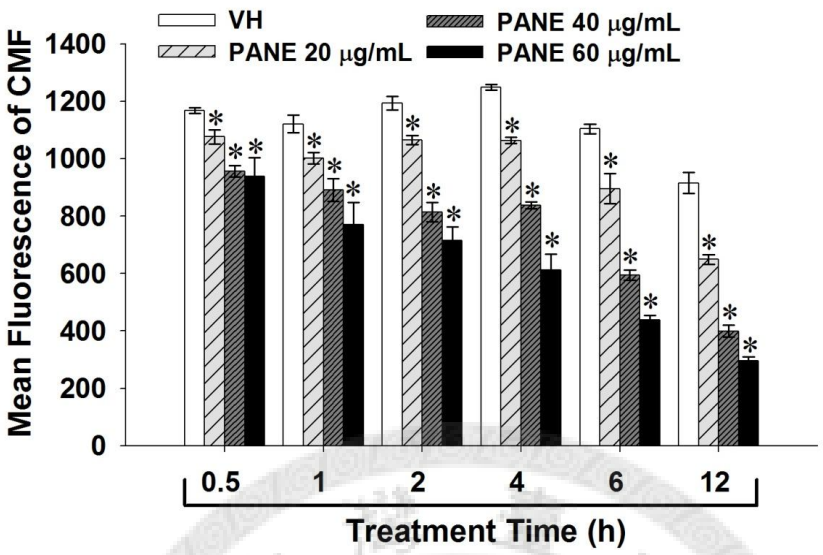
Data are expressed as the mean ± SE of pooled data from 3 independent experiments. N.D., not determined.

4.3-2 Effect of areca-derived procyanidins on the level of intracellular thiols in splenocytes

Intracellular thiols, in particular glutathione, play a critical role in maintaining the redox balance and regulating various functions in mammalian cells. Previous results have shown that ANE-induced suppression of lymphocyte reactivity was attenuated in the presence of NAC and glutathione. The influence of PANE and oligomeric procyanidins on the level of thiols in splenic lymphocytes was therefore investigated. Cells were treated with PANE (20-60 μg/mL), or fractionated procyanidins from dimers to decamers (40 or 60 μg/mL of each) for 12 h, and the cellular thiols were detected by flow cytometry. In consistent with the observed pro-apoptotic effect, PANE induced a time- and concentration-dependent diminishment in cellular thiols (Fig. 24A). In addition, pentamers to decamers were effective in diminishing cellular thiols, whereas dimers to tetramers were inactive (Fig. 24B). As quantified by the mean fluorescence intensity, the magnitude of thiol diminishment was gradually increased in cells treated with 60 μg/mL of oligomeric procyanidins from pentamers to nonamers, demonstrating a trend of chain length-dependent effect (Fig. 24B). The role of thiol diminishment in procyanidin-mediated lymphocyte apoptosis was further investigated. Splenic lymphocytes were treated with pentamers (10-100 μg/mL) in the absence or presence

of NAC (4 mM), and apoptosis and cellular thiols were measured. Notably, pentamers (40-100 μg/mL)-induced apoptosis was significantly attenuated in the presence of NAC (Fig. 25A). Likewise, a partial but significant restoration of pentamers-induced thiol diminishment was also observed (Fig. 25B). In addition, the effect of NAC (4 mM) on the apoptosis and thiol diminishment induced by highly oligomeric procyanidins from pentamers to nonamers (40 μg/mL of each) was examined. The results showed that the effects induced by each of the oligomers were partially but significantly attenuated in the presence of NAC (Table 2 and 3).

(A) PANE



(B) Oligomeric procyanidins

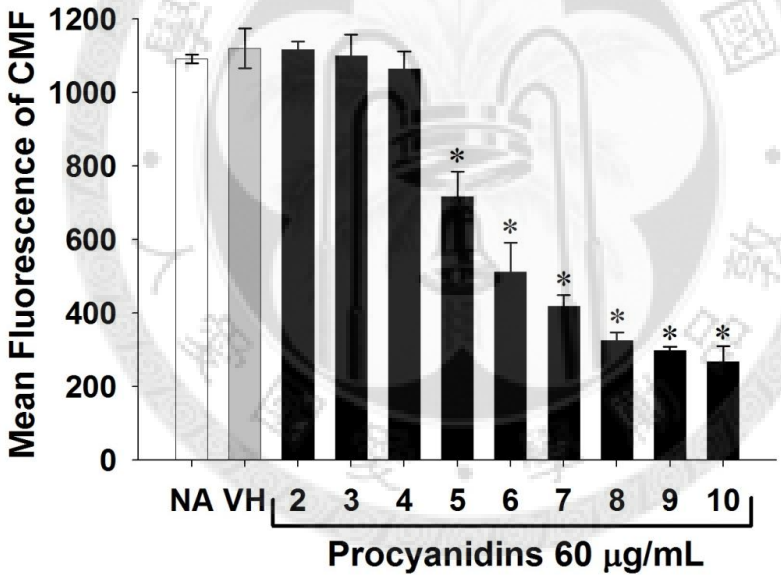
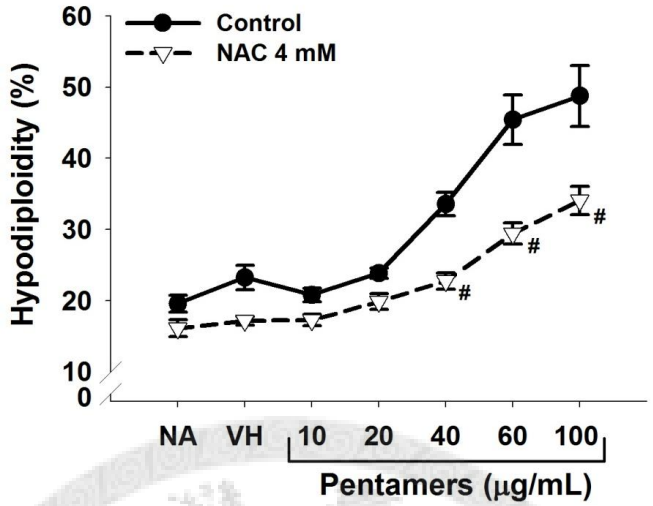


Figure 24. Effect of areca-derived oligomeric procyanidins on the intracellular level of total thiols in splenocytes.

(A) Splenocytes were treated with PANE (20-60 μg/mL) and/or VH (0.1% DMSO) for 0.5-12 h. (B) Splenocytes were either left untreated (NA) or treated with oligomeric procyanidins from dimers to dacamers (60 μg/mL) and/or VH for 12 h. Cells were incubated with CMF-DA (25 μM) for 15 min and the single cell fluorescence of 10,000 cells was measured by flow cytometry. The mean fluorescence of CMF was expressed as the mean ± SE of triplicate cultures. Results are representative of three independent experiments. *$p < 0.05$ as compared with the VH group.

(A) Cell cycle



(B) CMF

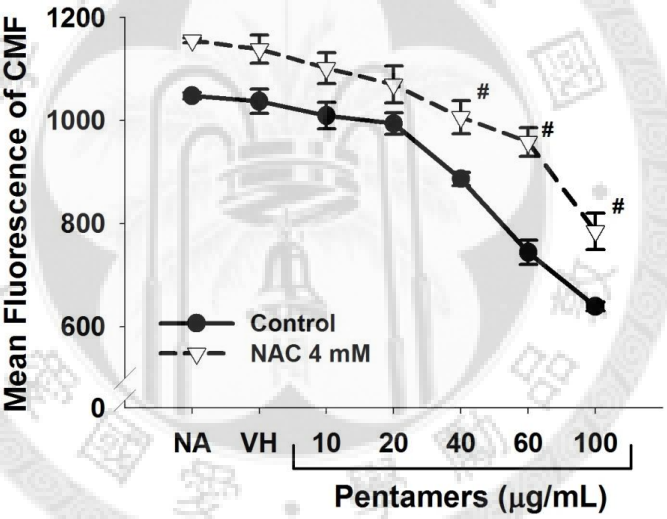


Figure 25. Attenuation of procyanidins pentamers-induced apoptosis and thiol diminishment in splenocytes by NAC.

(A) Splenocytes (5×10^6 cells/mL) were either left untreated (NA) or treated with PANE-derived procyanidins pentamers (10-100 μg/mL) and/or VH (0.1% DMSO) for 12 h. in the absence (control) or presence of NAC (4 mM). (A) The apoptosis was measured by cell cycle analysis. (B) The level of cellular thiols in splenocytes was determined by CMF-DA staining. Cells were incubated with CMF-DA (25 μM) for 15 min and the single cell fluorescence of 10,000 cells for each sample was measured by flow cytometry. The mean fluorescence of CMF was expressed as the mean ± SE of pooled data from six independent experiments. $^{\#}p < 0.05$ as compared with the matched group without NAC.

Table 2. Procyanidins (40 μg/mL)-mediated induction of apoptosis (%).

Antioxidants	VH	Pentamers	Hexamers	Heptamers	Octamers	Nonamers
Control	15.9 ± 0.1	33.6 ± 1.6	40.3 ± 0.5	39.9 ± 0.6	52.1 ± 1.1	46.9 ± 0.3
NAC 4 mM	18.0 ± 0.4	22.8 ± 1.1*	30.6 ± 1.0*	30.4 ± 0.1*	35.9 ± 0.7*	40.4 ± 0.6*

Splenic lymphocytes were treated with 40 μg/mLof procyanidins from pentamers to nonamers and/or VH (0.1% DMSO) for 12 h in the absence (control) or presence of NAC (4 mM). The apoptosis of cells was measured by cell cycle analysis. Data are expressed as the mean ± SE of pooled data from three independent experiments. *$p < 0.05$ as compared with the matched control.

Table 3. Procyanidins (40 μg/mL)-mediated diminishment of cellular thiols (mean fluorescence intensity of CMF).

Antioxidants	VH	Pentamers	Hexamers	Heptamers	Octamers	Nonamers
Control	932.3 ± 10.1	737.9 ± 19.2	568.1 ± 36.7	525.4 ± 62.3	437.6 ± 4.5	440.7 ± 17.4
NAC 4 mM	959.4 ± 18.7	818.3 ± 27.0*	698.3 ± 5.5*	668.9 ± 10.1*	540.5 ± 25.9*	657.1 ± 5.3*

Splenic lymphocytes were treated with 40 μg/mL of procyanidins from pentamers to nonamers and/or VH (0.1% DMSO) for 12 h in the absence (control) or presence of NAC. The level of cellular thiols was measured by flow cytometry. Data are expressed as the mean ± SE of pooled data from three independent experiments. *$p < 0.05$ as compared with the matched control.

4.4 ANE and PANE enhances the development of CD11b$^+$Gr-1$^+$ cells with the characteristics of myeloid-derived suppressor cells in OVA-sensitized BALB/c mice

4.4-1 Induction of CD11b$^+$Gr-1$^+$ cells by ANE and PANE *in vivo*

Treatment of ovalbumin (OVA)-sensitized mice with ANE and PANE induced splenomegaly, as evidenced by a marked increase in spleen index in ANE- and PANE-treated groups compared to the VH control (Table 4). The population of splenic CD4$^+$, CD8$^+$ and B220$^+$ cells was significantly decreased in ANE (50 mg/kg) and PANE (25 mg/kg)-treated groups, whereas the population of CD11b$^+$ myeloid cells was robustly increased in PBMC and splenocytes of ANE-treated mice in a dose-dependent manner (Table 4). In contrast, arecoline (5 mg/kg) had no effect on spleen index and the cellularity (Table 4). In addition, ANE and PANE dramatically increased the population of CD11b$^+$Gr-1$^+$ cells in splenocytes and PBMC in a dose-dependent fashion (Fig. 27B and 27C). Greater than 90% of the splenic CD11b$^+$ cells co-expressed Gr-1 in the ANE (50 mg/kg)-treated group (Fig. 27D). Morphological examination revealed that the splenic CD11b$^+$ cells included both granulocytic and monocytic cells (Fig. 27E).

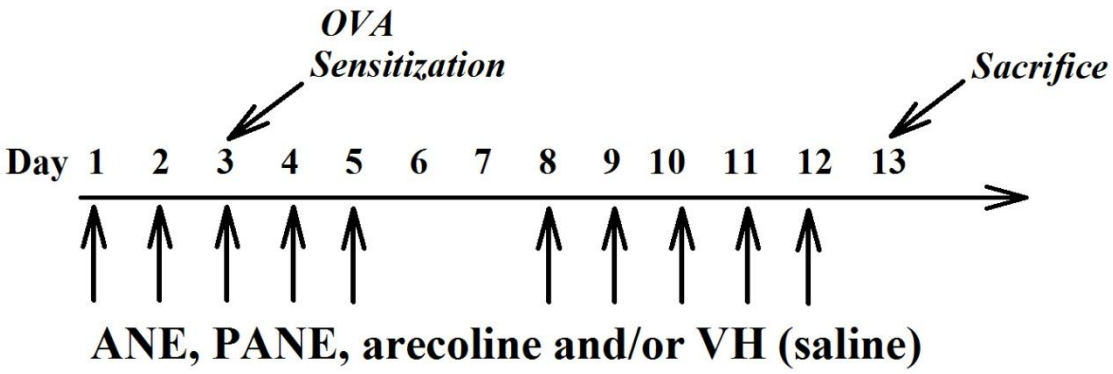


Figure 26. Protocol for OVA sensitization and administration of ANE, PANE or arecoline to BALB/c mice.

