

行政院國家科學委員會專題研究計畫 成果報告

檳榔成分對 T 淋巴球細胞激素基因表現之作用與機轉研究

計畫類別：個別型計畫

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計畫主持人：詹東榮

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行政院國家科學委員會補助專題研究計畫 成果報告
 期中進度報告

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共同主持人：

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中華民國 93 年 10 月 31 日

一、摘要

流行病學研究顯示嚼食檳榔是罹患口腔鱗狀上皮細胞癌的危險因子，實驗證據指出口腔鱗狀上皮細胞癌的致病原因和病人免疫機能的惡化密切相關。本研究的主要目的即在研究檳榔成分對 T 細胞增生、活化和細胞激素基因表現的影響並探討其可能的作用機制，小鼠脾臟細胞先加入檳榔萃取物(ANE)、檳榔鹼、arecaidine 或黃樟素處理，再以 PMA/Io 或 CD3 合併 CD28 單株抗體(CD3/CD28)刺激。ANE 處理顯著的抑制脾臟細胞的代謝活性以及 IL-2 和 IFN- γ 的分泌，但對 IL-4 則只有輕微的抑制效果。檳榔鹼、arecaidine 或黃樟素(1-100 μ M)的處理對脾臟細胞分泌細胞激素並無影響。ANE 對 CD3/CD28 刺激所誘發的脾臟細胞表現細胞激素 IL-2 和 IFN- γ 的 mRNA 也具有顯著抑制作用，這項作用和上述 ANE 抑制細胞激素分泌的結果相吻合。除了脾臟細胞，ANE 對 EL4 細胞的活化和細胞激素的分泌也呈現出類似的抑制作用，ANE 並會導致 EL4 細胞出現 DNA laddering 的現象，顯示細胞受到 ANE 的作用而發生細胞凋亡的現象。綜合上述結果，本研究顯示 ANE 對 T 細胞的增生和 Th1 細胞激素的表現具有顯著的抑制作用，這個作用並非是檳榔鹼或 arecaidine 所造成，實驗證據也顯示 ANE 抑制細胞激素製造的可能機轉之一是經由誘發 T 細胞發生細胞凋亡。

ABSTRACT

Betel quid chewing is a risk factor for oral squamous cell carcinoma (SCC). Experimental evidence suggests that the pathogenesis of SCC is associated with immune deterioration. The objective of the present studies was to investigate the effect of areca nut extract (ANE) on T-cell proliferation, activation and cytokine expression, and its underlying mechanisms. Murine splenocytes were pretreated with ANE, arecoline, arecaidine or safrole followed by stimulation with phorbol-12-myristate-13-acetate plus ionomycin (PMA/Io) or anti-CD3 plus anti-CD28 monoclonal antibodies (CD3/CD28). ANE pretreatment markedly attenuated both the cell metabolic activity, and the production of interleukin (IL)-2 and interferon (IFN)- γ . In contrast, ANE pretreatment only slightly inhibited IL-4 secretion. Pretreatment of cells with arecoline, arecaidine, or safrole (1-100 μ M) did not affect the cytokine production. Consistent with the effect on cytokine production, ANE pretreatment markedly suppressed the steady state mRNA expression of IL-2 and IFN- γ by CD3/CD28-stimulated splenocytes. The inhibitory effects of ANE on splenocyte activation and cytokine production were also demonstrated in EL4 cells. Furthermore, ANE treatment induced the formation of DNA ladder in EL4 cells, indicating apoptosis of cells in the presence of ANE. Collectively, the present studies demonstrated that Th1 cytokine expression by T cells was attenuated by ANE pretreatment, which was apparently not mediated by arecoline and arecaidine. The results also suggest that the suppression of cytokine production by ANE is, at least in part, mediated by induction of T-cell apoptosis.

