รายงานวิจัยฉบับสมบูรณ์

การใช้ไซโตไคน์อินดิวซ์คิลเลอร์เซลล์ในการรักษาโรคหอบหืด

APPLICATION OF CYTOKINE-INDUCED KILLER CELLS FOR TREATMENT OF ALLERGIC ASTHMA: A NOVEL IMMUNOTHERAPY FOR ALLERGIC ASTHMA

โดย ดร.นพ.กิติพงศ์ สุนทราภา และคณะ

กรกฎาคม 2559

สัญญาเลขที่ MRG5580129

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มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด

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ปัจจุบันมีการรายงานถึงการใช้ไซโตไคน์อินดิวซ์คิลเลอร์เซลล์ในการรักษาโรคมะเร็งมายาวนานกว่า 20 ปี ไซโตไคน์อินดิวซ์คิลเลอร์เซลล์แสดงคุณสมบัติของเซลล์เม็ดเลือดขาวทั้งชนิด NK cells และ Th1 cells ซึ่งการทดลองนี้จะอาศัยคุณสมบัติของ Th1 cells เพื่อไปปรับสมดุลของระบบภูมิคุ้มกันในผู้ป่วยโรคหอบหืด ซึ่งมีสาเหตุมาจากการตอบสนองต่อสิ่งกระตุ้นของเซลล์เม็ดเลือดขาวชนิด Th2 cells ที่มากเกินไป การเพาะเลี้ยงไซโตไคน์อินดิวซ์คิลเลอร์เซลล์ในหลอดทดลอง โดยทำการกระตุ้นเซลล์จากต่อมไทมัสด้วย interferon gamma (IFN-γ), anti-CD3 monoclonal antibody และ interleukin (IL)-2 ให้พัฒนาไปเป็นไซโตไคน์อินดิวซ์คิลเลอร์เซลล์ แล้วนำไปฉีดเข้าหนูเม้าส์ที่อยู่ระหว่างการกระตุ้นด้วยโปรตีนไข่ขาวให้เป็นโรคหอบหืด ผลการทดลองพบว่าไซโตไคน์อินดิวซ์คิลเลอร์เซลล์สามารถลดการอักเสบของหลอดลมได้ โดยพบการลดลงของeosinophil ในน้ำล้างหลอดลมถุงลมปอด (Bronchoalveolar lavage fluid), ลดระดับ Th2 cytokines (IL-5, IL-13) ในน้ำเลือด, ลดการสะสมของเซลล์เม็ดเลือดขาวและเซลล์ที่ทำหน้าที่เป็นต่อมหลั่งเมือก (goblet cells) รอบๆหลอดลม การศึกษานี้เป็นครั้งแรกในการประยุกต์ใช้ไซโตไคน์อินดิวซ์คิลเลอร์เซลล์ในการรักษาโรคหอบหืดจากภูมิแพ้

คำหลัก: ไซโตไคน์อินดิวซ์คิลเลอร์เซลล์, โรคหอบหืดจากภูมิแพ้, น้ำล้างหลอดลมถุงลมปอด

Abstract

Project Code: MRG5580129

Project Title: Application of cytokine-induced killer cells for treatment of allergic asthma: a novel immunotherapy for allergic asthma.

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The effectiveness of CIK cells for treatment of cancers have long been appreciated for more than two decades. Here, we report for the first time that CIK cells can be used to treat allergic asthma. Adopting from an established protocol for culturing CIK cells with some modifications, we generated CIK cells ex vivo from mouse T cells, and injected them intravenously into ovalbumin-induced asthmatic mice. Their curability was examined by evaluating bronchoalveolar lavage cellularity, Th2 cytokines levels and lung histology, all of which are important parameters for determining asthmatic severity. Interestingly, without any obvious adverse effects, to the degree comparable to those treated with a corticosteroid, asthmatic mice treated with CIK cells showed significant reductions in all the parameters, eosinophilic inflammation of the lungs (BALF cellularity), Th2 cytokines (IL-5, IL-13) levels in the serum, inflammatory cell infiltration around pulmonary arterioles, and mucus production in the airways. Thus, our study provides a first proof of applying CIK cells in the treatment of allergic asthma.