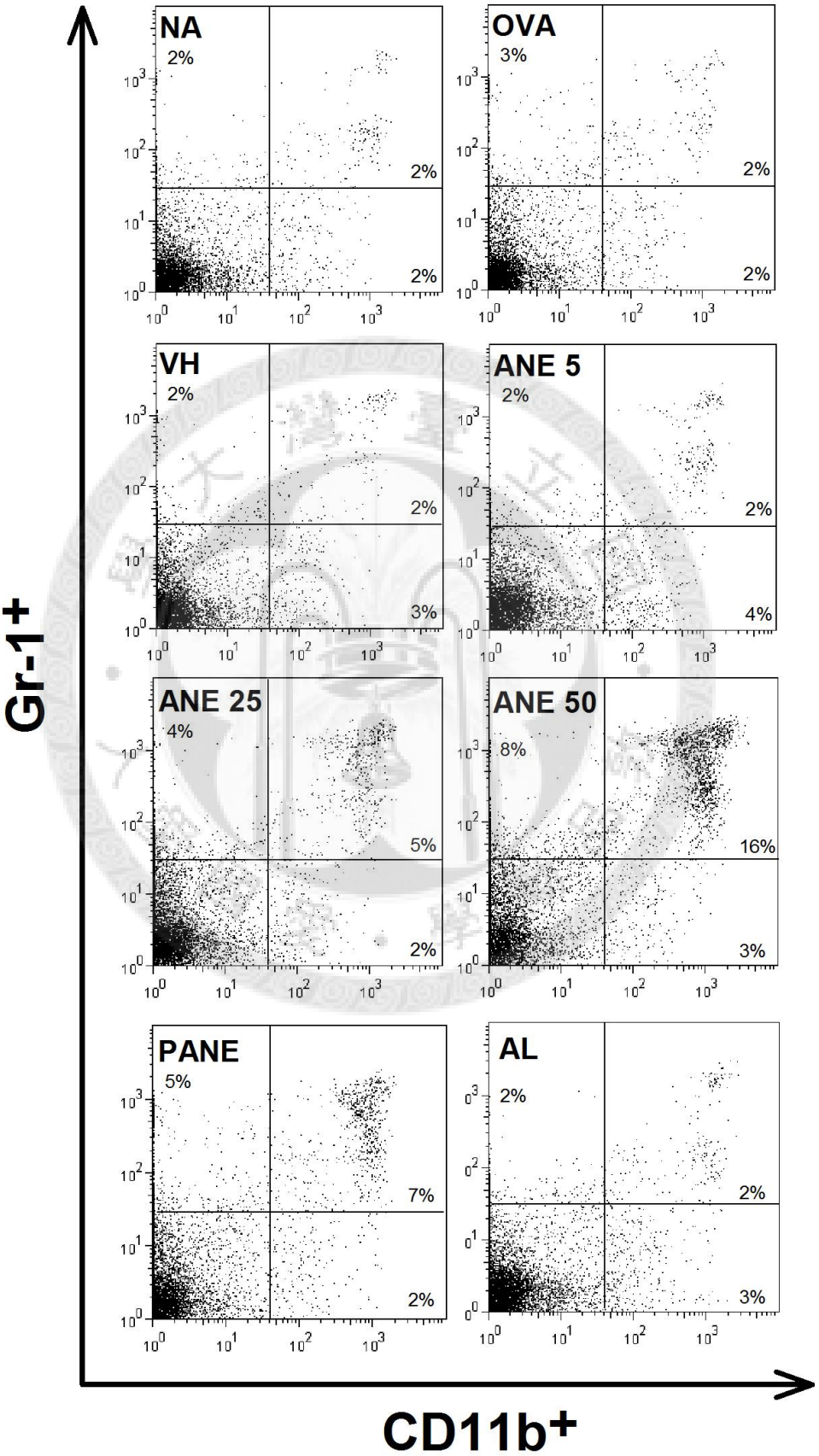
Mice were randomly divided into the following groups: naïve (NA), OVA-sensitized (OVA), vehicle-treated and OVA-sensitized (VH), and ANE-treated and OVA-sensitized (ANE). The mice (4–5 animals/group) were either left untreated (naïve; NA), or daily administered by intraperitoneal (IP) injection with ANE (5, 25, 50 mg/kg; 0.1 mL/mouse), PANE (25 mg/kg), arecoline (5 mg/kg) and/or vehicle (VH; saline) on day 1–5 and day 8–12. Except for the NA group, mice were sensitized with OVA 6 h after the ANE administration on day 3 by IP injection with 0.1 mL per mouse of sensitization solution containing 100 μg OVA and 1 mg alum in saline. The mice were sacrificed on day 13, and their blood samples and spleens were harvested for further experimentation.

Table 4. The change of body weight, spleen index and cellularity of splenocytes and PBMC in ANE- and PANE-treated mice.

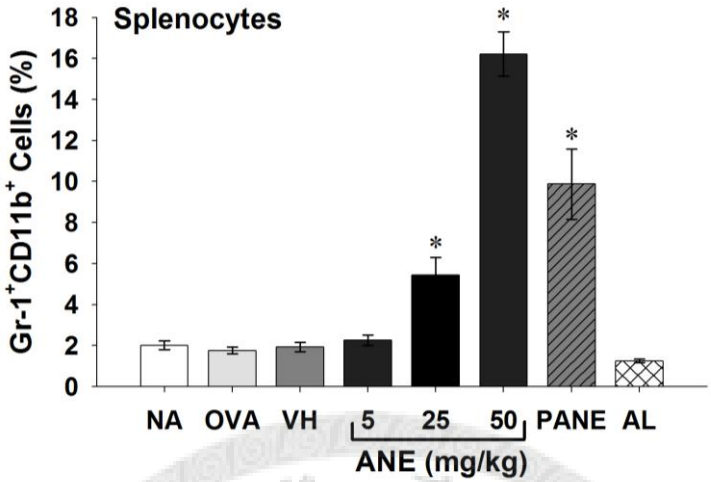
	NA	OVA	VH	ANE			PANE	Arecoline
				5 mg/kg	25 mg/kg	50 mg/kg	25 mg/kg	5 mg/kg
Body Weight (g)								
Day 1	25.8 ± 0.6	25.8 ± 0.3	26.8 ± 0.9	25.0 ± 0.4	25.3 ± 0.7	25.0 ± 0.7	25.3 ± 1.6	23.0 ± 0.6
Day14	27.8 ± 0.6	26.8 ± 0.3	25.8 ± 0.8	24.8 ± 0.6	24.7 ± 0.7	23.3 ± 0.3	22.3 ± 1.0	22.3 ± 0.3
Spleen Weight (mg)	93.3 ± 4.6	106.6 ± 5.0	105.5 ± 2.9	110.7 ± 5.0	**121.7 ± 3.4***	**168.6 ± 10.9***	**177.2 ± 10.9***	89.4 ± 2.8
Spleen Index	3.7 ± 0.1	4.1 ± 0.2	4.1 ± 0.2	4.3 ± 0.1	**5.2 ± 0.1***	**7. 7 ± 0. 5***	**7. 5 ± 0. 5***	3.9 ± 0.1
Spleen Cellularity (%)								
$CD4^+$	24.4 ± 0.8	23.2 ± 0.5	23.4 ± 1.1	23.2 ± 0.5	**20.2 ± 0.4***	**11.6 ± 1.5***	**14.9 ± 0.4***	24.8 ± 0.5
$CD8^+$	10.8 ± 0.4	10.7 ± 0.7	10.5 ± 0.6	10.8 ± 0.3	9.7 ± 0.3	**6.4 ± 0.1***	**7.3 ± 0.2***	10.9 ± 0.3
$B220^+$	54.0 ± 1.1	53.8 ± 1.1	51.8 ± 0.7	52.7 ± 0.8	**48.5 ± 0.6***	**28.2 ± 3.5***	**41.0 ± 0.5***	49.0 ± 0.2
$CD11b^+$	4.4 ± 0.5	3.9 ± 0.3	4.1 ± 0.3	4.2 ± 0.4	**6.6 ± 0.6***	**15.5 ± 1.1***	**9.2 ± 1.5***	3.1 ± 0.4
PBMC Cellularity (%)								
$CD4^+$	38.1 ± 1.7	36.7 ± 3.8	36.8 ± 2.7	37.6 ± 3.7	32.3 ± 2.9	**21.8 ± 4.0***	**25.6 ± 3.2***	33.7 ± 0.9
$CD8^+$	6.5 ± 0.5	7.4 ± 1.2	8.6 ± 0.7	7.5 ± 1.2	7.9 ± 0.7	**5.9 ± 0.9***	**5.2 ± 0.2***	8.3 ± 0.7
$B220^+$	28.6 ± 1.0	26.5 ± 0.6	29.1 ± 2.1	23.5 ± 1.8	22.1 ± 2.7	**15.7 ± 1.5***	**16.0 ± 2.8***	28.9 ± 2.0
$CD11b^+$	12.6 ± 1.3	15.5 ± 4.3	14.1 ± 1.2	**29.2 ± 1.7***	**43.0 ± 0.8***	**52.7 ± 4.2***	**48.3 ± 0.5***	13.5 ± 2.0

*, $p < 0.05$ as compared to the VH group

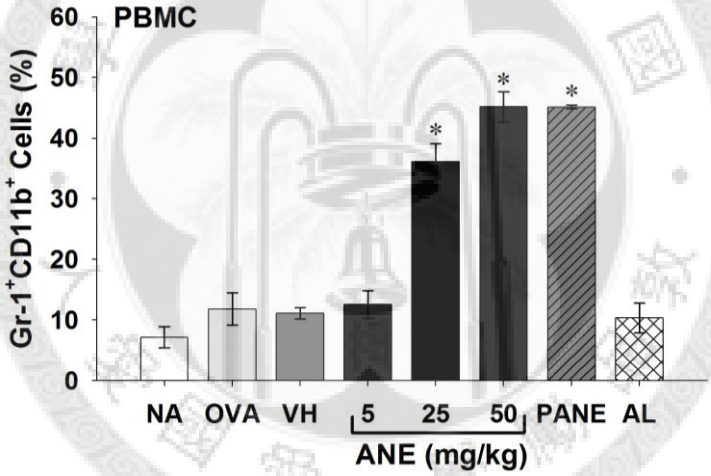
(A)


NA
2%
2%
2%
OVA
3%
2%
2%
VH
2%
2%
3%
ANE 5
2%
2%
4%
ANE 25
4%
5%
2%
ANE 50
8%
16%
3%
PANE
5%
7%
2%
AL
2%
2%
3%
Gr-1+
CD11b+

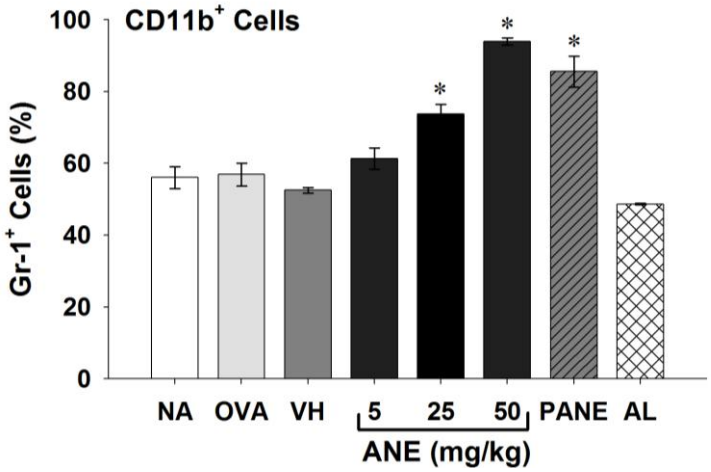
(B) Splenocytes



(C) PBMC



(D) Splenic CD11b+ Cells



(E)

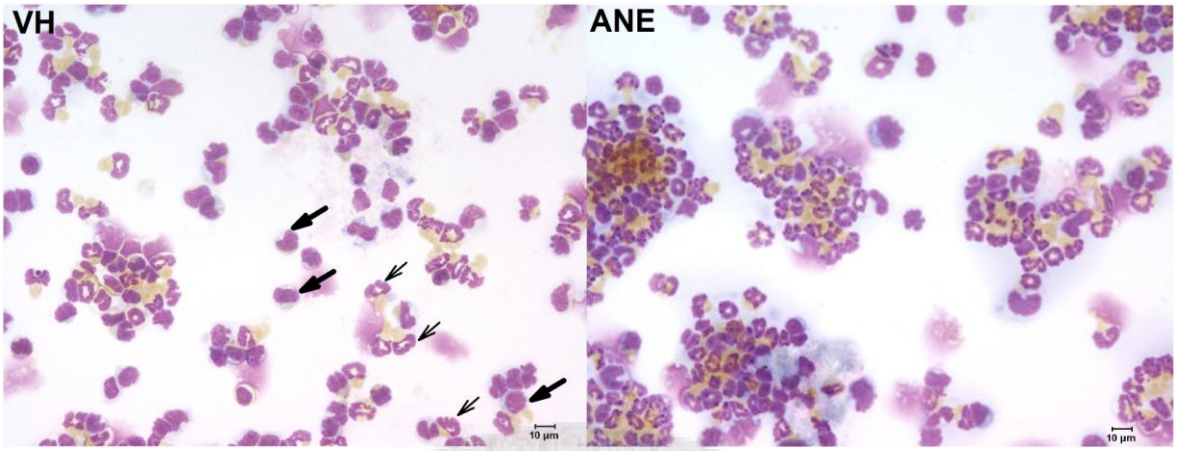


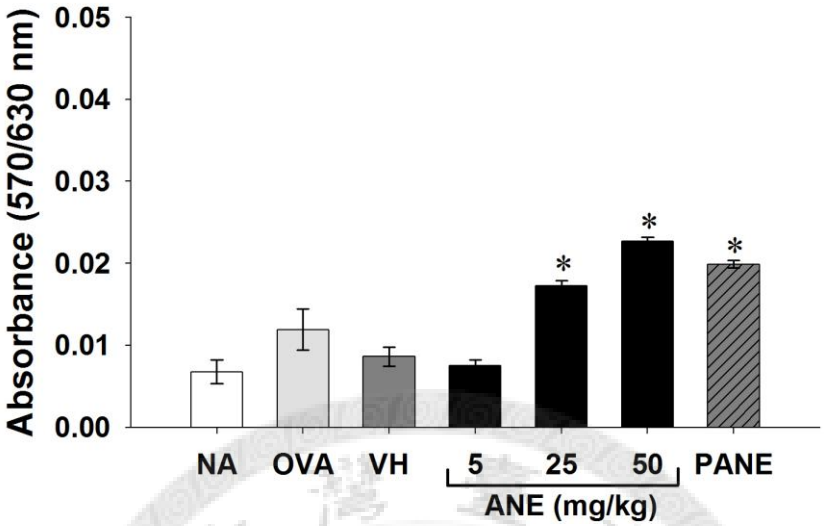
Figure 27. Induction of CD11b$^+$Gr-1$^+$ cells in splenocytes and PBMC of mice administered with ANE and PANE.

Mice were treated with ANE, PANE (25 mg/kg) and arecoline (AL; 5 mg/kg) as described in Fig. 26. The population of CD11b$^+$Gr-1$^+$ cells in splenocytes and PBMC was determined by flow cytometry. (A) Representative dot plots of CD11b$^+$Gr-1$^+$ cells were shown in panel A. (B) The percentage of CD11b$^+$Gr-1$^+$ cells in splenocytes was quantified. (C) The percentage of CD11b$^+$Gr-1$^+$ cells in PBMC was quantified. (D) Splenic CD11b$^+$ cells were purified by positive cell sorting using anti-CD11b magnetic beads. The percentage of Gr-1$^+$ cells in the splenic CD11b$^+$ cells was quantified. The data are expressed as mean ± SE of triplicate samples. The Results are representative of three independent experiments. *, $p < 0.05$ as compared to the VH group. (E) Splenic CD11b$^+$ cells isolated from the VH group and ANE (25 mg/kg)-treated mice were centrifuged onto slides, and stained with Diff-Quick. Open arrows indicate granulocytic cells with ring-shaped nuclei. Close arrows indicate monocytic cells.

