Journal of Food and Drug Analysis, Vol. 15, No. 1, 2007, Pages 48-54 藥物食品分析 第十五卷 第一期

Interleukin-10 Administration Inhibits TNF-α and IL-1β, but Not IL-6, Secretion of LPS-Stimulated Peritoneal Macrophages

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(Received: July 6, 2006; Accepted: September 27, 2006)

ABSTRACT

We hypothesize that exogenous administration of anti-inflammatory cytokine interleukin (IL)-10 would affect the secretion of pro-inflammatory cytokines such as IL-1β, IL-6, or tumor necrosis factor (TNF)–α using lipopolysaccharide (LPS)-stimulated macrophages exuded from the peritoneal cavity of female BALB/c mice. The LPS-stimulated macrophages were incubated for 48 hr to investigate the secretions of pro- and anti-inflammatory cytokine. The results indicated that the secretion levels of IL-1β, IL-6, IL-10, and TNF–α by LPS-stimulated macrophages elevated in a time dependent manner during 48-hr incubation period. The amount of cytokines secretion by LPS-stimulated macrophages varied: IL-6 > TNF-α > IL-10 > IL-1β. However, the secretion of IL-10 started to decrease at 18 hr of incubation. Therefore, the macrophage cultures were extra-administrated with various concentrations (0, 75, 150, and 225 pg/mL) of exogenous IL-10 after 18-hr incubation in order to evaluate the effect of exogenous IL-10 administration on inflammation. IL-10, IL-1β, IL-6, and TNF–α in LPS-stimulated macrophage cultures before and after exogenous IL-10 administration were determined by ELISA. The results demonstrated that exogenous IL-10 administration inhibited IL-1β (21.3-38.6%) and TNF–α (44.7-66.8%) secretion. However, the administration did not inhibit IL-6 secretion. Low dose (75 pg/mL) of exogenous IL-10 exerted maximal effects. IL-10 levels in LPS-stimulated macrophage cultures after exogenous IL-10 administration increased from 18.4% to 35.5%. Furthermore, only low dose of exogenous IL-10 administration increased endogenous IL-10 secretion (by 10.4%), while higher doses of exogenous IL-10 inhibited endogenous IL-10 production (from 31.7% to 41.9%). The results suggest that low dose administration of exogenous IL-10 might exhibit anti-inflammatory effects via inhibiting TNF-α and IL-1β, but not IL-6, secretion and increasing endogenous IL-10 production by LPS-stimulated peritoneal macrophages.

Key words: interleukin (IL)-10, IL-1β, IL-6, tumor necrosis factor (TNF)–α, lipopolysaccharide (LPS)-stimulated peritoneal macrophages

INTRODUCTION

Interleukin (IL)-10 is regarded as a pleiotropic cytokine. Five novel cytokines, IL-19, IL-20, IL-22, IL-24, and IL-25, with limited primary sequence identity and probable structural homology to IL-10 have been identified recently[1]. IL-10 is an 18.7 kD polypeptide produced by a variety of human immune cells, including T helper type 1 (Th1), and Th2 cells, B cells, monocytes, macrophages, dendritic cells (DC), mast cells, and eosinophils[2]. Much evidence has accumulated over past few years implicating the anti-inflammatory role of IL-10. Currently, data suggest that IL-10 inhibits Th1- and Th2-type immune responses. However, IL-10 favors humoral immunity and cytotoxic T lymphocyte function by acting directly on relevant cells and influencing immunomodulatory cells such as T regulatory, DC, and natural killer cells[3]. Therefore, IL-10 is involved in the inflammatory and immune reactions, and has potent anti-inflammatory and immunosuppressive activities on myeloid cells[4]. IL-10 regulates potential molecular pathways via the mitogen-activated protein kinase (MAPK) components to mediate the inflammation and cell death[5]. Thus, IL-10 is postulated to play a crucial role in acute and chronic inflammatory diseases, such as allergic diseases[6], experimental pancreatitis[7], and ozone-induced lung injury[8]. The functions of IL-10 and its applications on the immunomodulation have attracted great attention.