關鍵詞：areca quid, immune, T cell activation, cytokine

二、計畫緣起與目的：

Areca quid (AQ) chewing is a popular habit in the Southeast Asia. In Taiwan, it has been estimated that the number of AQ chewers is more than 2 million (1). Epidemiological studies indicate that AQ chewing has a strong impact on orodental health. AQ chewing is considered to be one of the major risk factors associated with oral SCC and oral precancerous lesions, such as oral submucous fibrosis (OSF) (2-4). It has been suggested that the pathogenesis of SCC is associated with deterioration in the immunological status (5,6). Deficiencies in both T and B cell-mediated immune responses have been reported in patients with oral cancers or OSF (6,7).

Both innate and acquired immunity are critical defense mechanisms against tumor formation *in vivo*. Among various types of immune cells, T cells play a pivotal role in mediating the anti-tumor function of the immune system (8,9). Cytotoxic T cells (CD8⁺ lymphocytes; T_c) are one of the major types of effector cells involved in cell-mediated immunity against malignant cells, whereas helper T cells (CD4⁺ lymphocytes; T_h) express a number of cytokines that are critical for mediating many immune responses. Analysis of lymphocyte subpopulation revealed an increase in the CD4⁺ to CD8⁺ lymphocyte ratio of the infiltrating mononuclear cells in lesions of both oral cancer and OSF patients (10,11). Depressed phytohemagglutinin-induced proliferative responses of peripheral blood lymphocytes and tumor infiltrating lymphocytes were found in oral SCC patients (12). Mitogen-stimulated proliferation and the expression of IFN- γ and IL-2 in T lymphocyte were diminished in patients with head and neck SCC (13). Likewise, alteration of cellular immunity and cytokine expression by immune cells has also been reported in patients with precancerous lesions (14,15). As AQ chewing is the main etiological factor for oral cancer and OSF, and the pathogenesis of these oral disorders is apparently associated with deterioration in T-cell functions, the objective of the present studies is to investigate the effect of AQ ingredients on T-cell activation and cytokine expression.

三、結果與討論：

Effect of ANE on cytokine secretion by splenocytes and EL4 cells: The production of IL-2 is a hallmark of T-cell activation. To study the effect of ANE on T-cell function, IL-2 production was firstly examined. Splensens of BALB/c mice (8-14 weeks old) were isolated aseptically and made into single cell suspensions. The splenocytes were cultured at 37°C in 5%CO₂ in complete RPMI 1640 medium, and received various treatments followed by stimulation with CD3/CD28 or PMA/Io. The supernatants of splenocyte cultures were harvested 24 and 48 hr after PMA/Io and CD3/CD28 stimulations, respectively. The amount of cytokines in the supernatants was assayed using ELISA. As illustrated in figure 1, naïve splenocytes produced large quantities of IL-2 in response to the stimulation with CD3/CD28 (2 μ g/mL of each antibody). Pretreatment of splenocytes with ANE (1-20 μ g/mL) for 30 min prior to CD3/CD28 stimulation significantly and concentration-dependently inhibited the production of IL-2 and IFN- γ . Likewise, pretreatment of splenocytes with ANE (1-60 μ g/mL) also produced an suppressive effect on the secretion of IL-2 and IFN- γ induced by PMA/Io (Fig. 2,3). In contrast, pretreatment with arecoline and arecaidine (1-100 μ M) did not influence the production of both cytokines by splenocytes (Fig. 2,3). The inhibitory effect of ANE on IL-2 production was also demonstrated in EL4 cells (data not shown). To further investigate the effect of ANE on T-cell cytokine expression, IL-4 in the supernatants was also determined. Notably, ANE, arecoline or arecaidine pretreatment did not affect the production of IL-4 by PMA/Io-stimulated splenocytes (Fig. 4).