4.4-2 Functional characteristics of ANE-induced $CD11b^+Gr\text{-}1^+$ cells

To investigate whether ANE modulate the reactivity of the $CD11b^+$ cells, the metabolic activity of splenic $CD11b^+$ cells was determined using an MTT assay. ANE and PANE markedly enhanced the metabolic activity of $CD11b^+$ cells with or without lipopolysaccharide (LPS) stimulation (Fig. 28A and 28B). It has been shown that MDSC may promote the development of regulatory T cells (T_{reg}) and down-regulate macrophage functions by the expression of the immunosuppressive cytokine IL-10 (Young et al., 1996). In addition, enhanced expression of iNOS and arginase-I, which deplete $_L$-arginine, a crucial amino acid required for T cell activation was associated with the immunosuppressive function of MDSC (Rodriguez et al., 2007). Therefore, the production of IL-10 by total splenocytes and splenic $CD11b^+$ cells was further examined. ANE treatment, in a dose-dependent manner, significantly enhanced the production of IL-10 by splenocytes and splenic $CD11b^+$ cells stimulated with LPS (5 μg/mL) (Fig. 29). ANE treatment enhanced the activity of arginase-I (Fig. 30) and the expression of iNOS and arginase-I by splenic $CD11b^+$ cells in a dose-dependent manner (Fig. 31).

(A) Without Stimulation



(B) LPS-stimulation

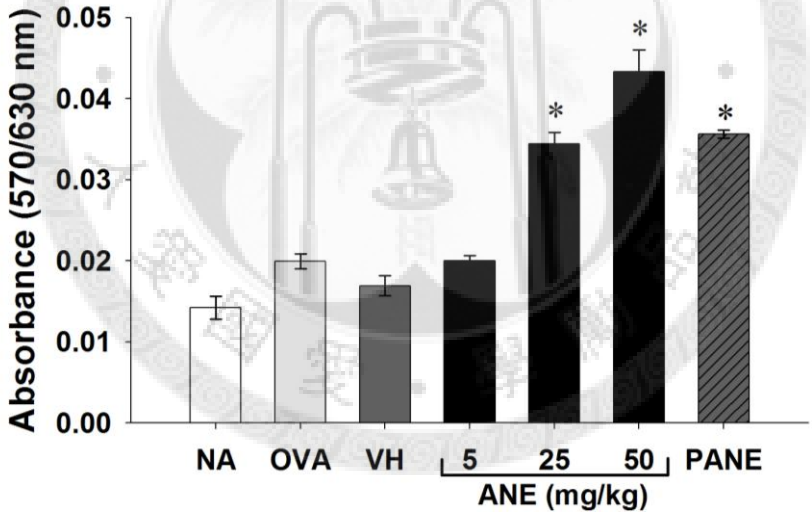
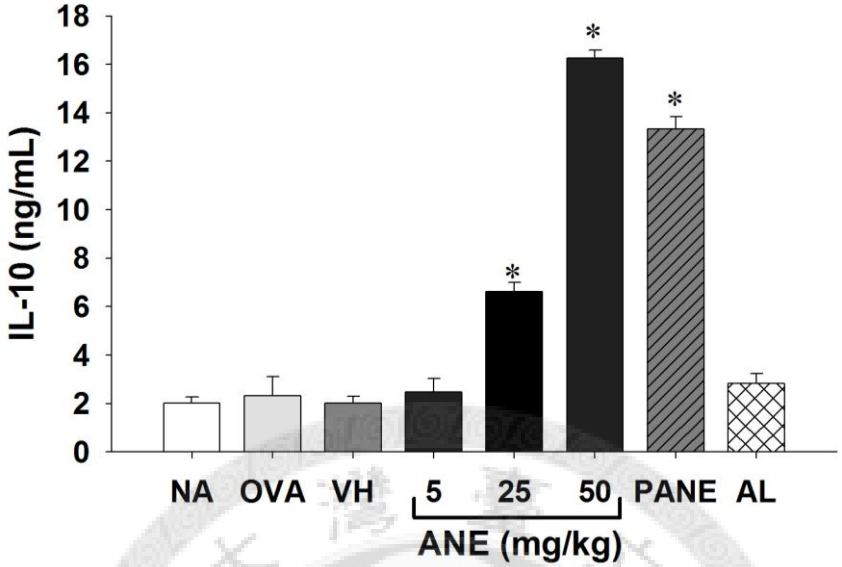


Figure 28. The effect of ANE on the metabolic activity of splenic CD11b$^+$ cells stimulated with or without LPS.

Mice were treated with ANE, PANE (25 mg/kg) and arecoline (AL; 5 mg/kg) as described in Fig. 26. CD11b$^+$ cells (2 × 10^5 cells/mL) were cultured without stimulation (A) or with LPS (B) stimulation (LPS; 5 μg/mL). The metabolic activity of viable cells was determined using an MTT assay. The data are expressed as the mean ± SE of triplicate samples per group. The results are representative of two independent experiments. *, $p < 0.05$ as compared to the VH group.

(A) Splenocytes



(B) Splenic CD11b^{+} Cells

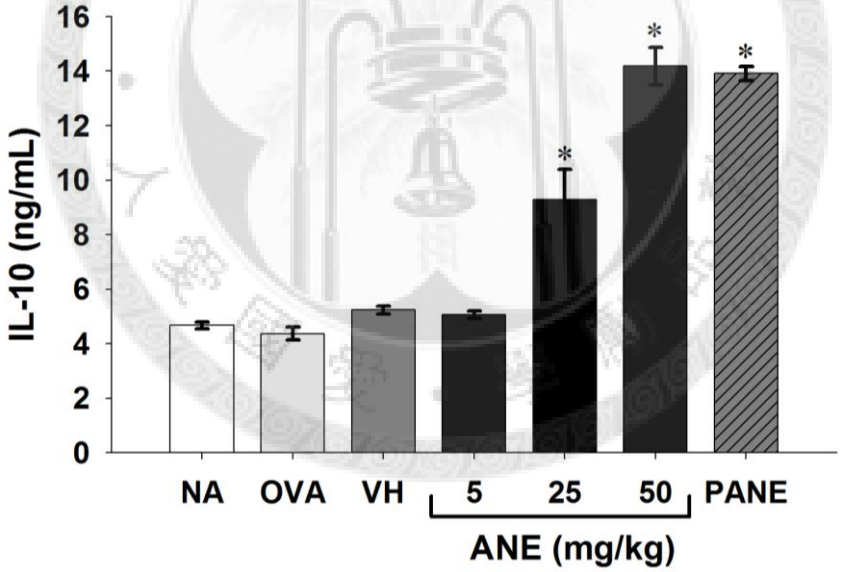


Figure 29. Enhancement of IL-10 production by splenocytes and splenic CD11b+ cells isolated from mice treated with ANE and PANE.

Mice were treated with ANE, PANE and arecoline as described in Fig. 26. (A) Splenocytes (5×10^6 cells/mL) and (B) splenic CD11b^{+} cells (5×10^5 cells/mL) were stimulated with LPS (5 μg/mL) for 48 h. The level of IL-10 in the supernatants was measured by ELISA. The data are expressed as the mean ± SE of quadruplicate samples. Results are representative of two independent experiments. *, $p < 0.05$ as compared to the VH group.

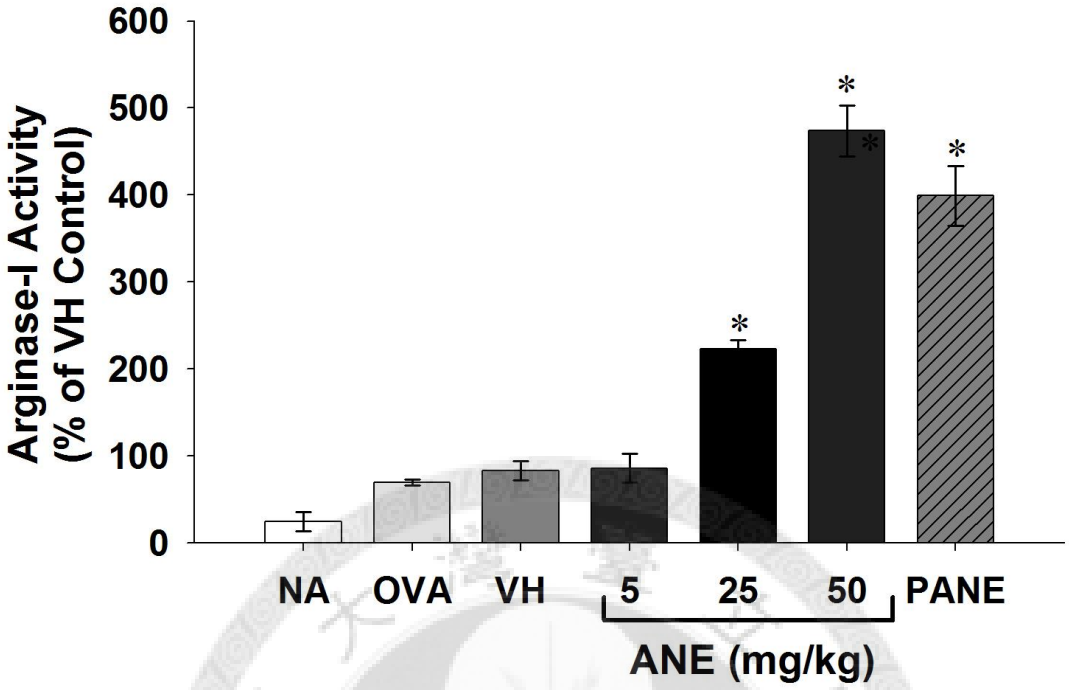


Figure 30. Elevation of arginase-I activity in splenic CD11b$^+$ cells isolated from mice treated with ANE and PANE.

Mice were treated as described in Fig. 26. Splenic CD11b$^+$ cells (2×10^6 cells) of ANE and PANE-treated mice were stimulated with LPS (5 μg/mL) for 18 h. Activity of arginase-I was determined by hydrolysis of L-arginine to urea. The urea concentration was measured at 540 nm.

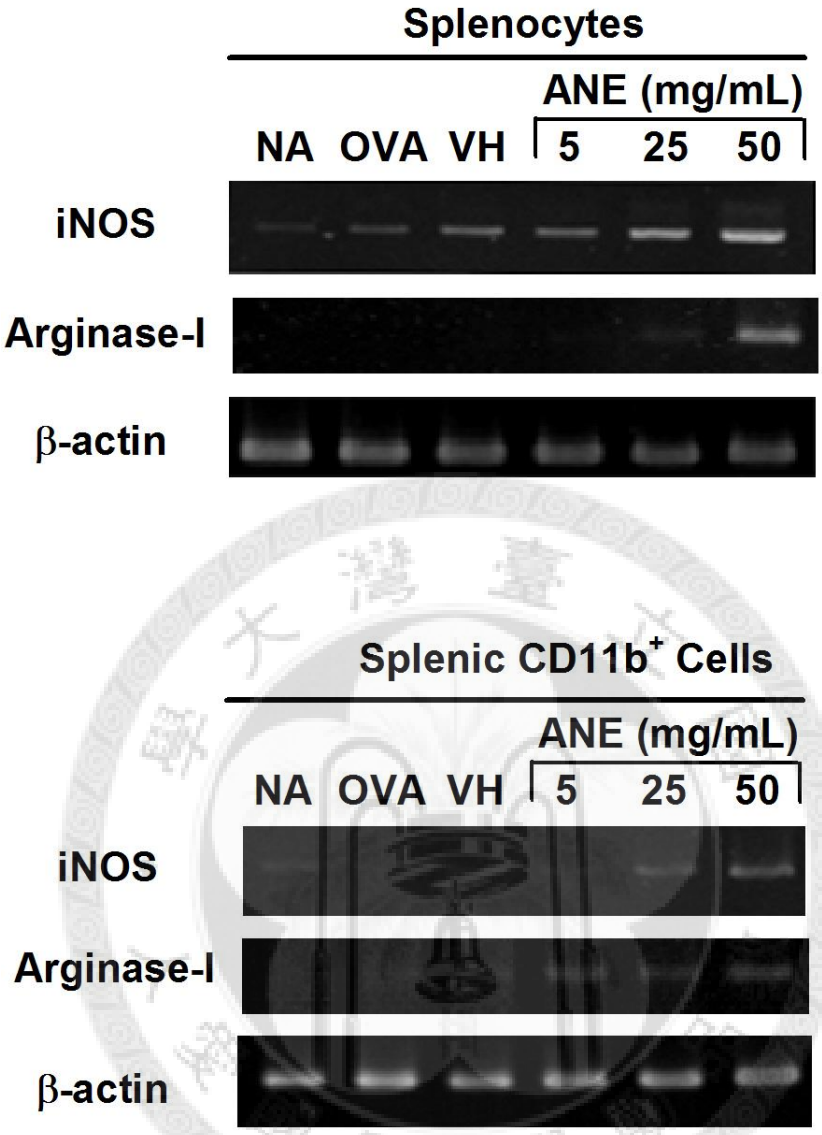


Figure 31. Induction of iNOS and arginiase-I mRNA expression by splenocytes and splenic CD11b$^+$ cells isolated from mice treated with ANE.

Splenocytes and splenic CD11b$^+$ cells were stimulated with LPS (5 μg/mL) for 24 h. The total RNA from the cells was extracted and the mRNA expression of iNOS, arginiase-I and β-actin was measured by RT-PCR. The level of housekeeping gene β-actin was used to normalize the expression of the mRNA for semi-quantification. Results are representative of three independent experiments.

Chapter 5. Discussion

5.1 ANE suppresses T-cell activation and IFN-γ production via the induction of oxidative stress

Although considerable clinical evidence indicates that deterioration of cell-mediated immunity is associated with the pathophysiology of areca-related oral diseases, the underlying mechanism responsible for the immune aberrations remains mostly unclear. The cytotoxic effect of areca constituents on oral epithelial cells, mucosal fibroblasts, gingival keratinocytes, CHO-K1 cells, lymphocytes, bone marrow cells and neutrophils has been reported (Bagchi et al., 2002; Chang et al., 2001a; Jeng et al., 1999; Jeng et al., 2003; Liu et al., 1996). However, little is known regarding how ANE affects the functional activities of T cells, the major immunocompetent cells found in OSF specimens. In confirmation of the previous report (Kumpawat et al., 2003), the present study demonstrated a significant cytotoxicity of ANE on primary splenocytes (Fig. 4). In addition, the production of IL-2 and IFN-γ was dramatically suppressed by ANE (Fig. 5 and 6), whereas IL-4 was unaltered (Fig. 7). These results demonstrated a differential effect of ANE on cytokine production by Th1 and Th2 cells. As Th1 cytokines play a pivotal role in cell-mediated immunity, the down-regulation of IL-2 and IFN-γ by ANE substantiates previous clinical findings demonstrating the deregulation of cell-mediated immunity

in OSF and oral cancer patients. The direct cytotoxicity and suppression of Th1 cytokine production induced by ANE may be one of the underlying mechanisms contributing to the observed immune deregulation.