Inflammation can be defined as the sequential release of mediators such as pro-inflammatory cytokines, including IL-1, tumor necrosis factor (TNF), interferon (IFN)-γ, IL-12, IL-18, and the granulocyte-macrophage colony-stimulating factor, and is settled by anti-inflammatory cytokines such as IL-4, IL-10, IL-13, IFN-α, and the transforming growth factor (TGF)-β[9]. Chronic or non-necessary inflammation may cause tissue damage such as atherosclerotic intimas in human coronary arteries, systemic lupus erythematosus, and autoimmune rheumatic diseases[10-11]. The mechanisms by which the

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inflammatory responses, such as the pro-inflammatory cytokine secretion and the inhibition by anti-inflammatory cytokine such as IL-10, is resolved might provide new targets in the treatment of acute or chronic inflammation *in vivo*.

IL-10 is a well known anti-inflammatory cytokine and plays an important role in suppressing the inflammatory response in many *in vitro* or *in vivo* experimental models. It has been reported that IL-10 inhibits the release of pro-inflammatory cytokines IL-6 and TNF-α by human monocyte/macrophage in response to polymethylmethacrylate (PMMA, spherical 1-10 μm) particle challenge *in vitro*[12]. IL-10 potently abrogates the proliferative response of human vascular smooth muscle to atherogenic mitogens such as TNF-α and basic fibroblast growth factor[13]. Li *et al*.[14] hypothesized that enhancing anti-inflammatory cytokine IL-10 may be a promising approach for acute coronary disease therapy.

Monocytes and macrophages can produce a large repertoire of cytokines and participate in the pathogenesis of granulomatous diseases[15], hepatitis[16] and damage neuro-inflammatory insults such as hypoxia-ischemia[17]. In some cases, IL-10 may provide protective effects. However, the timing of IL-10 production/administration and pro-inflammatory cytokines secretion by activated macrophages remain to be further investigated. To elucidate the pharmacological effects of IL-10 on pro-inflammatory cytokine action and production, lipopolysaccharide (LPS)-stimulated peritoneal macrophages from BALB/c mice were cultured *in vitro* in this study. The LPS, a component of the gram negative cell wall, was used to stimulate peritoneal macrophages and to induce the production of inflammatory cytokines via the stimulation of Toll-like receptors in macrophages. Here, we report the time dependent production of pro- and anti-inflammatory cytokines upon exogenous IL-10 administration and endogenous IL-10 production by LPS-stimulated peritoneal macrophages.

MATERIALS AND METHODS

I. *Animals*

The female BALB/cByJNarl mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, ROC and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University, College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The mice were housed and kept on a chow diet (laboratory standard diet). The animal room was kept on a 12-hr-light and 12-hr-dark cycle. Constant temperature (25 ± 2°C) and relative humidity (50-60%) were maintained. After acclimation for 4 weeks, the mice were sacrificed to obtain peritoneal macrophages.

II. *Peritoneal Macrophages Isolation and Cultures*

Briefly, the adult female BALB/c mice were anesthetized with diethyl ether, exsanguinated using a retro-orbital venous plexus puncture and immediately euthanized by CO_2 inhalation. Peritoneal cells were prepared by lavaging the peritoneal cavity twice with 5 mL of sterile Hank's balanced salts solution through peritoneum. The peritoneal lavage fluid was centrifuged at 200 ×g for 10 min at 4°C. The cell pellets were collected and resuspended in TCM medium (TCM, a multipurpose serum replacement from CELOX Laboratories). The peritoneal adherent cells (mostly macrophages) from each animal were adjusted to 2×10^6 cells/ mL in TCM medium by hemocytometry and trypan blue dye exclusion method. Peritoneal adherent cells (0.50 mL/well) in the presence of stimulus, LPS (Sigma L-2654, 0.5 mL/well, 10 μg/mL) were plated in 48-well plates. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for up to 48 hr. The culture supernatants were collected respectively at 0, 6, 12, 18, 24, 48 hr after incubation. The plates were centrifuged at 200 ×g for 10 min and the supernatants were collected and stored at -80°C for later cytokine assays.

III. *IL-10 Administration*

To measure the effects of exogenous IL-10 administration on the secretion of pro-inflammatory cytokines and endogenous anti-inflammatory cytokines, the LPS-stimulated peritoneal macrophage cultures were individually administrated with various amounts (final concentrations 0, 75, 150, and 225 pg/mL) of IL-10 (mouse IL-10, R&D Systems DY417) after 18-hr incubation. The plates were incubated at 37°C and the culture supernatants were collected as described above, at the incubation time of 0, 6, 12, 18, 24 and 48 hr, respectively, before and after exogenous IL-10 administration.