Effect of ANE on cytokine mRNA expression by splenocytes: As ANE exhibited marked effects on IL-2 and IFN- γ production by splenocytes, the steady state mRNA expression of T-cell cytokines in ANE-pretreated splenocytes was determined. A RNase protection assay (RPA) was employed, which allowed to quantify the mRNA expression of 9 cytokines simultaneously, including IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15 and IFN- γ . As shown in figure 5, activation of splenocytes with CD3/CD28 for 6 hr induced the mRNA expression of IL-2, 4, 5, 6, 10, 13, and IFN- γ . Using the housekeeping genes L32 and GAPDH as reference standards, the

level of cytokine mRNA expression can be quantified. Consistent with the effect on cytokine production, ANE (10-50 μ g/mL) pretreatment concentration-dependently inhibited the mRNA expression of IL-2 and IFN- γ by CD3/CD28-stimulated splenocytes. ANE at the highest employed concentration (50 μ g/mL) also suppressed the expression of IL-4 mRNA. However, the magnitude of suppression on IL-4 mRNA was less than that of IL-2 and IFN- γ (Fig. 5A, B). Pretreatment of cells with arecoline did not influence the expression of cytokine mRNA (Fig. 5).

Inhibition of proliferation, metabolic activity and viability of splenocytes and EL4 cells by

ANE: Previous reports have shown that ANE can induce cytotoxicity in oral epithelial cells and keratinocytes (16,17). Therefore, the present studies investigated whether ANE affected the viability and proliferation of T cells. As shown in figure 6, MTT assay demonstrated that ANE (20-40 μ g/mL) pretreatment significantly suppressed the metabolic activity of splenocytes stimulated with PMA/Io, whereas arecoline and arecaidine were inactive. These results are in consistent with the inhibitory effect of ANE on IL-2 production. Similar results were also observed in EL4 cells. Pretreatment of cells with ANE markedly suppressed the metabolic activity (Fig. 7), viability and proliferation (Fig. 8) of EL4 cells under the conditions where IL-2 production was attenuated. Moreover, treatment with ANE for 24 hr induced DNA ladder formation in EL4 cells as demonstrated by electrophoresis (Fig. 9). Taken together, the present studies demonstrated that ANE produced suppressive effects on Th1 (IL-2 and IFN- γ) cytokine production, cell proliferation, metabolic activity and viability. One of the possible underlying mechanisms was via the induction of apoptosis as evidenced by DNA laddering. Further mechanistic studies is ongoing to decipher the mechanism of action of the immunosuppressive effect by ANE.

四、計畫成果自評：

The present studies demonstrated that ANE produced a direct inhibitory effect on the proliferation of murine T cells and Th1 cytokine production. As T cells are critically involved in the host defense mechanisms against tumor formation, these results are of scientific significance indicating a potential consequence of immunotoxicity resulted from betel quid chewing, which may be one of the factors responsible for the pathogenesis of oral cancer occurred in betel quid chewers.

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FIGURE LEGENDS:

Fig. 1. Inhibition by ANE of IL-2 and IFN- γ production by CD3/CD28 activated splenocytes. Splenocytes were either untreated (NA), or pretreated with ANE (1-20 $\mu\text{g}/\text{mL}$) for 30 min followed by treatment with anti-CD3 plus anti-CD28 antibodies (CD3/CD28; 2 $\mu\text{g}/\text{mL}$ of each antibody). After 48 hr of culture, supernatants were harvested and IL-2 and IFN- γ assayed by ELISA. Data are expressed as the mean \pm standard error of triplicate cultures. *, $p < 0.05$ as compared to the CD3/CD28 group. *ND*, cytokine proteins were below the level of quantification. Results are representative of three independent experiments.

Fig. 2. The effect of ANE, arecoline and arecaidine on IL-2 production by PMA/Io activated splenocytes. Splenocytes were either untreated (NA), or pretreated with ANE (1-60 $\mu\text{g}/\text{mL}$), arecoline (1-100 μM), or arecaidine (1-100 μM) for 30 min followed by stimulation with PMA/Io (PI; 80 nM/1 μM). After 24 hr of culture, supernatants were harvested and IL-2 assayed by ELISA. Data are expressed as the mean \pm standard error of triplicate cultures. *, $p < 0.05$ as compared to the PI group. Results are representative of three independent experiments.