IFN-γ, a major cytokine produced by Th1 cells and other immune cells, plays a pivotal role in both adaptive and innate immune responses. For example, IFN-γ skews the immune responses toward a Th1 phenotype. IFN-γ induces IL-12 production in phagocytes, and the coordination of IFN-γ and IL-12 mediates a positive feedback loop to amplify cell-mediated immunity. Up-regulation of MHC class I by IFN-γ increased the recognition of peptides by Tc cells and promoted the induction of cell-mediated immunity (Boehm et al., 1997). IFN-γ enhances the expression of MHC class II and costimulatory molecules in professional antigen-presenting cells, thereby promoting antigen-specific activation of Th cells (Mach et al., 1996). Moreover, the killing activity of NK cells and the microbicidal ability of phagocytes are increased by IFN-γ (Billiau and Matthys, 2009). IFN-γ modulates the trafficking of specific immune cells to inflammation sites through increasing expression of adhesion molecules and chemokines, including IFN-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), and vascular cell adhesion molecule-1 (VCAM-1) (Schroder et al., 2004). It has been suggested that IFN-γ plays a key role in cancer immunosurveillance (Ikeda et al., 2002; Shankaran et al., 2001). For example, Meth

A-induced tumors grew more rapidly in the mice treated with a neutralizing antibody against IFN-γ (Dighe et al., 1994). Overexpression of dominant negative IFNGR1 (encoding the ligand-binding chain of the IFN-γ receptor) mutants resulted in unresponsiveness of tumor cells to IFN-γ and an enhanced cell growth (Coughlin et al., 1998; Dighe et al., 1994). The incidence of lymphoma is increased in IFN-γ-knockout mice (Street et al., 2001). Collectively, the downregulation of IFN-γ may compromise cell-mediate immunity and promote infection and tumor promotion.

In addition, IFN-γ exhibits anti-fibrotic activity via the inhibition of collagen synthesis (Lee et al., 1991; Nguyen et al., 1994). Down-regulation of IFN-γ by leukocytes was observed in OSF patients (Haque et al., 2000), and intralesional injections with IFN-γ improved the mouth opening in OSF patients (Haque et al., 2001). These reports suggest that an altered expression of IFN-γ may be involved in the pathophysiology of areca-related oral diseases. Notably, the present study demonstrated that direct treatment of splenocytes with ANE remarkably suppressed IFN-γ production (Fig. 5). In light of the anti-fibrotic and the antitumor activities of IFN-γ, it is speculated that ANE-mediated attenuation of IFN-γ production may lead to the suppression of the host mechanisms against the development of tumor and fibrosis, contributing to the progression of the oral diseases in areca chewers.

It has been documented that oxidative stress plays a pivotal role in the

pathogenesis of oral diseases associated with areca chewing. Several studies have shown that ANE and lime enhance the generation of reactive oxygen species (ROS) and cause oxidative DNA damage under alkaline conditions (pH 9.5). ROS may be involved in the tumor initiation process via the induction of gene mutation and genotoxicity (Nair et al., 1987; Nair et al., 1990). The induction of ROS by ANE was enhanced by $Cu2^{+}$, Fe^{2+} and Fe^{3+}, but inhibited by Mn^{2+} (Nair et al., 1987; Nair et al., 1990). The generation of ROS is likely due to the autoxidation of areca polyphenols, such as tannins and catechin, under alkaline condition, following by quinone/semiquinone radical- and iron-catalyzed Haber-Weiss and Fenton reactions (Nair et al., 2004). As nucleotide modification and 8-hydroxy-2-deoxyguanosine (8-OH-dG) formation can be induced by ROS leading to the development of initiated mutated cells, 8-OH-dG is therefore considered as a biomarker for carcinogenesis (Marnett, 2000). Interestingly, an increased generation of ROS in the oral cavity of volunteers chewing areca quid has been demonstrated, and ANE increases the formation of 8-OH-dG (Liu et al., 1996; Nair et al., 1995). In addtion, ANE has been shown to induce H_2O_2 formation in CHO-K1 and JB6 epithelial cells (Lin et al., 2003; Liu et al., 1996). ANE exposure caused oxidative DNA damage in human keratinocytes, as evidenced by the increased formation of 8-OH-dG (Lai and Lee, 2006). Long term exposure of human HaCaT epithelial cells to ANE caused oxidative

DNA damage (Lai and Lee, 2006). Together these results indicate that exposure to areca constituents leads to ROS generation and DNA damage, thereby contributing to the AQ-associated oral carcinogenesis.

Results from the present study also suggest that oxidative stress is critically involved in ANE-mediated effects on splenocytes. The presence of NAC markedly attenuated ANE-mediated cytotoxicity and inhibition of IL-2 and IFN-γ (Fig. 4-6). NAC is an antioxidant that acts as scavenger for ROS and it is rapidly metabolized into intracellular glutathione, which maintains the cellular redox balance. To further investigate the role of oxidative stress in ANE effects, the direct influence of ANE on the level of cellular ROS was determined, and the results showed that ANE (10-40 μg/mL) augmented intracellular ROS levels (Fig. 8) and induced a concomitant decrease in the intracellular glutathione content in splenic T-cells preincubated with NAC (Fig. 9). Collectively, these data clearly demonstrated that the cytotoxicity and suppression of Th1 cytokine production by ANE were mediated, at least in part, by the induction of oxidative stress.

ROS play a crucial role in the regulation of T-cell activation, energy and apoptosis. The activation of T cells through the T-cell receptor induces a low but significant level of ROS that are involved in the signaling transduction for T-cell proliferation (Williams and Kwon, 2004). In contrast, high levels of ROS induce T

cell apoptosis (Tripathi and Hildeman, 2004). The balance of the redox status is critical for T-cell reactivity. Exogenous H_2O_2 has been shown to modulate cytokine production and cell death of leukocytes. The suppression of IFN-γ production by H_2O_2 was observed at concentrations slightly less than that required to induce cell death (Malmberg et al., 2001). Interestingly, the present study also revealed a similar pattern of concentration-dependency by ANE on cytotoxicity and IFN-γ production in splenocytes. The sub-cytotoxic concentrations of ANE (10-20 μg/mL) suppressed IFN-γ production in parallel with ROS induction (Fig. 5 and 8), whereas concentrations of ANE > 20 μg/mL caused cytotoxicity. These results suggest a role for ROS in ANE-mediated cytotoxicity and attenuation of IFN-γ production by splenocytes.

5.2 Involvement of the mitochondrion-dependent pathway and oxidative stress in the apoptosis of murine splenocytes induced by ANE

In oral cancer patients, induction of T cell apoptosis and down-expression of T cell receptor ζ-chain were correlated with the high levels of FasL expressed by tumor cells (Reichert et al., 2002). Immunosuppressive factors, such as TGF-β, PGE_2 and IL-10, which may be produced by cancer cells or immune cells, attenuated the functional activity of T cells and induced cell death (Chang et al., 2004; Reichert et al.,

2002; Rice et al., 1992). In areca chewers and oral cancer patients, the frequency of sister chromatid exchanges (SCE) and chromosome aberrations (CA) in peripheral blood lymphocytes were elevated, indicating the genotoxic effect of areca constituents on lymphocytes (Adhvaryu et al., 1991). These data provide evidence to show how tumor cells and areca ingredients may cause T cell dysfunction. However, a potential direct effect by areca ingredients cannot be ruled out. Administration of ANE increased the SCE and CA in mouse bone marrow cells (Kumpawat et al., 2003). It was reported that ANE inhibited the phagocytic activity of neutrophils (Hung et al., 2006) and the reactivity of mast cells (Lee et al., 2004). ANE (10-80 μg/mL) dose-dependently induced HaCaT cell apoptosis (Lai and Lee, 2006). Apoptosis is an important mechanism to control the development and homeostasis of T cells. An up-regulation of apoptosis in T cells may lead to immunosuppression. As previous results have shown the cytotoxicity of ANE in splenocytes (Fig. 4), the effect of ANE on the apoptosis of splenocytes was investigated. As expected, ANE markedly induced splenocyte apoptosis as measured by cell cycle analysis and morphological examination (Fig. 10-12). These data provide a potential mechanism for the above results showing the attenuation of splenocyte reactivity by ANE.

The biochemical pathways involved in ANE-mediated lymphocyte apoptosis was further examined. Pretreatment of splenocytes with various caspase inhibitors

significantly reverses ANE-mediated cell apoptosis, indicating the involvement of caspase-dependent pathways (Fig. 14). Mitochondrion is critical component of the intrinsic apoptosis pathway. In many cells, the change in mitochondrial membrane potential ($\Delta\psi_m$) is one of the first irreversible steps in apoptotic signaling, which subsequently generates ROS and activates caspases (Holtzman et al., 2000). It has been reported that the deregulation of $\Delta\psi_m$ induced by ANE treatment was associated with ANE-mediated cytotoxicity (Chang et al., 2001a). In the present study, treatment of splenocytes with ANE rapidly induced $\Delta\psi_m$ depolarization (Fig. 15), followed by the cytochrome *c* release (Fig. 16) and caspase-9 activation (Fig. 17), indicating the activation of mitochondrion-dependent pathway. Notably, ANE disrupted $\Delta\psi_m$ in oral KB cells, resulting in cell cycle arrest but not apoptosis (Chang et al., 2001a). Moreover, head and neck squamous cell carcinoma was resistant to apoptosis induced by mitochondrial membrane-depolarizing agents (Zhao et al., 2008). The failure to activate apoptosis after DNA injury in oral cells may be one potential mechanism leading to carcinogenesis. It was apparent that ANE might induce a differential cytotoxic effect between oral and immune cells. In responses to ANE, lymphocytes might undergo apoptosis resulting in immunosuppression, whereas oral cells were arrested, and potentially underwent transformation. This difference may be a possible mechanism involved in the pathophysiology or areca-associated oral diseases.

Mitochondrion participates in the synthase of ATP, cell redox status, control of pH, osmotic pressure and calcium homeostasis to maintain normal cell function (Wallace et al., 1997). In addition, mitochondrion is a sensitive target attacked by oxidants, lipophilic actions and electrophiles (Wallace et al., 1997). Deregulation of $\Delta\psi_m$ has been shown to inhibit the proliferation of lymphocytes and splenocytes (Daniele and Holian, 1976). Moreover, mitochondrion is the major source of the endogenous cellular ROS; the rupture of mitochondrion membrane may cause the release of ROS and intensify the oxidative damage to cells. ROS play an important role in the pathogenesis of areca-related oral diseases as mentioned above. The present study therefore investigated the role of ROS in ANE-mediated apoptosis. The results showed that ANE markedly enhanced ROS production in the apoptotic population of ANE-treated splenocytes (Fig. 18). Time-course analysis revealed that ANE (20 μg/mL)-mediated $\Delta\psi_m$ depolarization occurred at 0.5 h post ANE treatment (Fig. 15), which preceded the enhancement of ROS production observed at 3 h post ANE treatment (Fig. 18). Of interest, ANE robustly induced the ROS production in the apoptotic population of splenocytes, whereas ANE slightly induced the ROS production in the non-apoptotic population (Fig. 18). On the base of these results, it is speculated that the increased ROS production might be from the depolarized mitochondrion of apoptotic splenocytes exposed to ANE. Moreover, ANE-induced

apoptosis, ROS formation, and caspase-9 activation were partially but significantly attenuated in the presence of *N*-acetyl-$_L$-cysteine (NAC), a thiol antioxidant capable of scavenging ROS (Fig. 19), substantiating that ROS played a critical role in ANE-mediated apoptosis of splenocytes. However, ANE-mediated $\Delta\psi_m$ depolarization was unaffected by NAC (Fig. 19D), suggesting that the $\Delta\psi_m$ depolarization was independent of the ROS production. This phenomenon is in line with the kinetic relationship between the $\Delta\psi_m$ disruption and the ROS production induced by ANE. It is also noticed that NAC does not completely reverse ANE-mediated apoptosis, suggesting other unidentified mechanisms than ROS production are involved and yet to be elucidated. In summary, the present study demonstrated that ANE exposure resulted in enhancement of apoptosis in splenocytes, which was associated with the activation of the intrinsic pathway and the production of ROS. The pro-apoptotic property of ANE in primary lymphocytes may be a critical mechanism responsible for the immune deterioration observed in AQ chewers with oral cancer.

5.3 Highly oligomeric procyanidins derived from areca nuts induce lymphocyte apoptosis via the depletion of intracellular thiols

Areca nuts have been shown to be a rich source of flavan-3-ol polyphenols containing monomers of (+)-catechin and (-)-epicatechin and their polymerized oligomers (Huang et al., 2010; Wu et al., 2007). Areca-derived tannin has been reported to induce SCE in bone marrow cells (Panigrahi and Rao, 1986). The polyphenol fraction of ANE induced more oral lesions in hamster cheek pouch than ANE did (Ranadive et al., 1979). Ripe ANE, which contains more polyphenols than tender ANE, induced more 8-OH-dG formation in CHO-K1 cells (Liu et al., 1996). A growing body of literature indicates that oligomeric procyanidins are immunoactive phytochemicals affecting the functionality of immune cells, such as lymphocytes (Akiyama et al., 2005; Kenny et al., 2007; Miyake et al., 2006; Yoshioka et al., 2008). Therefore, areca-derived polyphenols may be the candidate responsible for the ANE-mediated cytotoxicity in splenocytes. The present study investigated the pro-apoptotic effect of areca-derived oligomeric procyanidins in primary splenocytes. Although the pro-apoptotic property of procyanidins has been well documented in a variety of transformed cells (Neuwirt et al., 2008; Ranadive et al., 1979), little is known pertaining to their influence on the apoptosis of primary cells, in particular, lymphocytes. Yet this issue is critical as lymphocyte-mediated immune responses

have been reported to be modulated by procyanidins (Akiyama et al., 2005; Miyake et al., 2006; Yoshioka et al., 2008). The present study showed that PANE (Fig. 20-21) and highly oligomeric procyanidins (Fig. 23) markedly induced apoptosis of splenic lymphocytes in a concentration- and time-dependent manner, whereas monomers and oligomers smaller than tetramers were inactive (Fig. 20 and 23). These data provide the evidence to show the pro-apoptotic property of areca-derived highly oligomeric procyanidins in primary lymphocytes, substantiating the role of areca-derived polyphenols in areca-mediated immunosuppression.