IV. *Determination of Cytokines Secretion by ELISA*

Cytokine (IL-1β, IL-6, IL-10, and TNF-α) levels in LPS-stimulated peritoneal macrophage cultures were determined using sandwich ELISA kits, respectively, according to the manufacturer's instructions [mouse DuoSet ELISA Development system (R&D Systems)]. The sensitivity of these cytokine assays was about 15.6 pg/mL.

V. *Statistical Analysis*

Data were expressed as mean ± S.E. and analyzed statistically using Dunnett's test of parametric type. Differences between the exogenous IL-10 administration groups and control were considered statistically significant if $P < 0.05$.

RESULTS

I. *Secretion Time Course of LPS-stimulated Peritoneal Macrophages*

To investigate the time course of the cytokine secretion in LPS-stimulated peritoneal macrophages, both anti-inflammatory (IL-10) and pro-inflammatory (IL-1β, IL-6, and TNF–α) cytokines were determined. The results showed that all the levels of IL-10, IL-1β, IL-6, and TNF–α secretion in the macrophages culture increased, although slightly fluctuated, in a time-dependent manner during the 48-hr incubation period (Figure 1). We found that the secretion amount varied: IL-6 > TNF-α > IL-10 > IL-1β; the secretion amounts of IL-6, TNF-α, IL-10, and IL-1β at 48-hr incubation time were 7.170 ± 0.248, 2.072 ± 0.801, 0.299 ± 0.085, and 0.181 ± 0.070 ng/mL respectively.

A maximal cytokine secretion rate, except for that of IL-10, in LPS-stimulated macrophages was found during the first 12-hr incubation. Although a steady cytokine secretion rate maintained after 24-hr incubation, the secretion amount of IL-10 decreased at the time of 18-hr incubation. To evaluate the effect of exogenous IL-10 administration on inflammation, 18 hr was chosen to be the time to administrate exogenous IL-10 to avoid the inhibition of cytokine synthesis at the beginning of endotoxin (LPS) treatment.

II. *Effects of IL-10 Administration on the IL-1β Secretion*

To determine if IL-10 demonstrates its anti-inflammatory effect via inhibiting IL-1β secretion *in vitro*, the exogenous IL-10 was added to the LPS-stimulated macrophages culture at the incubation time of 18 hr. The results showed that exogenous IL-10 in concentrations of 75 pg/mL (low dose), 150 pg/mL (medium dose), and 225 pg/mL (high dose) respectively inhibited the secretions of pro-inflammatory cytokine IL-1β (Figure 2). Although the exogenous IL-10 inhibited IL-1β secretion, the inhibitory manner was not dose-dependent. The low administration dose (75 pg/mL) had a maximum inhibitory effect and decreased IL-1β secretion by 38.6% (from 181 ± 70 pg/mL to 111 ± 65 pg/mL) compared to the control (0 pg/mL IL-10 added) after 30 hr of exogenous IL-10 administration. The medium and high administration doses (150 and 225 pg/mL) decreased IL-1β secretion by 32.6% (from 181 ± 70 to 149 ± 67 pg/mL) and 21.3% (from 181 ± 70 to 143 ± 72 pg/mL), respectively.

III. *Effects of IL-10 Administration on the IL-6 Secretion*

The effects of exogenous IL-10 administration on IL-6 secretion in LPS-stimulated macrophages are shown in Figure 3. The results revealed that low, medium, and high doses of exogenous IL-10 slightly, but not significantly ($P > 0.05$) increased IL-6 secretion by 10.3% (from 7.17 ± 0.25 to 7.91 ± 1.16 ng/mL), 8.7% (from 7.17 ± 0.25 to 7.79 ± 2.42 ng/mL), and 17.0% (from 7.17 ± 0.25 ng/mL to 8.39 ± 2.50 ng/mL), respectively, compared to the control after 30 hr of exogenous IL-10 administration.