Fig. 3. The effect of ANE, arecoline and arecaidine on IFN- γ secretion by PMA/Io activated splenocytes. Splenocytes were either untreated (NA), or pretreated with ANE (1-60 $\mu\text{g}/\text{mL}$), arecoline (1-100 μM), or arecaidine (1-100 μM) for 30 min followed by stimulation with PI (80 nM/ 1 μM). After 24 hr of culture, supernatants were harvested and IFN- γ assayed by ELISA. Data are expressed as the mean \pm standard error of triplicate cultures. *, $p < 0.05$ as compared to the PI group. *ND*, IFN- γ protein was below the level of quantification. Results are representative of three independent experiments.

Fig. 4. The effect of ANE, arecoline and arecaidine on IL-4 secretion by PMA/Io activated splenocytes. Splenocytes were either untreated (NA), or pretreated with ANE (1-60 $\mu\text{g}/\text{mL}$), arecoline (1-100 μM), or arecaidine (1-100 μM) for 30 min followed by stimulation with PI (80 nM/ 1 μM). After 24 hr of culture, supernatants were harvested and IL-4 assayed by ELISA. Data are expressed as the mean \pm standard error of triplicate cultures. *ND*, IFN- γ protein was below the level of quantification. Results are representative of three independent experiments.

Fig. 5. The effect of ANE and arecoline on cytokine mRNA expression by splenocytes. Splenocytes were either untreated (NA), or pretreated with ANE (1-50 $\mu\text{g}/\text{mL}$) or arecoline (10-100 μM) for 30 min followed by stimulation with CD3/CD28 (2 $\mu\text{g}/\text{mL}$ of each antibody) for 6 hr. The total RNA was isolated and cytokine mRNA expression was determined by RNase protection assays. Densitometry of phosphoimages were analyzed using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA). Values of interested mRNA densities were normalized to the values of density of the housekeeping genes L32 and GAPDH. Data are expressed as the percentage of the normalized value of the CD3/CD28 control group. Results are representative of two independent experiments.

Fig. 6. The effect of ANE, arecoline and arecaidine on the metabolic activity of splenocytes. Splenocytes were either untreated (NA), or pretreated with ANE (1-60 $\mu\text{g}/\text{mL}$), arecoline (1-100 μM), or arecaidine (1-100 μM) for 30 min followed by stimulation with PI (80 nM/1 μM). After 24 hr of culture, a MTT stock solution (5 mg/mL in PBS) was added to each well (10 $\mu\text{L}/\text{well}$) and incubated for 4 hr. The formed formazan was then dissolved with 0.1N acid-isopropanol, and the absorbance at 570 and 630 nm was measured. Data are expressed as the mean \pm standard error of triplicate cultures. *, $p < 0.05$ as compared to the PI control group. Results are representative of two independent experiments.

Fig. 7. The effect of ANE and arecoline on the metabolic activity of EL4 T cells. EL4 T cells (2×10^5 cells/mL) were either untreated (NA), or treated with ANE (1-60 $\mu\text{g}/\text{mL}$) or arecoline (1-100 μM). After 24 hr of culture, a MTT stock solution (5 mg/mL in PBS) was added to each well (10 $\mu\text{L}/\text{well}$) and incubated for 4 hr. The formed formazan was then dissolved with 0.1N acid-isopropanol, and the absorbance at 570 and 630 nm was measured. Data are expressed as the mean \pm standard error of triplicate cultures. *, $p < 0.05$ as compared to the NA group. Results are representative of three independent experiments.

Fig. 8. The effect of ANE on the growth and viability of EL4 cell. EL4 cells (2×10^5 cells/mL) were either untreated (NA) or pretreated with ANE (1-60 $\mu\text{g}/\text{mL}$). After 24 h of culture, cell viability and total amount of EL4 cells was determined by trypan blue. Data are expressed as the means \pm standard error of triplicate cultures. *, $P < 0.05$ as compared to the NA group. Results are representative of three independent experiments.