Accumulating evidence describes the immunomodulatory effect of procyanidins on T cell-mediated immune reactions. The expression of cytokines including IL-2, IL-4 and IL-5 by PHA-stimulated PBMC was suppressed by procyanidins (Mao et al., 2002). Intake of 10% cocoa diet for 3 weeks in rats has been shown to decrease the production of IL-4 and antibodies, including IgM, IgG and IgA, suggesting that cocoa procyanidins may down-regulate Th2 activity and interfere with B cell functions (Ramiro-Puig et al., 2007). Highly oligomeric procyanidins purified from Jatoba herb have been demonstrated to ameliorate encephalomyelitis and collagen-induced arthritis in mice via suppression of Th1 cell-mediated immunity, including suppression of IFN-γ production, inhibition of macrophage maturation and a decrease number of $CD4^+$ T cells (Miyake et al., 2008; Miyake et al., 2006). The

anti-inflammatory and T-cell regulatory effects of procyanidins have been demonstrated in animal models of inflammatory bowel disease and type IV hypersensitivity (Yoshioka et al., 2008). Apple and areca polyphenols strongly inhibited histamine release and degranulation in basophilic leukemia (RBL-2H3) cells, demonstrating the anti-allergic potential of procyanidins (Kanda et al., 1998; Lee et al., 2004). Unripe apple polyphenols have been shown to inhibit the development of food allergies and to ameliorate active systemic anaphylaxis by decreasing IgE production and histamine release (Akiyama et al., 2005). The regulation of cellular redox status may be one of the possible mechanisms by which polyphenols modulate cell functions. Procyanidins may confer anti-inflammatory benefits via scavenging reactive oxygen and nitrogen species (Cos et al., 2003; Lotito et al., 2000), interfering with hyaluronidase and cyclooxygenases activity (Huang et al., 2010; Kanda et al., 1998; Zhang et al., 2006), preventing NF-κB activation (Erlejman et al., 2008; Park et al., 2000), and modulating pro-inflammatory cytokine production (Mao et al., 2002; Rahman et al., 2006; Ramiro-Puig et al., 2007). However, both antioxidant and pro-oxidant effects of polyphenols have been demonstrated. On one hand, polyphenols may act as antioxidant to improve cell survival (Caddeo et al., 2008; Kenny et al., 2007); on the other hand, polyphenols may induce apoptosis and prevent tumor growth by its pro-oxidant activities (Hadi et al., 2007; Lambert et al., 2005).

These data are in parallel with a previous report showing the induction of apoptosis by a single high concentration (500 μg/mL) of grape procyanidins in primary cardiomyocytes (Shao et al., 2006). However, the effective concentrations (≥20 μg/mL) of areca procyanidins in lymphocytes was much lower than that of grape procyanidins in cardiomyocytes. It is apparent that immune cells, such as lymphocytes, are sensitive targets for procyanidins. The importance of oligomer chain length in several biological effects induced by procyanidins has been previously reported (Kenny et al., 2007; Lotito et al., 2000; Miyake et al., 2006; Pierini et al., 2008). In particular, the activity of Jatoba-derived procyanidins to suppress experimental autoimmune encephalomyelitis required at least five degrees of polymerization (Miyake et al., 2006), which is consistent with my data on lymphocyte apoptosis. Recent studies further demonstrated that the pro-apoptotic effect of apple procyanidins in osophageal adenocarcinoma, melanoma, and mammary tumor cells correlated positively with the degree of polymerization (Miura et al., 2008; Pierini et al., 2008). Together these results indicate that the oligomer chain length of procyanidins is a critical factor dictating the pro-apoptotic and immunomodulatory activity of procyanidins.

Mechanistic studies revealed a marked diminishment in the cellular thiols in lymphocytes exposed to the active oligomers (Fig. 24). The chain length-dependency

for thiol diminishment matched with apoptosis induction, in which pentamers were the threshold of molecular size for both effects. The involvement of thiol diminishment in the apoptotic response was further confirmed by my results showing the attenuation of areca procyanidins-induced apoptosis in the presence of NAC (Fig. 25, Table 2-3). The concomitant attenuation of both apoptosis and thiol diminishment by NAC suggests an oxidative stress-dependent mechanism. Notably, both antioxidant and pro-oxidant effects by grape procyanidins have been reported in primary cardiomyocytes (Shao et al., 2003; Shao et al., 2006). A high concentration (500 μg/mL) of procyanidins induced apoptosis via oxidative stress, whereas lower concentrations (10-100 μg/mL) attenuated oxidant-mediated injury, suggesting that the contrasting effects of procyanidins on the cellular redox status may be due to the different concentrations. The data presented here showed that areca-derived highly oligomeric procyanidins exerted a marked pro-apoptotic effect in primary lymphocytes, which may be a potential mechanism contributing to the immunosuppressive effects induced by areca-derived procyanidins. The pro-apoptotic activity of procyanidins is dictated by the oligomer chain length with 5 degrees of polymerization being the threshold for the effect. In addition, it is proposed an oxidative stress-dependent mechanism for the pro-apoptotic effect, as supported by the results that the active oligomers induced a diminishment of cellular thiols, and that

NAC attenuated procyanidin-induced thiol diminishment and apoptosis.

5.4 Induction of myeloid derived suppressor cells by ANE and PANE *in vivo*

Considerable evidence from clinical studies suggests a close association between immune deterioration and the pathophysiology of areca-related oral diseases (Chang et al., 2005; Haque et al., 2000). Although several reports have shown the induction of $CD34^+$ immunoinhibitory myeloid cells in head and neck cancer patients (Pak et al., 1995; Young et al., 1996) and $CD11b^+Gr\text{-}1^+$ cells in mice implanted with oral carcinoma cells (Tanaka et al., 2007), it is presently unclear if areca nut constituents influence the development of MDSC. The present study investigated the effect of ANE and polyphenols-enriched ANE (PANE) on the induction of $CD11b^+$ myeloid cells in splenocytes and PBMC in antigen-sensitized mice (Table 4). The results showed that ANE and PANE markedly induced splenomegaly and increased the population of $CD11b^+$ cells in splenocytes (Table 4). Moreover, ANE and PANE increased the population of $CD11b^+Gr\text{-}1^+$ cells, commonly defined as murine MDSC (Marigo et al., 2008; Youn et al., 2008) in both splenocytes and PBMC (Fig. 27). MDSC are known to facilitate immunosuppression via the up-regulation of several critical proteins, including IL-10, iNOS and arginase-I (Ostrand-Rosenberg and Sinha, 2009). To further investigate the phenotype of the $CD11b^+Gr\text{-}1^+$ cells induced by ANE

and PANE, it was firstly showed that the metabolic activity of splenic $CD11b^+$ cells was increased in ANE- and PANE-treated groups (Fig. 28). Next, the production of IL-10 by LPS-stimulated splenocytes and splenic $CD11b^+$ cells was significantly enhanced by ANE and PANE treatment (Fig. 29). Furthermore, the arginase-I activity of splenic $CD11b^+$ cells and the mRNA expression of iNOS and arginase-I by splenocytes and splenic $CD11b^+$ cells were markedly augmented by ANE and PANE treatments (Fig. 30-31). Collectively, these results demonstrated that ANE and PANE promoted the induction of immature myeloid cells with a functional profile of MDSC. It is speculated that the development of the immature myeloid cells by areca constituents may be one of the underlying mechanisms contributing to the immune dysfunction reported in patients with areca-related oral diseases.

MDSC are a heterogeneous population of cells, comprising of granulocytes, monocytes/macrophages, DC at various stages of differentiation (Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009; Youn et al., 2008). Murine MDSC are characterized by the double expression of CD11b and Gr-1 surface markers (Ostrand-Rosenberg and Sinha, 2009; Youn et al., 2008). Normally, few $CD11b^+Gr\text{-}1^+$ cells are present in bone marrow, blood and secondary lymphoid organs, and the cells lack immunosuppressive activity and quickly differentiate to mature myeloid cells (Gabrilovich and Nagaraj, 2009). In pathological conditions, including

cancer, various infectious diseases, chronic inflammation, sepsis, trauma, bone marrow transplantation and some autoimmune diseases, the maturation of $CD11b^{+}Gr\text{-}1^{+}$ cells was hampered, resulting in the expansion of the immature MDSC (Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009; Youn et al., 2008). A significant increase in the proportion of the $CD11b^{+}Gr\text{-}1^{+}$ cells from < 5% to > 15% has been reported in many tumor models (Youn et al., 2008). In addition, a significant correlation between circulating MDSC and clinical cancer stage has been reported (Diaz-Montero et al., 2009). The present study showed that the population of $CD11b^{+}Gr\text{-}1^{+}$ cells in splenocytes of normal mice was approximately 2%, which was increased to 5-17% in mice treated with ANE and PANE (Fig. 27A and 27B). Based on the marked induction of $CD11b^{+}Gr\text{-}1^{+}$ cells by ANE, it is speculated that ANE-induced accumulation of immature myeloid cells may contribute to the dysfunction of anti-tumor immunity in patients with areca-related oral diseases (Pak et al., 1995; Young et al., 1996).

MDSC robustly suppresses the function of nonspecific and antigen-specific T cells via multiple mechanisms involving the production of IL-10 and up-regulation of arginase-I and iNOS, the main immune-related enzymes metabolizing $_{L}$-arginine to produce urea and $_{L}$-ornithine, and NO and $_{L}$-citrulline, respectively (Bronte and Zanovello, 2005; Huang et al., 2006). $_{L}$-Arginine is an essential amino acid for T cells.

The depletion of $_{L}$-arginine by MDSC-derived arginase-I induces T-cell anergy, which is commonly found in cancer patients (Capuano et al., 2009; Ochoa et al., 2007). The depletion of $_{L}$-arginine by arginase-I may induce T cell arrest in G0-G1 phase and interfere with the expression of CD3 ζ-chain, which compromises T cell functions (Rodriguez et al., 2007; Rodriguez et al., 2004). It has been reported that iNOS expressed by MDSC is involved in the disturbance of T cell activation pathways through the reduction of tyrosine phosphorylation of Jak3 and STAT5 mediated by NO (Bingisser et al., 1998). Interestingly, the supra-physiological activity of arginase-I may increase superoxide production in MDSC (Bronte et al., 2003). The combination of superoxide and NO further forms peroxynitrites that is able to enhance T cell apoptosis and interfere with cellular signaling (Bronte et al., 2003; Ochoa et al., 2007; Szabo et al., 2007). In addition, IL-10 produced by MDSC is responsible for the generation of regulatory T cells and the suppression of the functions of DC, macrophages and T cells (Sinha et al., 2007). The production of IL-10 by MDSC was enhanced by pro-inflammatory cytokines, such as IL-1β. IL-10 is capable of skewing immunity toward a type 2 response and suppressing the production of IL-12 by macrophage (Bunt et al., 2009; Sinha et al., 2007). Collectively, these reports demonstrated the MDSC exhibited critical immunosuppressive functions by generation of immunosuppressive mediators,

including IL-10, ROS and RNS and up-regulation of arginase-I and iNOS. The present study revealed that ANE dose-dependently induced the production of IL-10, the activity of areginase-1 and the mRNA expression of iNOS and arginase-I by splenocytes and $CD11b^{+}Gr\text{-}1^{+}$ cells stimulated with LPS (Fig. 29-31). Together with the expression of CD11b and Gr-1, two commonly known markers for murine MDSC, these results suggest that ANE enhanced the development of immature myeloid cells with a functional profile of MDSC.

The induction of $CD11b^{+}Gr\text{-}1^{+}$ cells may also intensify the host immune tolerance caused by chronic inflammation or immunosuppressive mediators produced by tumor cells. Inflammation, a part of immune defense mechanism involving in the release of ROS, RNS and proinflammatory mediators, is thought to aggravate carcinogenesis by causing cell and DNA damage and creating a microenvironment to promote cell replication, angiogenesis and tissue repair (Whiteside, 2008). Pro-inflammatory cytokines, such as IL-1β, IL-6 and PGE_2 have been described to promote MDSC expansion, providing the evidence that inflammation may down-regulate immune surveillance and antitumor immunity by induction of MDSC (Bunt et al., 2007; Ostrand-Rosenberg and Sinha, 2009). In addition, IFN-γ, IL-4 and IL-13 activated several different signaling pathways in MDSC that involve STAT1, STAT6 and NF-κB (Gabrilovich and Nagaraj, 2009). It has been shown that high

levels of IFN-γ expressed by antigen-stimulated T cells can activate MDSC to generate iNOS and arginase-I and maintain a prolonged activation of MDSC, resulting in immunosuppression under certain pathological conditions (Gallina et al., 2006; Rossner et al., 2005; Serafini et al., 2004). Furthermore, the combination of two signals, LPS and IFN-γ, boosted the generation and activation of splenic MDSC and blocked the differentiation of immature myeloid precursors into DC (Greifenberg et al., 2009). Taken together, the overactive antigen-specific T cells may release an abundant amount of IFN-γ to trigger the generation and activation of MDSC. Notably, previous results from our laboratory demonstrated that ANE exacerbated inflammatory responses in delay-type hypersensitivity and robustly enhanced the expression of IFN-γ by antigen-stimulated splenocytes in ovalbumin-sensitized mice, demonstrating the proinflammatory property of ANE *in vivo* (Wang, in press). Therefore, a potential mechanism responsible for the enhanced MDSC expansion by ANE may be attributed to its proinflammatory effects.

In summary, the present studies demonstrated that directly exposure of antigen-sensitized mice to ANE and areca-derived polyphenols resulted in the induction of immature $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells capable of expressing the immunosuppressive cytokine IL-10 and the l-arginine metabolizing enzymes arginase-I and iNOS. The induction of MDSC may be a critical mechanism

contributing to the immunosuppressive effect of areca constituents and the immune deregulation previous reported in patients with areca-related oral diseases.

Chapter 6. Summary and conclusions

Alteration of cell-mediated immune responses has been shown to be closely associated with the pathophysiology of areca-related oral diseases. Although the changes of lymphocyte subsets and functional activities in patients with OSF and oral cancer have been documented, the direct influence of areca nut constituents on the reactivity of T cells remains a puzzle. The present study investigated the immunomodulatory effect of areca nut extract (ANE), polyphenols-enriched ANE (PANE) and areca-derived procyanidins on T cells *in vitro*, as well as the effect of ANE and PANE on the development of myeloid-derived suppressor cells (MDSC) *in vivo*. Firstly, ANE induced a marked cytotoxic effect, and suppressed the production of IL-2 and IFN-γ by splenocytes, which was mediated, at least in part, by the induction of oxidative stress. Secondly, ANE induced the depolarization of mitochondrial membrane potential, release of cytochrome *c*, activation of caspase-9 and enhancement of cellular ROS in association with ANE-mediated splenocyte apoptosis. Thirdly, PANE and the fractionated oligomeric procyanidins from pentamers to decamers were active in inducing splenocyte apoptosis and the diminishment of intracellular thiols, indicating that the highly oligomeric procyanidins derived from areca nut induced a chain length-dependent pro-apoptotic effect in splenocytes. Lastly, ANE and PANE administration to antigen-sensitized

mice enhanced the generation of $CD11b^{+}Gr\text{-}1^{+}$ cells that exhibited a functional profile of MDSC. Collectively, these results provide mechanistic insights to the potential mechanisms by which areca ingredients compromise cell-mediated immunity.