IV. *Effects of IL-10 Administration on the TNF-α Secretion*

The results showed that exogenous IL-10 administration in concentrations of 75, 150, and 225 pg/mL,

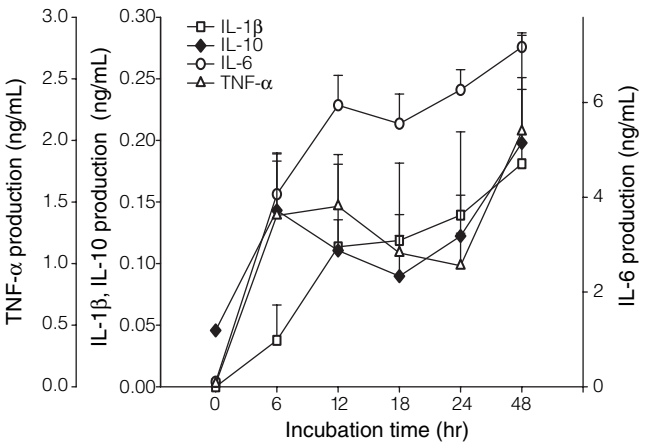

Figure 1. The time courses of IL-10, IL-1β, IL-6 and TNF-α production using LPS-stimulated peritoneal macrophage cultures from female BALB/c mice. The secretions from the cell cultures were determined after LPS (10 μg/mL) addition for 0, 6, 12, 18, 24 and 48 hr, respectively. Data are represented as mean ± SE (n = 3).

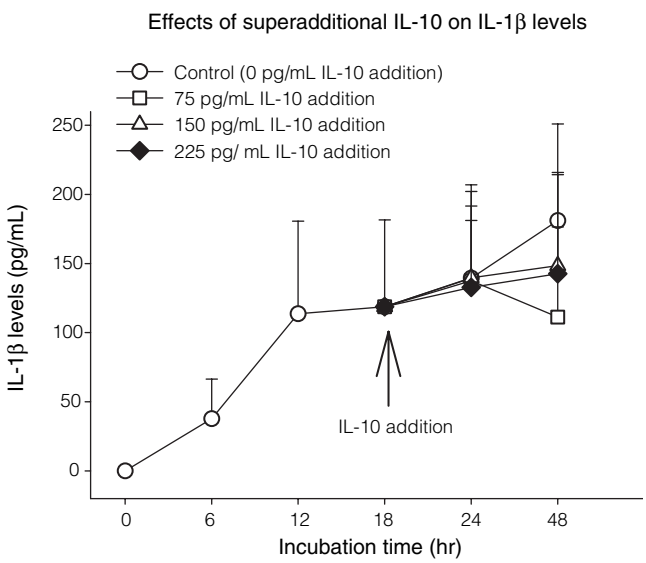

Figure 2. Exogenous IL-10 administration effects on the IL-1β secretion using LPS-stimulated peritoneal macrophage cultures from female BALB/c mice. The secretions of IL-1β from the cell cultures were determined at the indicated incubation time 0, 6, 12, 18, 24 and 48 hr, respectively. The exogenous IL-10 was added to the cell cultures after 18-hr incubation. Data are represented as mean ± SE (n = 3).

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respectively, inhibited the secretions of pro-inflammatory cytokine TNF-α in LPS-stimulated macrophages (Figure 4). Although the exogenous IL-10 administration inhibited TNF-α secretion, the inhibitory manner was not dose-dependent either. The low dose of exogenous IL-10 administration had a maximal inhibitory effect and decreased TNF-α secretion by 66.8% (from 2.07 ± 0.80 ng/mL to 0.77 ± 0.47 ng/mL) compared to the control after 30 hr of exogenous IL-10 administration. The medium and high administration doses decreased TNF-α secretion by 44.7% (from 2.07 ± 0.80 ng/mL to 1.15 ± 0.50 ng/mL) and 51.8% (from 2.07 ± 0.80 ng/mL to 1.00 ± 0.50 ng/mL) during the same time course.