Fig. 9. Induction of genomic DNA fragmentation in EL4 cells by ANE. EL4 cells (2×10^5 cells/mL) were either untreated (NA) or pretreated with ANE (1-40 $\mu\text{g}/\text{mL}$). After 24 h of culture, cells were harvested and genomic DNA isolated. The concentration of DNA was determined spectrophotometrically at 260 nm and loaded into a 1.5% agarose gel (5 $\mu\text{g}/\text{lane}$), followed by electrophoresis at a voltage of 100 V. Gels were then stained with ethidium bromide and photographed under UV light illumination. Results are representative of three independent experiments.

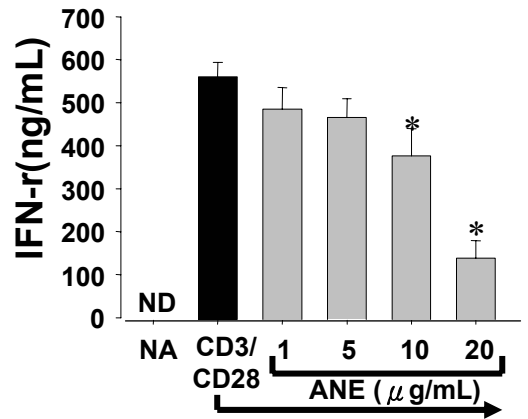
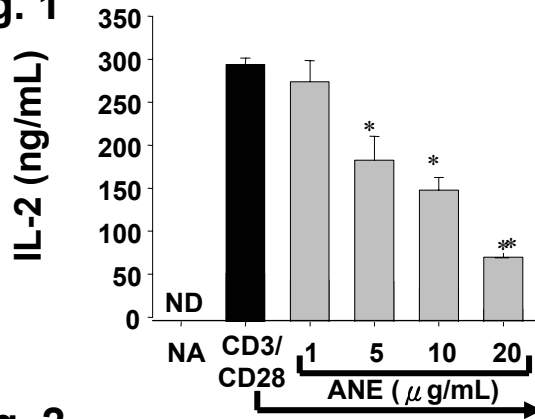
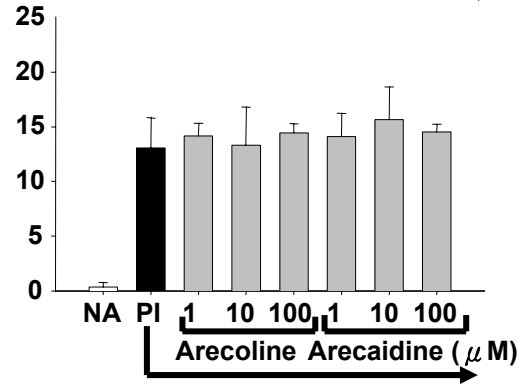
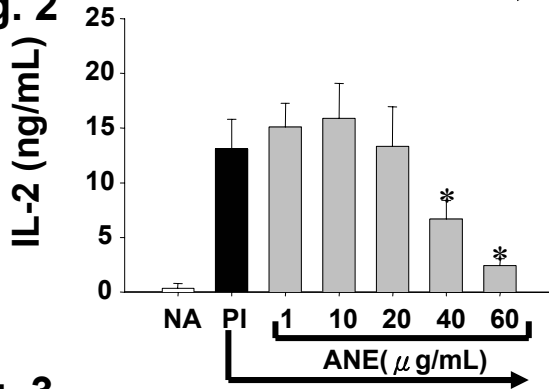
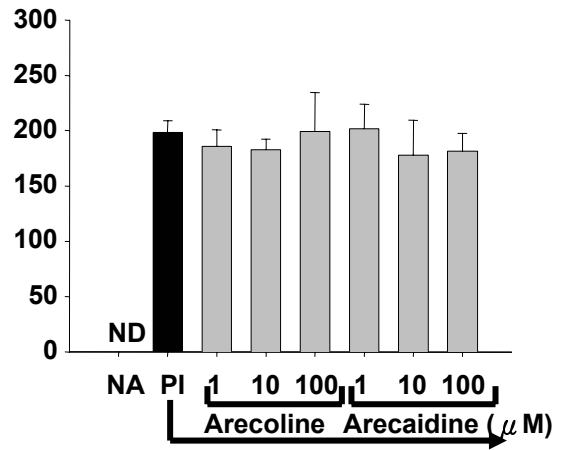
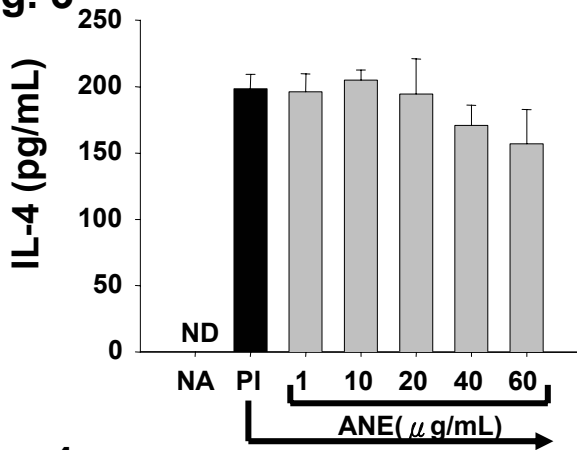
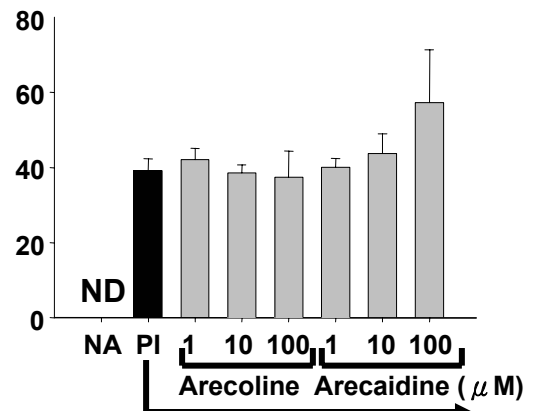
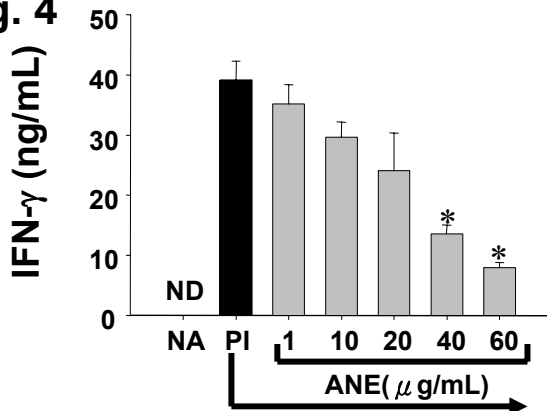
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Fig. 5