Chapter 7. Future perspectives

It is presently unclear what ingredients contained in ANE are responsible for the reported immunomodulatory effects on lymphocytes and neutrophils. In the results, areca alkaloids are unlikely to play a significant role, as the concentrations of arecoline and arecaidine corresponding to the effective concentrations of ANE did not affect splenocyte apoptosis and cytokine expression. Highly oligomeric procyanidins derived from areca nut were effective in inducing apoptosis and causing thiol diminishment in splenocytes, demonstrating that areca procyanidins were immunoactive. Literature reports have demonstrated the cytotoxic and pro-oxidant effects of plant-derived procyanidins in various cell culture systems. Hop-derived procyanidins induced the generation of H_2O_2 in colon cancer cells (Chung et al., 2009). High concentrations of grape seed procyanidins induced the production of nitric oxide and cell death in chick cardiomyocytes (Shao et al., 2006). The generation of radicals by auto-oxidation of areca-derived procyanidins has been reported in CHO-K1 cells (Liu et al., 1996). Furthermore, the free hydroxyl groups of flavanol-3-ol (at position 3 of the C-ring and at position 7 of the A-ring) have been suggested to be genotoxic to cells (Lozano et al., 2006), indicating the electron transfer capacity of catechin may be a factor contributing to the pro-oxidant and cytotoxic effect of procyanidins (Lizarraga et al., 2007; Lozano et al., 2006). Further

studies investigating the relationship between the chain length and auto-oxidation of areca-derived oligomeric procyanidins are required to further address this issue.

Tumor-induced MDSC have been described to mediate immune suppression in cancer patients, such as head and neck cancer and hepatocellular carcinomas (Korangy et al., 2010; Pak et al., 1995). To my knowledge, the relationship between the generation of MDSC and the pathophysiology of areca-associated oral diseases has not been reported yet. In addition to tumors, inflammation has been reported to promote the development of MDSC (Greifenberg et al., 2009; Ostrand-Rosenberg and Sinha, 2009). As areca components have been shown to enhance the production of proinflammatory cytokine by keratinocytes and PBMC (Chang et al., 2009; Jeng et al., 2003), it is speculated that ANE-mediated enhancement of MDSC development may be resulted from the proinflammatory property of ANE. To date, it is unclear whether the various inflammatory mediators induce the development of MDSC through independent or overlapping pathways. In addition, little is known regarding chemical-induced generation of MDSC. It has been reported that glucocorticoids induce a subset of monocytes that are $CD11b^{+}Gr\text{-}1^{+}$ cells, expressing IL-4Rα and IL-10, with the characteristics of MDSC. To further investigate which proinflammatory cytokines are involved in the generation of MDSC by ANE and the underlying signaling transduction pathway may provide additional evidence to

decipher how ANE regulate the development of MDSC. Although I demonstrated the enhancement of MDSC development by ANE, the role of these cells in tumor progression, especially for oral cancer, and the host immune competency has not been investigated. Further studies are required to more comprehensively address how ANE-enhanced MDSC regulate the tumor cell growth and the host immunity *in vivo*.

References

Adhvaryu, S.G., Dave, B.J., Trivedi, A.H., 1991. Cytogenetic surveillance of tobacco-areca nut (mava) chewers, including patients with oral cancers and premalignant conditions. Mutat Res 261, 41-49.

Agarwal, A., Rani, M., Saha, G.K., Valarmathi, T.M., Bahadur, S., Mohanti, B.K., Das, S.N., 2003. Disregulated expression of the Th2 cytokine gene in patients with intraoral squamous cell carcinoma. Immunol Invest 32, 17-30.

Ahmed, S., Rahman, A., Hull, S., 1997. Use of betel quid and cigarettes among Bangladeshi patients in an inner-city practice: prevalence and knowledge of health effects. Br J Gen Pract 47, 431-434.

Akiyama, H., Sato, Y., Watanabe, T., Nagaoka, M.H., Yoshioka, Y., Shoji, T., Kanda, T., Yamada, K., Totsuka, M., Teshima, R., Sawada, J., Goda, Y., Maitani, T., 2005. Dietary unripe apple polyphenol inhibits the development of food allergies in murine models. FEBS Lett 579, 4485-4491.

Bagchi, M., Balmoori, J., Bagchi, D., Stohs, S.J., Chakrabarti, J., Das, D.K., 2002. Role of reactive oxygen species in the development of cytotoxicity with various forms of chewing tobacco and pan masala. Toxicology 179, 247-255.

Balaram, P., Pillai, M.R., Abraham, T., 1987. Immunology of premalignant and malignant conditions of the oral cavity. II. Circulating immune complexes. J Oral Pathol 16, 389-391.

Balaram, P., Pillai, M.R., Abraham, T., Nalinakumari, K.R., Meenattoor, G., Kannan, S., Hareendran, N.K., Nair, M.K., 1992. Interferon and interleukin-2 induced spontaneous cell-mediated cytotoxicity: a preliminary evaluation. J Cancer Res Clin Oncol 118, 408-410.

Baniyash, M., 2004. TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response. Nat Rev Immunol 4, 675-687.

Barker, J.N., Mitra, R.S., Griffiths, C.E., Dixit, V.M., Nickoloff, B.J., 1991. Keratinocytes as initiators of inflammation. Lancet 337, 211-214.

Billiau, A., Matthys, P., 2009. Interferon-gamma: a historical perspective. Cytokine Growth Factor Rev 20, 97-113.

Bingisser, R.M., Tilbrook, P.A., Holt, P.G., Kees, U.R., 1998. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. J Immunol 160, 5729-5734.

Boehm, U., Klamp, T., Groot, M., Howard, J.C., 1997. Cellular responses to interferon-gamma. Annu Rev Immunol 15, 749-795.

Boise, L.H., Minn, A.J., Thompson, C.B., 1995. Receptors that regulate T-cell susceptibility to apoptotic cell death. Ann N Y Acad Sci 766, 70-80.

Bromley, S.K., Burack, W.R., Johnson, K.G., Somersalo, K., Sims, T.N., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., Dustin, M.L., 2001. The immunological synapse. Annu Rev Immunol 19, 375-396.

Bronte, V., Serafini, P., De Santo, C., Marigo, I., Tosello, V., Mazzoni, A., Segal, D.M., Staib, C., Lowel, M., Sutter, G., Colombo, M.P., Zanovello, P., 2003. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. J Immunol 170, 270-278.

Bronte, V., Zanovello, P., 2005. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 5, 641-654.

Bunt, S.K., Clements, V.K., Hanson, E.M., Sinha, P., Ostrand-Rosenberg, S., 2009. Inflammation enhances myeloid-derived suppressor cell cross-talk by signaling through Toll-like receptor 4. J Leukoc Biol 85, 996-1004.

Bunt, S.K., Yang, L., Sinha, P., Clements, V.K., Leips, J., Ostrand-Rosenberg, S., 2007. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res 67, 10019-10026.

Caddeo, C., Teskac, K., Sinico, C., Kristl, J., 2008. Effect of resveratrol incorporated in liposomes on proliferation and UV-B protection of cells. Int J Pharm 363, 183-191.

Canniff, J.P., Harvey, W., 1981. The aetiology of oral submucous fibrosis: the stimulation of collagen synthesis by extracts of areca nut. Int J Oral Surg 10, 163-167.

Canniff, J.P., Harvey, W., Harris, M., 1986. Oral submucous fibrosis: its pathogenesis and management. Br Dent J 160, 429-434.

Capuano, G., Rigamonti, N., Grioni, M., Freschi, M., Bellone, M., 2009. Modulators of arginine metabolism support cancer immunosurveillance. BMC Immunol 10, 1.

Chang, L.Y., Wan, H.C., Lai, Y.L., Kuo, Y.F., Liu, T.Y., Chen, Y.T., Hung, S.L., 2009. Areca nut extracts increased expression of inflammatory cytokines, tumor necrosis factor-alpha, interleukin-1beta, interleukin-6 and interleukin-8, in peripheral blood mononuclear cells. J Periodontal Res 44, 175-183.

Chang, L.Y., Wan, H.C., Lai, Y.L., Liu, T.Y., Hung, S.L., 2006. Enhancing effects of areca nut extracts on the production of interleukin-6 and interleukin-8 by peripheral blood mononuclear cells. J Periodontol 77, 1969-1977.

Chang, M.C., Chiang, C.P., Lin, C.L., Lee, J.J., Hahn, L.J., Jeng, J.H., 2005. Cell-mediated immunity and head and neck cancer: with special emphasis

on betel quid chewing habit. Oral Oncol 41, 757-775.

Chang, M.C., Ho, Y.S., Lee, J.J., Kok, S.H., Hahn, L.J., Jeng, J.H., 2002. Prevention of the areca nut extract-induced unscheduled DNA synthesis of gingival keratinocytes by vitamin C and thiol compounds. Oral Oncol 38, 258-265.

Chang, M.C., Ho, Y.S., Lee, P.H., Chan, C.P., Lee, J.J., Hahn, L.J., Wang, Y.J., Jeng, J.H., 2001a. Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. Carcinogenesis 22, 1527-1535.

Chang, M.C., Wu, H.L., Lee, J.J., Lee, P.H., Chang, H.H., Hahn, L.J., Lin, B.R., Chen, Y.J., Jeng, J.H., 2004. The induction of prostaglandin E2 production, interleukin-6 production, cell cycle arrest, and cytotoxicity in primary oral keratinocytes and KB cancer cells by areca nut ingredients is differentially regulated by MEK/ERK activation. J Biol Chem, 279, 50676-50683.

Chang, Y.C., Hu, C.C., Lii, C.K., Tai, K.W., Yang, S.H., Chou, M.Y., 2001b. Cytotoxicity and arecoline mechanisms in human gingival fibroblasts in vitro. Clin Oral Investig 5, 51-56.

Chen, C.C., Huang, J.F., Tsai, C.C., 1995. In vitro production of interleukin-6 by human gingival, normal buccal mucosa, and oral submucous fibrosis fibroblasts treated with betel-nut alkaloids. Gaoxiong Yi Xue Ke Xue Za Zhi 11, 604-614.

Chen, C.L., Chi, C.W., Liu, T.Y., 2002. Hydroxyl radical formation and oxidative DNA damage induced by areca quid in vivo. J Toxicol Environ Health A 65, 327-336.

Cheynier, V., 2005. Polyphenols in foods are more complex than often thought. Am J Clin Nutr 81, 223S-229S.

Chiang, C.P., Chang, M.C., Lee, J.J., Chang, J.Y., Lee, P.H., Hahn, L.J., Jeng, J.H., 2004. Hamsters chewing betel quid or areca nut directly show a decrease in body weight and survival rates with concomitant epithelial hyperplasia of cheek pouch. Oral Oncol 40, 720-727.

Chiang, C.P., Hsieh, R.P., Chen, T.H., Chang, Y.F., Liu, B.Y., Wang, J.T., Sun, A., Kuo, M.Y., 2002a. High incidence of autoantibodies in Taiwanese patients with oral submucous fibrosis. J Oral Pathol Med 31, 402-409.

Chiang, C.P., Wu, H.Y., Liu, B.Y., Wang, J.T., Kuo, M.Y., 2002b. Quantitative analysis of immunocompetent cells in oral submucous fibrosis in Taiwan. Oral Oncol 38, 56-63.

Chou, C.Y., Cheng, S.Y., Liu, J.H., Cheng, W.C., Kang, I.M., Tseng, Y.H., Shih, C.M., Chen, W., 2009. Association between betel-nut chewing and chronic

kidney disease in men. Public Health Nutr 12, 723-727.

Chung, W.G., Miranda, C.L., Stevens, J.F., Maier, C.S., 2009. Hop proanthocyanidins induce apoptosis, protein carbonylation, and cytoskeleton disorganization in human colorectal adenocarcinoma cells via reactive oxygen species. Food Chem Toxicol 47, 827-836.

Cohen, J.J., Duke, R.C., Fadok, V.A., Sellins, K.S., 1992. Apoptosis and programmed cell death in immunity. Annu Rev Immunol 10, 267-293.

Corraliza, I.M., Campo, M.L., Soler, G., Modolell, M., 1994. Determination of arginase activity in macrophages: a micromethod. J Immunol Methods 174, 231-235.

Cos, P., Hermans, N., Calomme, M., Maes, L., De Bruyne, T., Pieters, L., Vlietinck, A.J., Vanden Berghe, D., 2003. Comparative study of eight well-known polyphenolic antioxidants. J Pharm Pharmacol 55, 1291-1297.

Coughlin, C.M., Salhany, K.E., Gee, M.S., LaTemple, D.C., Kotenko, S., Ma, X., Gri, G., Wysocka, M., Kim, J.E., Liu, L., Liao, F., Farber, J.M., Pestka, S., Trinchieri, G., Lee, W.M., 1998. Tumor cell responses to IFNgamma affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. Immunity 9, 25-34.

D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., Masella, R., 2007. Polyphenols, dietary sources and bioavailability. Ann Ist Super Sanita 43, 348-361.

Dammer, R., Wurm, E.M., Niederdellmann, H., Fleischmann, H., Knuchel, R., 1997. [Immunocytochemical venous blood studies in patients with manifest oral cavity carcinomas, oral precancerous conditions, benign tumors and in chronic alcoholic patients]. Mund Kiefer Gesichtschir 1, 95-103.

Daniele, R.P., Holian, S.K., 1976. A potassium ionophore (valinomycin) inhibits lymphocyte proliferation by its effects on the cell membrane. Proc Natl Acad Sci U S A 73, 3599-3602.

Dasgupta, R., Saha, I., Pal, S., Bhattacharyya, A., Sa, G., Nag, T.C., Das, T., Maiti, B.R., 2006. Immunosuppression, hepatotoxicity and depression of antioxidant status by arecoline in albino mice. Toxicology 227, 94-104.

Diaz-Montero, C.M., Salem, M.L., Nishimura, M.I., Garrett-Mayer, E., Cole, D.J., Montero, A.J., 2009. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 58, 49-59.

Dighe, A.S., Richards, E., Old, L.J., Schreiber, R.D., 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative

IFN gamma receptors. Immunity 1, 447-456.

Doherty, P.C., 1993. Cell-mediated cytotoxicity. Cell 75, 607-612.

Dolcetti, L., Marigo, I., Mantelli, B., Peranzoni, E., Zanovello, P., Bronte, V., 2008. Myeloid-derived suppressor cell role in tumor-related inflammation. Cancer Lett 267, 216-225.

Dong, C., Flavell, R.A., 2001. Th1 and Th2 cells. Curr Opin Hematol 8, 47-51.

Erlejman, A.G., Jaggers, G., Fraga, C.G., Oteiza, P.I., 2008. TNF-alpha-induced NF-kappaB activation and cell oxidant production are modulated by hexameric procyanidins in Caco-2 cells. Arch Biochem Biophys 476, 186-195.

Fesus, L., 1992. Apoptosis. Immunol Today 13, A16-17.

Gabrilovich, D.I., Nagaraj, S., 2009. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 9, 162-174.

Gallina, G., Dolcetti, L., Serafini, P., De Santo, C., Marigo, I., Colombo, M.P., Basso, G., Brombacher, F., Borrello, I., Zanovello, P., Bicciato, S., Bronte, V., 2006. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. J Clin Invest 116, 2777-2790.