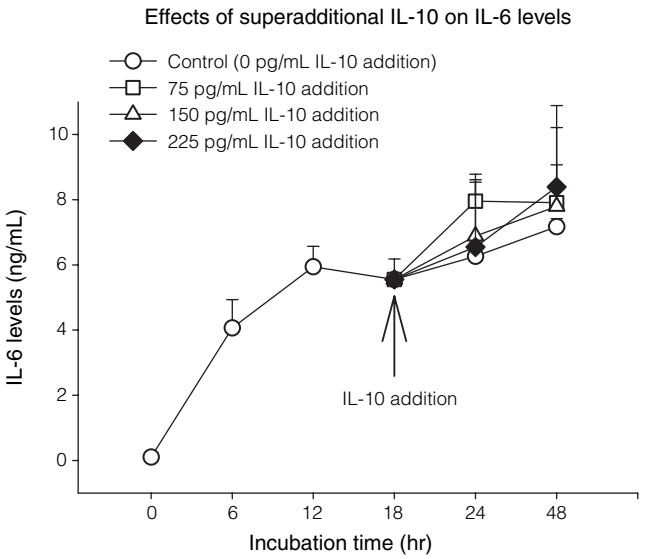

Figure 3. Effects of exogenous IL-10 administration effects on IL-6 secretion using LPS-stimulated peritoneal macrophage cultures from female BALB/c mice. The secretions of IL-6 from the cell cultures were determined at the indicated incubation time 0, 6, 12, 18, 24 and 48 hr, respectively. The exogenous IL-10 was added to the cell cultures after 18 hr incubation. Data are represented as mean ± SE (n = 3).

V. *Effects of IL-10 Administration on the IL-10 Secretion*

The effects of exogenous IL-10 administration on IL-10 secretion by LPS-stimulated macrophages are shown in Figure 5. The results revealed that low, medium, and high doses of exogenous IL-10 administration seemed to increase IL-10 concentrations in the LPS-stimulated macrophages by 35.5% (from 299 ± 86 pg/mL to 405 ± 66 pg/mL), 18.4% (from 299 ± 86 pg/mL to 354 ± 56 pg/mL), and 33.2% (from 299 ± 86 pg/mL to 399 ± 32 pg/mL), respectively, compared to control after 30 hr of exogenous IL-10 administration. To determine the net endogenous IL-10 secretion by LPS-stimulated macrophages, the concentrations of exogenous IL-10 added to the medium at 18 hr were individually subtracted from those of LPS-stimulated macrophages after 30 hr of exogenous IL-10 administration. Net changes of endogenous IL-10 secretions by LPS-stimulated macrophages upon extra-administration of low, medium, and

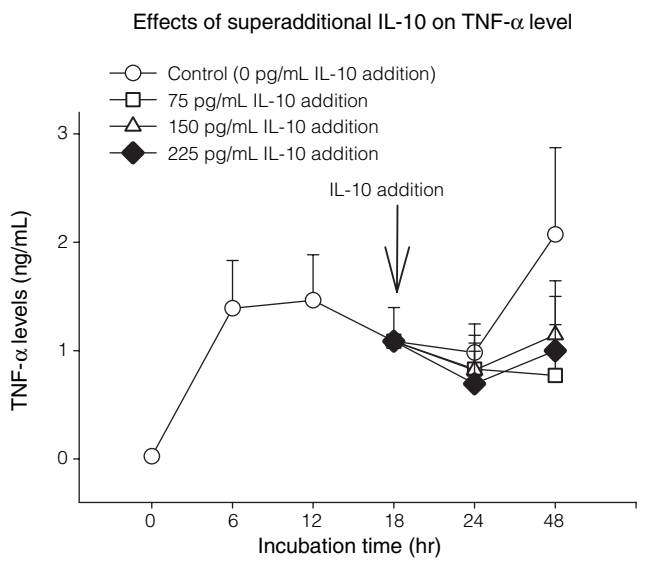

Figure 4. Exogenous IL-10 administration effects on the TNF-α secretion using LPS-stimulated peritoneal macrophage cultures from female BALB/c mice. The secretions of TNF-α from the cell cultures were determined at the indicated incubation time 0, 6, 12, 18, 24 and 48 hr, respectively. The exogenous IL-10 was added to the cell cultures after 18 hr incubation. Data are represented as mean ± SE (n = 3).