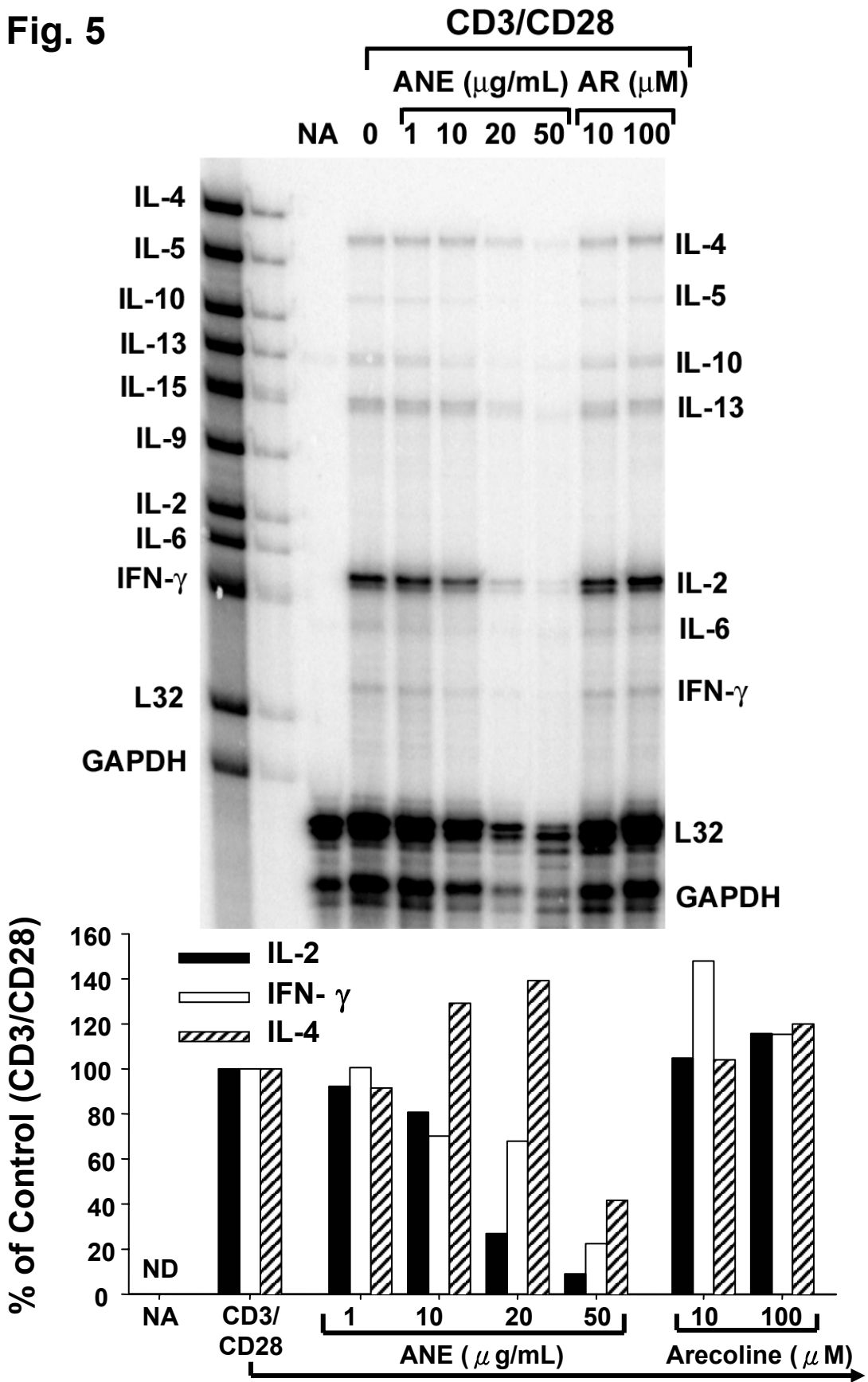


Fig. 6

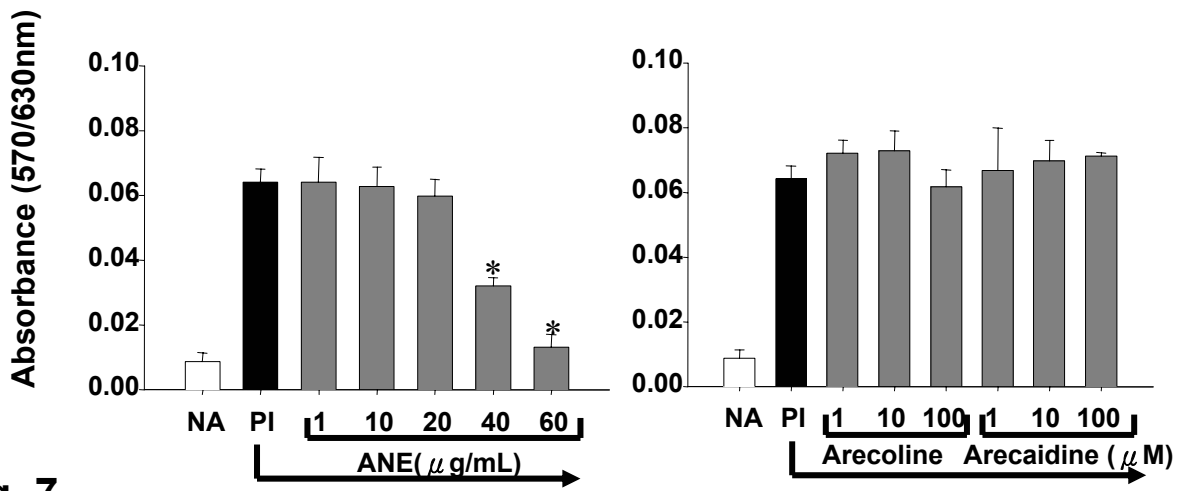


Fig. 7