Gastman, B.R., Atarshi, Y., Reichert, T.E., Saito, T., Balkir, L., Rabinowich, H., Whiteside, T.L., 1999. Fas ligand is expressed on human squamous cell carcinomas of the head and neck, and it promotes apoptosis of T lymphocytes. Cancer Res 59, 5356-5364.

Greifenberg, V., Ribechini, E., Rossner, S., Lutz, M.B., 2009. Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treatment impairs DC development. Eur J Immunol 39, 2865-2876.

Gu, L., House, S.E., Wu, X., Ou, B., Prior, R.L., 2006. Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. J Agric Food Chem 54, 4057-4061.

Gupta, P.C., Warnakulasuriya, S., 2002. Global epidemiology of areca nut usage. Addict Biol 7, 77-83.

Hadi, S.M., Bhat, S.H., Azmi, A.S., Hanif, S., Shamim, U., Ullah, M.F., 2007. Oxidative breakage of cellular DNA by plant polyphenols: a putative mechanism for anticancer properties. Semin Cancer Biol 17, 370-376.

Haque, M.F., Harris, M., Meghji, S., Barrett, A.W., 1998. Immunolocalization of cytokines and growth factors in oral submucous fibrosis. Cytokine 10, 713-719.

Haque, M.F., Harris, M., Meghji, S., Speight, P.M., 1997. An immunohistochemical study of oral submucous fibrosis. J Oral Pathol Med 26, 75-82.

Haque, M.F., Meghji, S., Khitab, U., Harris, M., 2000. Oral submucous fibrosis patients have altered levels of cytokine production. J Oral Pathol Med 29, 123-128.

Haque, M.F., Meghji, S., Nazir, R., Harris, M., 2001. Interferon gamma (IFN-gamma) may reverse oral submucous fibrosis. J Oral Pathol Med 30, 12-21.

Heath, W.R., Carbone, F.R., 2001. Cross-presentation, dendritic cells, tolerance and immunity. Annu Rev Immunol 19, 47-64.

Heimdal, J.H., Aarstad, H.J., Klementsen, B., Olofsson, J., 1998. Disease stage related in vitro responsiveness of peripheral blood T-lymphocytes in patients with head and neck carcinoma. Acta Otolaryngol 118, 887-891.

Hildeman, D.A., Mitchell, T., Kappler, J., Marrack, P., 2003. T cell apoptosis and reactive oxygen species. J Clin Invest 111, 575-581.

Ho, W.H., Lee, Y.Y., Chang, L.Y., Chen, Y.T., Liu, T.Y., Hung, S.L., 2010. Effects of areca nut extract on the apoptosis pathways in human neutrophils. J Periodontal Res 45,412-420.

Holtzman, M.J., Green, J.M., Jayaraman, S., Arch, R.H., 2000. Regulation of T cell apoptosis. Apoptosis 5, 459-471.

Hsu, H.J., Chang, K.L., Yang, Y.H., Shieh, T.Y., 2001. The effects of arecoline on the release of cytokines using cultured peripheral blood mononuclear cells from patients with oral mucous diseases. Kaohsiung J Med Sci 17, 175-182.

Huang, B., Pan, P.Y., Li, Q., Sato, A.I., Levy, D.E., Bromberg, J., Divino, C.M., Chen, S.H., 2006. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res 66, 1123-1131.

Huang, P.L., Chi, C.W., Liu, T.Y., 2010. Effects of Areca catechu L. containing procyanidins on cyclooxygenase-2 expression in vitro and in vivo. Food Chem Toxicol 48, 306-313.

Huang, P.L., Chi, C.W., Liu, T.Y., 2010. Effects of Areca catechu L. containing procyanidins on cyclooxygenase-2 expression in vitro and in vivo. Food Chem Toxicol 48, 306-313.

Hung, S.L., Cheng, Y.Y., Peng, J.L., Chang, L.Y., Liu, T.Y., Chen, Y.T., 2005. Inhibitory effects of areca nut extracts on phagocytosis of Actinobacillus actinomycetemcomitans ATCC 33384 by neutrophils. J Periodontol 76, 373-379.

Hung, S.L., Lee, Y.Y., Liu, T.Y., Peng, J.L., Cheng, Y.Y., Chen, Y.T., 2006. Modulation of phagocytosis, chemotaxis, and adhesion of neutrophils by areca nut extracts. J Periodontol 77, 579-585.

IARC, 2004. Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. IARC Monogr Eval Carcinog Risks Hum 85, 1-334.

Ikeda, H., Old, L.J., Schreiber, R.D., 2002. The roles of IFN gamma in protection against tumor development and cancer immunoediting. Cytokine Growth Factor Rev 13, 95-109.

Jeng, J.H., Chang, M.C., Hahn, L.J., 2001. Role of areca nut in betel quid-associated chemical carcinogenesis: current awareness and future perspectives. Oral Oncol 37, 477-492.

Jeng, J.H., Hahn, L.J., Lin, B.R., Hsieh, C.C., Chan, C.P., Chang, M.C., 1999. Effects of areca nut, inflorescence piper betle extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. J Oral Pathol Med 28, 64-71.

Jeng, J.H., Ho, Y.S., Chan, C.P., Wang, Y.J., Hahn, L.J., Lei, D., Hsu, C.C., Chang, M.C., 2000. Areca nut extract up-regulates prostaglandin production, cyclooxygenase-2 mRNA and protein expression of human oral keratinocytes. Carcinogenesis 21, 1365-1370.

Jeng, J.H., Kuo, M.L., Hahn, L.J., Kuo, M.Y., 1994. Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. J Dent Res 73, 1043-1049.

Jeng, J.H., Lan, W.H., Hahn, L.J., Hsieh, C.C., Kuo, M.Y., 1996. Inhibition of the migration, attachment, spreading, growth and collagen synthesis of human gingival fibroblasts by arecoline, a major areca alkaloid, in vitro. J Oral Pathol Med 25, 371-375.

Jeng, J.H., Wang, Y.J., Chiang, B.L., Lee, P.H., Chan, C.P., Ho, Y.S., Wang, T.M., Lee, J.J., Hahn, L.J., Chang, M.C., 2003. Roles of keratinocyte inflammation in oral cancer: regulating the prostaglandin E2, interleukin-6 and TNF-alpha production of oral epithelial cells by areca nut extract and arecoline. Carcinogenesis 24, 1301-1315.

Kaminski, N.E., Koh, W.S., Yang, K.H., Lee, M., Kessler, F.K., 1994. Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylate cyclase by a pertussis toxin-sensitive G-protein coupled mechanism. Biochem Pharmacol 48, 1899-1908.

Kanda, T., Akiyama, H., Yanagida, A., Tanabe, M., Goda, Y., Toyoda, M., Teshima, R., Saito, Y., 1998. Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. Biosci Biotechnol Biochem 62, 1284-1289.

Kenny, T.P., Keen, C.L., Schmitz, H.H., Gershwin, M.E., 2007. Immune effects of cocoa procyanidin oligomers on peripheral blood mononuclear cells. Exp

Biol Med (Maywood) 232, 293-300.

Kikuchi, K., Kusama, K., Sano, M., Nakanishi, Y., Ishige, T., Ohni, S., Oinuma, T., Nemoto, N., 2006. Vascular endothelial growth factor and dendritic cells in human squamous cell carcinoma of the oral cavity. Anticancer Res 26, 1833-1848.

Kim, H.M., Oh, G.T., Hong, D.H., Hyun, B.H., Cha, Y.N., Yoo, B.S., Han, S.B., 1996. Facilitation of apoptosis by autologous serum and related immunosuppression in the splenocyte culture. Immunopharmacology 34, 39-50.

Knudson, C.M., Brown, N.M., 2008. Mitochondria potential, bax "activation," and programmed cell death. Methods Mol Biol 414, 95-108.

Ko, Y.C., Chiang, T.A., Chang, S.J., Hsieh, S.F., 1992. Prevalence of betel quid chewing habit in Taiwan and related sociodemographic factors. J Oral Pathol Med 21, 261-264.

Ko, Y.C., Huang, Y.L., Lee, C.H., Chen, M.J., Lin, L.M., Tsai, C.C., 1995. Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. J Oral Pathol Med 24, 450-453.

Korangy, F., Hochst, B., Manns, M.P., Greten, T.F., 2010. Immune responses in hepatocellular carcinoma. Dig Dis 28, 150-154.

Kumpawat, K., Deb, S., Ray, S., Chatterjee, A., 2003. Genotoxic effect of raw betel-nut extract in relation to endogenous glutathione levels and its mechanism of action in mammalian cells. Mutat Res 538, 1-12.

Laad, A., Kode, J., Chavan, S., Rao, R., Fakih, A.R., Chiplunkar, S., 1996. Limiting dilution analysis of proliferating and cytotoxic lymphocytes in the peripheral blood and tumours of oral cancer patients. Eur J Cancer B Oral Oncol 32B, 337-342.

Lai, K.C., Lee, T.C., 2006. Genetic damage in cultured human keratinocytes stressed by long-term exposure to areca nut extracts. Mutat Res 599, 66-75.

Lambert, J.D., Hong, J., Yang, G.Y., Liao, J., Yang, C.S., 2005. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am J Clin Nutr 81, 284S-291S.

Lee, J.H., Chang, S.H., Park, Y.S., Her, E., Lee, H.Y., Park, J.W., Han, J.W., Kim, Y.M., Choi, W.S., 2004. In-vitro and in-vivo anti-allergic actions of Arecae semen. J Pharm Pharmacol 56, 927-933.

Lee, P.H., Chang, M.C., Chang, W.H., Wang, T.M., Wang, Y.J., Hahn, L.J., Ho, Y.S., Lin, C.Y., Jeng, J.H., 2006. Prolonged exposure to arecoline arrested human KB epithelial cell growth: regulatory mechanisms of cell cycle and apoptosis. Toxicology 220, 81-89.

Lee, S.S., Yang, S.F., Tsai, C.H., Chou, M.C., Chou, M.Y., Chang, Y.C., 2008. Upregulation of heme oxygenase-1 expression in areca-quid-chewing-associated oral squamous cell carcinoma. J Formos Med Assoc 107, 355-363.

Lee, Y.C., Park, M.H., Baek, N.H., 1991. Effect of gamma-interferon on fibroblast proliferation and collagen synthesis after glaucoma filtering surgery in white rabbits. Korean J Ophthalmol 5, 59-67.

Lin, M.H., Chou, F.P., Huang, H.P., Hsu, J.D., Chou, M.Y., Wang, C.J., 2003. The tumor promoting effect of lime-piper betel quid in JB6 cells. Food Chem Toxicol 41, 1463-1471.

Lin, S.C., Lu, S.Y., Lee, S.Y., Lin, C.Y., Chen, C.H., Chang, K.W., 2005. Areca (betel) nut extract activates mitogen-activated protein kinases and NF-kappaB in oral keratinocytes. Int J Cancer 116, 526-535.

Lin, W.Y., Chiu, T.Y., Lee, L.T., Lin, C.C., Huang, C.Y., Huang, K.C., 2008. Betel nut chewing is associated with increased risk of cardiovascular disease and all-cause mortality in Taiwanese men. Am J Clin Nutr 87, 1204-1211.

Lin, W.Y., Pi-Sunyer, F.X., Liu, C.S., Li, T.C., Li, C.I., Huang, C.Y., Lin, C.C., 2009. Betel nut chewing is strongly associated with general and central obesity in Chinese male middle-aged adults. Obesity (Silver Spring) 17, 1247-1254.

Liu, T.Y., Chen, C.L., Chi, C.W., 1996. Oxidative damage to DNA induced by areca nut extract. Mutat Res 367, 25-31.

Lizarraga, D., Lozano, C., Briede, J.J., van Delft, J.H., Tourino, S., Centelles, J.J., Torres, J.L., Cascante, M., 2007. The importance of polymerization and galloylation for the antiproliferative properties of procyanidin-rich natural extracts. FEBS J 274, 4802-4811.

Lotito, S.B., Actis-Goretta, L., Renart, M.L., Caligiuri, M., Rein, D., Schmitz, H.H., Steinberg, F.M., Keen, C.L., Fraga, C.G., 2000. Influence of oligomer chain length on the antioxidant activity of procyanidins. Biochem Biophys Res Commun 276, 945-951.

Lozano, C., Julia, L., Jimenez, A., Tourino, S., Centelles, J.J., Cascante, M., Torres, J.L., 2006. Electron-transfer capacity of catechin derivatives and influence on the cell cycle and apoptosis in HT29 cells. FEBS J 273, 2475-2486.

Mach, B., Steimle, V., Martinez-Soria, E., Reith, W., 1996. Regulation of MHC class II genes: lessons from a disease. Annu Rev Immunol 14, 301-331.

Malmberg, K.J., Arulampalam, V., Ichihara, F., Petersson, M., Seki, K., Andersson, T., Lenkei, R., Masucci, G., Pettersson, S., Kiessling, R., 2001. Inhibition of activated/memory (CD45RO(+)) T cells by oxidative stress associated

with block of NF-kappaB activation. J Immunol 167, 2595-2601.

Mao, T.K., Van de Water, J., Keen, C.L., Schmitz, H.H., Gershwin, M.E., 2002. Effect of cocoa flavanols and their related oligomers on the secretion of interleukin-5 in peripheral blood mononuclear cells. J Med Food 5, 17-22.

Marigo, I., Dolcetti, L., Serafini, P., Zanovello, P., Bronte, V., 2008. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunol Rev 222, 162-179.

Marnett, L.J., 2000. Oxyradicals and DNA damage. Carcinogenesis 21, 361-370.

Mathew, A.G., Govindarajan, V.S., 1964. Polyphenolic substances of arecanut. II. Changes during maturation and ripening. Phytochemistry 3, 657-665.

Miura, T., Chiba, M., Kasai, K., Nozaka, H., Nakamura, T., Shoji, T., Kanda, T., Ohtake, Y., Sato, T., 2008. Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3. Carcinogenesis 29, 585-593.

Miyake, M., Ide, K., Sasaki, K., Matsukura, Y., Shijima, K., Fujiwara, D., 2008. Oral administration of highly oligomeric procyanidins of Jatoba reduces the severity of collagen-induced arthritis. Biosci Biotechnol Biochem 72, 1781-1788.

Miyake, M., Sasaki, K., Ide, K., Matsukura, Y., Shijima, K., Fujiwara, D., 2006. Highly oligomeric procyanidins ameliorate experimental autoimmune encephalomyelitis via suppression of Th1 immunity. J Immunol 176, 5797-5804.

Mohamad, N., Gutierrez, A., Nunez, M., Cocca, C., Martin, G., Cricco, G., Medina, V., Rivera, E., Bergoc, R., 2005. Mitochondrial apoptotic pathways. Biocell 29, 149-161.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65, 55-63.

Mougiakakos, D., Choudhury, A., Lladser, A., Kiessling, R., Johansson, C.C., in 2010 Regulatory T cells in cancer. Adv Cancer Res 107, 57-117.