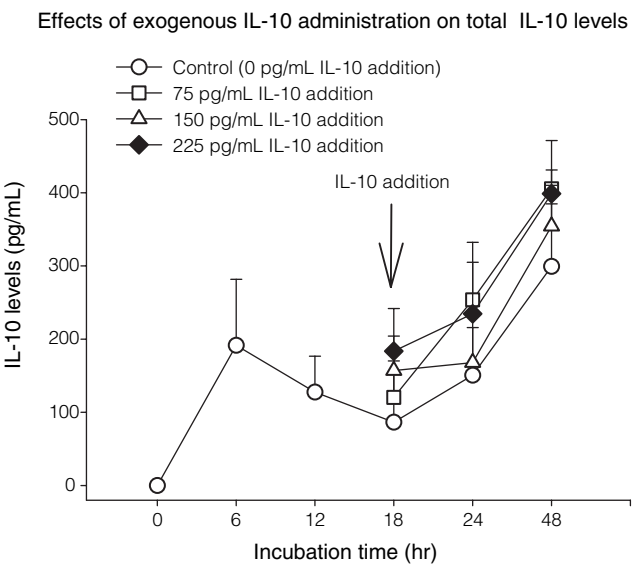

Figure 5. Exogenous IL-10 administration effects on the total IL-10 levels using LPS-stimulated peritoneal macrophage cultures from female BALB/c mice. The secretions of IL-10 from the cell cultures were determined at the indicated incubation time 0, 6, 12, 18, 24 and 48 hr, respectively. The exogenous IL-10 was added to the cell cultures after 18 hr incubation. Data are represented as mean ± SE (n = 3).

high doses of exogenous IL-10 were from 299 to 330 pg/mL (increase by 10.4%), from 299 to 204 pg/mL (decrease by 31.7%), and from 299 to 174 pg/mL (decrease by 41.9%), respectively.

DISCUSSION

Monocytes/macrophages play an important role in the persistence of chronic inflammation and local tissue destruction in diseases such as rheumatoid arthritis, atherosclerosis, brain, and bronchoalveolar inflammation[18-20]. However, the time course of cytokine secretion and anti-inflammatory regulation by inflamed macrophages itself is still not fully understood. In this study, we established an *in vitro* model using LPS-stimulated primary peritoneal macrophages from female BALB/c mice to evaluate the interaction between pro-inflammatory and anti-inflammatory cytokines. We found that LPS, an endotoxin, stimulated peritoneal macrophges to produce both pro-inflammatory cytokines (IL-1β, IL-6, and TNF–α) and anti-inflammatory cytokine (IL-10) *in vitro* (Figure 1). However, exogenous IL-10 administration significantly affected the cytokines secretion and may relieve the inflammation status of LPS-stimulated peritoneal macrophages via decreasing the secretion of pro-inflammatory cytokines, such as IL-1β and TNF-α (Figures 2 and 4), and increasing the secretion of anti-inflammatory cytokine, such as IL-10 (Figure 5). MacKenzie *et al.*[21] reported that LPS stimulates monocyte/macrophage cells in primary culture from trout *Oncorhynchus mykiss* and activate the LPS-dependent TNF-α expression[21]. Peritoneal macrophages from mice treated with LPS produce IL-10 and the IL-10 production is promoted by the serine/threonine kinase Akt pathway[22]. In this study, total IL-10 level (including exogenous and endogenous IL-10) was significantly ($P < 0.05$) negatively correlated, respectively, with IL-1β and TNF-α. levels in the LPS-stimulated macrophages culture. This study provides clues of the secretion time course and interaction between pro- and anti-inflammatory cytokines in LPS-stimulated macrophages in nature.

Inflammation progress consists of complicated release of mediators and recruiting circulating leukocytes to the inflammatory site. However, in some cases, the inflammatory response is alleviated by the release of endogenous anti-inflammatory mediators and the accumulation of intracellular negative regulatory factors[9]. In this study, although pro-inflammatory cytokines IL-1β, IL-6, and TNF–α were produced by LPS-stimulated peritoneal macrophages, an endogenous anti-inflammatory cytokine IL-10 might be produced by the same cells (Figure 1). Sumiyoshi *et al.*[10] reported that the IL-10 expression is positively correlated with oxidized low density lipoprotein deposition in the atherosclerotic intimas of human coronary arteries. The results suggest that the inflammatory balance modulated by pro- and anti-inflammatory cytokines produced by LPS-stimulated macrophages does exist.