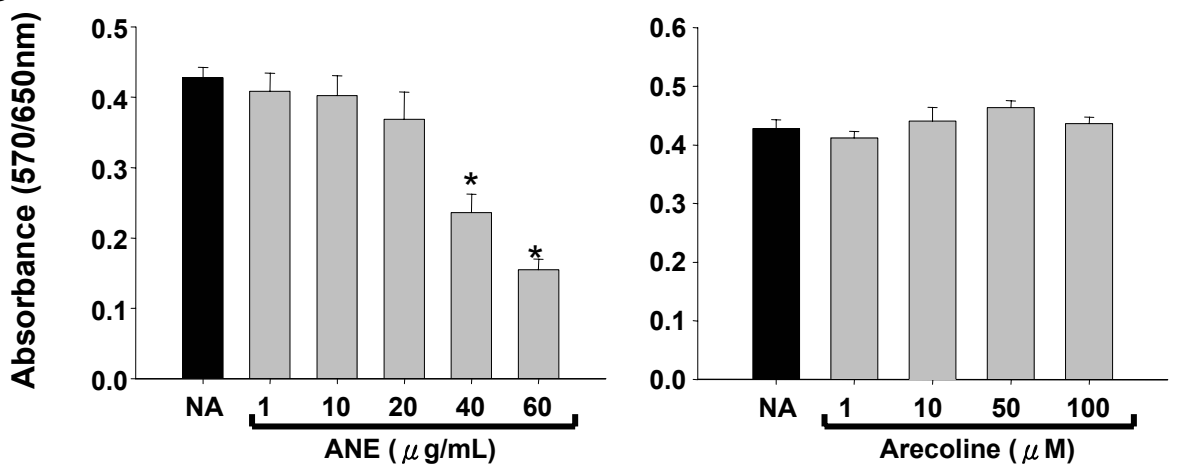


Fig. 8

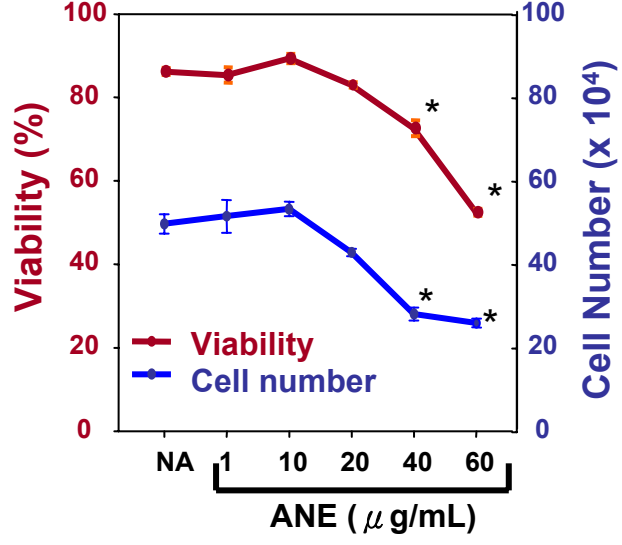


Fig. 9