Murali, P.S., Somasundaram, R., Rao, R.S., Fakih, A.R., Gangal, S.G., 1989. Interleukin-2 mediated regulation of mitogen-activated T cell reactivity from different lymphoid sources in patients with squamous cell carcinoma of the oral cavity. J Oral Pathol Med 18, 327-332.

Nagaraj, S., Gupta, K., Pisarev, V., Kinarsky, L., Sherman, S., Kang, L., Herber, D.L., Schneck, J., Gabrilovich, D.I., 2007. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat Med 13, 828-835.

Nair, U., Bartsch, H., Nair, J., 2004. Alert for an epidemic of oral cancer due to use of

the betel quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. Mutagenesis 19, 251-262.

Nair, U.J., Floyd, R.A., Nair, J., Bussachini, V., Friesen, M., Bartsch, H., 1987. Formation of reactive oxygen species and of 8-hydroxydeoxyguanosine in DNA in vitro with betel quid ingredients. Chem Biol Interact 63, 157-169.

Nair, U.J., Friesen, M., Richard, I., MacLennan, R., Thomas, S., Bartsch, H., 1990. Effect of lime composition on the formation of reactive oxygen species from areca nut extract in vitro. Carcinogenesis 11, 2145-2148.

Nair, U.J., Nair, J., Friesen, M.D., Bartsch, H., Ohshima, H., 1995. Ortho- and meta-tyrosine formation from phenylalanine in human saliva as a marker of hydroxyl radical generation during betel quid chewing. Carcinogenesis 16, 1195-1198.

Neuwirt, H., Arias, M.C., Puhr, M., Hobisch, A., Culig, Z., 2008. Oligomeric proanthocyanidin complexes (OPC) exert anti-proliferative and pro-apoptotic effects on prostate cancer cells. Prostate 68, 1647-1654.

Nguyen, K.D., Hoang, A.T., Lee, D.A., 1994. Transcriptional control of human Tenon's capsule fibroblast collagen synthesis in vitro by gamma-interferon. Invest Ophthalmol Vis Sci 35, 3064-3070.

Nonaka, G., Hsu, F.L., Nishioka, I., 1981. Structures of dimeric, trimeric, and tetrameric procyanidins from Areca catechu L. J Chem Soc Chem Commun, 781-783.

O'Connor, B.P., Gleeson, M.W., Noelle, R.J., Erickson, L.D., 2003. The rise and fall of long-lived humoral immunity: terminal differentiation of plasma cells in health and disease. Immunol Rev 194, 61-76.

O'Donnell, R.K., Mick, R., Feldman, M., Hino, S., Wang, Y., Brose, M.S., Muschel, R.J., 2007. Distribution of dendritic cell subtypes in primary oral squamous cell carcinoma is inconsistent with a functional response. Cancer Lett 255, 145-152.

Ochoa, A.C., Zea, A.H., Hernandez, C., Rodriguez, P.C., 2007. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. Clin Cancer Res 13, 721s-726s.

Opferman, J.T., Korsmeyer, S.J., 2003. Apoptosis in the development and maintenance of the immune system. Nat Immunol 4, 410-415.

Ostrand-Rosenberg, S., Sinha, P., 2009. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 182, 4499-4506.

Pak, A.S., Wright, M.A., Matthews, J.P., Collins, S.L., Petruzzelli, G.J., Young, M.R., 1995. Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions

within cancers that secrete granulocyte-macrophage colony-stimulating factor. Clin Cancer Res 1, 95-103.

Panigrahi, G.B., Rao, A.R., 1982. Chromosome-breaking ability of arecoline, a major betel-nut alkaloid, in mouse bone-marrow cells in vivo. Mutat Res 103, 197-204.

Panigrahi, G.B., Rao, A.R., 1984. Induction of in vivo sister chromatid exchanges by arecaidine, a betel nut alkaloid, in mouse bone-marrow cells. Cancer Lett 23, 189-192.

Panigrahi, G.B., Rao, A.R., 1986. Study of the genotoxicity of the total aqueous extract of betel nut and its tannin. Carcinogenesis 7, 37-39.

Park, Y.C., Rimbach, G., Saliou, C., Valacchi, G., Packer, L., 2000. Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF-alpha secretion, and NF-kappaB-dependent gene expression in RAW 264.7 macrophages. FEBS Lett 465, 93-97.

Pierini, R., Kroon, P.A., Guyot, S., Ivory, K., Johnson, I.T., Belshaw, N.J., 2008. Procyanidin effects on oesophageal adenocarcinoma cells strongly depend on flavan-3-ol degree of polymerization. Mol Nutr Food Res 52, 1399-1407.

Pillai, M.R., Balaram, P., Abraham, T., Nair, M.K., 1987. Lymphocyte populations in premalignant lesions and cancer of the oral cavity. Neoplasma 34, 469-479.

Pillai, M.R., Balaram, P., Kannan, S., Sudha, L., Nalinakumari, K.R., Hareendran, N.K., Nair, M.K., 1990. Interferon activation of latent natural killer cells and alteration in kinetics of target cell lysis: clinical implications for oral precancerous lesions. Oral Surg Oral Med Oral Pathol 70, 458-461.

Prior, R.L., Gu, L., 2005. Occurrence and biological significance of proanthocyanidins in the American diet. Phytochemistry 66, 2264-2280.

Rahman, I., Biswas, S.K., Kirkham, P.A., 2006. Regulation of inflammation and redox signaling by dietary polyphenols. Biochem Pharmacol 72, 1439-1452.

Rajendran, R., Sugathan, C.K., Remani, P., Ankathil, R., Vijayakumar, T., 1986. Cell mediated and humoral immune responses in oral submucous fibrosis. Cancer 58, 2628-2631.

Ramiro-Puig, E., Perez-Cano, F.J., Ramirez-Santana, C., Castellote, C., Izquierdo-Pulido, M., Permanyer, J., Franch, A., Castell, M., 2007. Spleen lymphocyte function modulated by a cocoa-enriched diet. Clin Exp Immunol 149, 535-542.

Ranadive, K.J., Gothoskar, S.V., Rao, A.R., Tezabwalla, B.U., Ambaye, R.Y., 1976.

Experimental studies on betel nut and tobacco carcinogenicity. Int J Cancer 17, 469-476.

Ranadive, K.J., Ranadive, S.N., Shivapurkar, N.M., Gothoskar, S.V., 1979. Betel quid chewing and oral cancer: experimental studies on hamsters. Int J Cancer 24, 835-843.

Reichert, T.E., Strauss, L., Wagner, E.M., Gooding, W., Whiteside, T.L., 2002. Signaling abnormalities, apoptosis, and reduced proliferation of circulating and tumor-infiltrating lymphocytes in patients with oral carcinoma. Clin Cancer Res 8, 3137-3145.

Rice, S.Q., Crane, I.J., Scully, C., Prime, S.S., 1992. Production of a suppressor of lymphocyte proliferation by two human oral carcinoma cell lines. Scand J Immunol 36, 443-452.

Rodriguez, P.C., Quiceno, D.G., Ochoa, A.C., 2007. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood 109, 1568-1573.

Rodriguez, P.C., Quiceno, D.G., Zabaleta, J., Ortiz, B., Zea, A.H., Piazuelo, M.B., Delgado, A., Correa, P., Brayer, J., Sotomayor, E.M., Antonia, S., Ochoa, J.B., Ochoa, A.C., 2004. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 64, 5839-5849.

Rossner, S., Voigtlander, C., Wiethe, C., Hanig, J., Seifarth, C., Lutz, M.B., 2005. Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide production in vitro. Eur J Immunol 35, 3533-3544.

Schroder, K., Hertzog, P.J., Ravasi, T., Hume, D.A., 2004. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75, 163-189.

Selvan, R.S., Selvakumaran, M., Rao, A.R., 1991. Influence of arecoline on immune system: II. Suppression of thymus-dependent immune responses and parameter of non-specific resistance after short-term exposure. Immunopharmacol Immunotoxicol 13, 281-309.

Serafini, P., Carbley, R., Noonan, K.A., Tan, G., Bronte, V., Borrello, I., 2004. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. Cancer Res 64, 6337-6343.

Shankaran, V., Ikeda, H., Bruce, A.T., White, J.M., Swanson, P.E., Old, L.J., Schreiber, R.D., 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410, 1107-1111.

Shao, Z.H., Becker, L.B., Vanden Hoek, T.L., Schumacker, P.T., Li, C.Q., Zhao, D., Wojcik, K., Anderson, T., Qin, Y., Dey, L., Yuan, C.S., 2003. Grape seed proanthocyanidin extract attenuates oxidant injury in cardiomyocytes. Pharmacol Res 47, 463-469.

Shao, Z.H., Hsu, C.W., Chang, W.T., Waypa, G.B., Li, J., Li, D., Li, C.Q., Anderson, T., Qin, Y., Schumacker, P.T., Becker, L.B., Hoek, T.L., 2006. Cytotoxicity induced by grape seed proanthocyanidins: role of nitric oxide. Cell Biol Toxicol 22, 149-158.

Sinha, P., Clements, V.K., Bunt, S.K., Albelda, S.M., Ostrand-Rosenberg, S., 2007. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. J Immunol 179, 977-983.

Snyderman, C.H., Heo, D.S., Johnson, J.T., D'Amico, F., Barnes, L., Whiteside, T.L., 1991. Functional and phenotypic analysis of lymphocytes in head and neck cancer. Arch Otolaryngol Head Neck Surg 117, 899-905.

Street, S.E., Cretney, E., Smyth, M.J., 2001. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. Blood 97, 192-197.

Szabo, C., Ischiropoulos, H., Radi, R., 2007. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat Rev Drug Discov 6, 662-680.

Tanaka, K., Jinhua, P., Omura, K., Azuma, M., 2007. Multipotency of CD11bhighGr-1+ immature myeloid cells accumulating in oral squamous cell carcinoma-bearing mice. Oral Oncol 43, 586-592.

Tatake, R.J., Krishnan, N., Rao, R.S., Fakih, A.R., Gangal, S.G., 1989. Lymphokine-activated killer-cell function of lymphocytes from peripheral blood, regional lymph nodes and tumor tissues of patients with oral cancer. Int J Cancer 43, 560-566.

Tilakaratne, W.M., Klinikowski, M.F., Saku, T., Peters, T.J., Warnakulasuriya, S., 2006. Oral submucous fibrosis: review on aetiology and pathogenesis. Oral Oncol 42, 561-568.

Tripathi, P., Hildeman, D., 2004. Sensitization of T cells to apoptosis--a role for ROS? Apoptosis 9, 515-523.

Tsai, J.F., Chuang, L.Y., Jeng, J.E., Ho, M.S., Hsieh, M.Y., Lin, Z.Y., Wang, L.Y., 2001. Betel quid chewing as a risk factor for hepatocellular carcinoma: a case-control study. Br J Cancer 84, 709-713.

Verastegui, E., Morales, R., Barrera, J.L., Mueller, A., Guzman, B., Meneses, A., Alfaro, G., 2002. Immunological approach in the evaluation of regional

lymph nodes of patients with squamous cell carcinoma of the head and neck. Clin Immunol 102, 37-47.

Verstraeten, S.V., Keen, C.L., Schmitz, H.H., Fraga, C.G., Oteiza, P.I., 2003. Flavan-3-ols and procyanidins protect liposomes against lipid oxidation and disruption of the bilayer structure. Free Radic Biol Med 34, 84-92.

Wallace, K.B., Eells, J.T., Madeira, V.M., Cortopassi, G., Jones, D.P., 1997. Mitochondria-mediated cell injury. Symposium overview. Fundam Appl Toxicol 38, 23-37.

Wang CC, Lin HL, Wey SP, Jan TR. Areca nut extract modulates antigen-specific immunity and augments inflammation in ovalbumin-sensitized mice. Immunopharm Immunot, in press.

Wang, C.C., Liu, T.Y., Wey, S.P., Wang, F.I., Jan, T.R., 2007. Areca nut extract suppresses T-cell activation and interferon-gamma production via the induction of oxidative stress. Food Chem Toxicol 45, 1410-1418.

Wang, C.K., Lee, W.H., 1996. Separation, characterization and biological activities of phenolics in areca fruit. J Agric Food Chem 44, 2014-2019.

Whiteside, T.L., 2008. The tumor microenvironment and its role in promoting tumor growth. Oncogene 27, 5904-5912.

Williams, M.S., Kwon, J., 2004. T cell receptor stimulation, reactive oxygen species, and cell signaling. Free Radic Biol Med 37, 1144-1151.

Williamson, G., Manach, C., 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. Am J Clin Nutr 81, 243S-255S.

Wu, Q., Yang, Y., Simon, J.E., 2007. Qualitative and quantitative HPLC/MS determination of proanthocyanidins in areca nut (Areca catechu). Chem Biodivers 4, 2817-2826.

Wustrow, T.P., Mahnke, C.G., 1996. Causes of immunosuppression in squamous cell carcinoma of the head and neck. Anticancer Res 16, 2433-2468.

Yang, J.A., Huber, S.A., Lucas, Z.J., 1979. Inhibition of DNA synthesis in cultured lymphocytes and tumor cells by extracts of betel nut, tobacco, and miang leaf, plant substances associated with cancer of the ororespiratory epithelium. Cancer Res 39, 4802-4809.

Yoshioka, Y., Akiyama, H., Nakano, M., Shoji, T., Kanda, T., Ohtake, Y., Takita, T., Matsuda, R., Maitani, T., 2008. Orally administered apple procyanidins protect against experimental inflammatory bowel disease in mice. Int Immunopharmacol 8, 1802-1807.

Youn, J.I., Nagaraj, S., Collazo, M., Gabrilovich, D.I., 2008. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 181,

5791-5802.

Young, M.R., Wright, M.A., Lozano, Y., Matthews, J.P., Benefield, J., Prechel, M.M., 1996. Mechanisms of immune suppression in patients with head and neck cancer: influence on the immune infiltrate of the cancer. Int J Cancer 67, 333-338.

Zhang, W.Y., Liu, H.Q., Xie, K.Q., Yin, L.L., Li, Y., Kwik-Uribe, C.L., Zhu, X.Z., 2006. Procyanidin dimer B2 [epicatechin-(4beta-8)-epicatechin] suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells. Biochem Biophys Res Commun 345, 508-515.

Zhao, M., Mydlarz, W.K., Zhou, S., Califano, J., 2008. Head and neck cancer cell lines are resistant to mitochondrial-depolarization-induced apoptosis. ORL J Otorhinolaryngol Relat Spec 70, 257-263.

Znaor, A., Brennan, P., Gajalakshmi, V., Mathew, A., Shanta, V., Varghese, C., Boffetta, P., 2003. Independent and combined effects of tobacco smoking, chewing and alcohol drinking on the risk of oral, pharyngeal and esophageal cancers in Indian men. Int J Cancer 105, 681-686.