An endogenous anti-inflammatory cytokine IL-10 might modulate the inflammatory responses via regulating the MAPK components which have emerged as potential signaling cascades in the regulation of a plethora of cell functions, including inflammation and cell death[5]. We hypothesized that exogenous IL-10 administration might enhance the anti-inflammatory effects of LPS-stimulated macrophages. The resolution of the effect of exogenous IL-10 on LPS-stimulated macrophages might establish a basis for the current therapeutical intervention concepts of IL-10 for different inflammatory diseases. In this study, the results revealed that the exogenous IL-10 administration significantly decreased the pro-inflammatory cytokine secretions, IL-1β and TNF-α, and increased the total IL-10, including exogenous and endogenous, level in the culture. The results suggest that exogenous IL-10 can inhibit IL-1β and TNF-α secretion by inflamed macrophages and might alleviate the inflammation status *in vitro* or *in vivo*. However, the effects of exogenous IL-10 administration on IL-1β, TNF-α, and IL-10 secretion did not demonstrate in a dose dependent manner. The low dose of exogenous IL-10 administration seemed to have a maximal effect. To further clarify the puzzle, the endogenous IL-10 secretion by LPS-stimulated macrophages after extra-administrated with exogenous IL-10 were determined. The changes of endogenous IL-10 production from LPS-stimulated macrophages after extra-administrated with low, medium, and high doses of exogenous IL-10 were +10.4%, -31.7%, and -41.9% (Figure 5). Low dose of exogenous IL-10 administration slightly induced the endogenous IL-10 secretion. However, higher doses of exogenous IL-10 inhibited the endogenous IL-10 secretion. The results suggest that the biological concentration of endogenous IL-10 is constitutively regulated. However, this study was not aimed to elucidate the detailed mechanism by which the stability or half-life of exogenous IL-10 was mentioned in the cell culture model. Determination of the endogenous IL-10 level by directly subtracting exogenous IL-10 levels added to the medium at 18 hr from total IL-10 levels may cause a bias on endogenous IL-10 levels. Certain cells modulate IL-10 secretion via negative-feedback or positive-feedback regulation[9]. The magnitude of the immune cell response generally depends on the dose of stimulant administered. However, extremely low or high stimulant doses may induce specific irresponsive states (acquired low-zone or high-zone tolerance)[23]. In this study, our results suggest that low dose (75 pg/mL) administration of exogenous IL-10 has maximal anti-inflammatory effects via decreasing the pro-inflammatory cytokines production, such as IL-1β and TNF-α, and increasing the endogenous anti-inflammatory cytokine, such as IL-10, in this *in vitro* model. However, the prac-

tical uses of exogenous IL-10 in the immunotherapy against inflammation and the regulation between endogenous and exogenous IL-10 levels remain to be explored.

IL-6 is known to be a multifunctional cytokine that modulates the immune response, hematopoiesis, acute phase response and inflammation[24]. Deregulation of IL-6 production is beneficial in the inflammation pathology or several disease processes, such as prostate cancer progression[25]. ST2, a member of IL-1 receptor family expressed in Th2 cells, negatively regulates LPS-induced IL-6 production via the inhibition of IκB degradation in THP-1 cells[26]. Trindade *et al.*[12] reported that IL-10 inhibits the release of the pro-inflammatory cytokines IL-6 and TNF-α in human monocyte/macrophage in response to PMMA particle challenge *in vitro*. In this study, LPS induced IL-6 production of peritoneal macrophages. However, in contrary to our hypothesis, exogenous IL-10 administration could not inhibit IL-6 secretion by LPS-stimulated macrophages. IL-6 is an acute phase pro-inflammatory cytokine and the secretion amount of IL-6 was much higher than that of IL-10 (about 20 folds) (Figure 1). Administration time point and concentrations of exogenous IL-10 intervention might cause a contray result of IL-6 secretion. The reason remains to be further elucidated.

In conclusion, this study demonstrated that exogenous IL-10 administration significantly inhibited IL-1β and TNF–α secretion but increased IL-10 levels in LPS-stimulated peritoneal macrophage cultures. The results suggest that low dose of exogenous IL-10 administration might exhibit anti-inflammatory effects via inhibiting TNF-α and IL-1β secretion and increasing endogenous IL-10 secretion by LPS-stimulated peritoneal macrophages. These results will be important and useful in the future exploitation of cytokine therapy and anti-inflammation evaluation.

ACKNOWLEDGEMENTS

This study was supported by a research grant NSC93-2313-B-005-033 from the National Science Council, Taipei, Taiwan, ROC.

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