Keywords: Cytokine-induced killer cells, Allergic asthma, Bronchoalveolar lavage fluid**,** Ovalbumin-induced asthmatic mice

CHAPTER 1

INTRODUCTION

Cytokine Induced Killer (CIK) cells, reported for the first time by Schmidt-Wolf *et al*., (1) are *ex vivo* expanded T cells that display phenotypic and functional characteristics of both natural killer (NK) cells and cytotoxic T cells (2). There are two main subsets of T cells in the human CIK cell culture, $CD3^+$ $CD56^+$ and $CD3^+$ $CD56^-$ cells (for murine CIK cell culture: $CD3^+$ $DX5^+$ / $CD3^+$ $NK1.1^+$ and $CD3^+$ $DX5^-$ / $CD3^+$ $NK1.1^-$) (3). Interestingly, the double positive cells are mainly $CD8^+$ T cells with a CD1d-independent, major histocompatibility complex (MHC)-unrestricted cytolytic activity against tumor targets (4). Upon natural killer group 2 member D (NKG2D) recognition, CIK antitumor activity is mediated through the release of a variety of cytotoxic mediators including perforin, granzyme, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (5). Besides anti-cancer activity, CIK cells also reduced graft-versus-host disease in an animal study (6). At present, CIK cells have proven to be safe for adoptive transfer, and are widely used as immunotherapy for various cancers including lymphoma, metastatic renal cancer, hepatocellular carcinoma and relapsing hematological malignancies (7). Almost all of the studies on CIK cells generated from human PBMC have focused on their antitumor effects (8). However, other applications of CIK cells and full elucidation of their differentiation process remain elusive. Moreover, murine CIK cells have been used for pre-clinical research, mostly adoptive immunotherapy for cancers (9). Again, the expansion of murine CIK cells is problematic for preclinical studies as the cells could be expanded for a brief time; hence, has to be infused by 14 days of culture (9). A variety of mouse models can provide clues to ascertain their usefulness in other diseases, so it would be very beneficial to generate murine T cell-derived CIK cells under optimal conditions.

Often the standard human CIK cell culture protocol is used to obtain murine CIK cells from various organs including the thymus, spleen and lymph nodes (4, 6, 9). As it has already been proven that the major effector population of human CIK cells, i.e., $CD3^+$ $CD56^+$, is differentiated from a $CD3^+CD56^-$ population, i.e., T cells (10). As the thymus contains more than 90% T cells, the use of murine thymocytes for CIK cell generation can eliminate the tedious and time-consuming T cell isolation process. Previously, Baker *et al*. also used thymocytes to generate CIK cells; however the yield of CIK cells from the thymus is relatively low, unlike that of the spleen (9).

Moreover, the well-established protocol to obtain human CIK cells derived from peripheral blood mononuclear cells (PBMC) includes signaling through rIFN-γ, anti-CD3 mAb and rIL-2 (11). IFN-γ administered at the start of culture increases T cell cytotoxicity and facilitates the acquisition of a T-helper (Th) 1 phenotype (12). Next, rIL-2 further promotes proliferation, survival and differentiation of T cells (13). Finally, anti-CD3 monoclonal antibody (mAb) supplied in the culture 24 hours (h) after the activation with rIFN-γ acts via T cell receptor (TCR) signaling and provides the first fundamental signalfor cell activation (14). In addition to TCR signaling, T cells require a second signal for full activation, and enhanced proliferation (15). The additive signal provided by CD28 costimulation has been shown to enhance T cell proliferation (16). The CD28 costimulatory signal in T and NKT cells is well known to act synergistically with the first TCR-derived signal (17). Moreover, the CD28 costimulatory signal was shown to enhance the expression of chemokine receptors by CIK cells. Those CIK cells showed increased trafficking into the tumor site (18). These observations demonstrated the importance of CD28 costimulatory signal in CIK cell activity. However, whether CD28 costimulatory signaling increases CIK cell proliferation and enhances cytotoxicity against tumor cells remains elusive. Also, whether enhanced cytotoxicity is due to increased number of CIK cell trafficking or their absolute potentiation of their cytotoxic ability is not clear.

Here, we generated murine thymocytes derived CIK cells in the presence or absence of the CD28 costimulatory signal, and monitored the phenotypeduring the process of culture. Then, we measured the cytotoxicity of hence generated CIK cells against murine leukemic cells. Finally, we investigated for the production of cytotoxic granules and proinflammatory cytokines to determine the potential mechanisms enhancing cytotoxicity of CIK cells by the additional CD28 costimulatory signals. Interestingly, CD28 costimulatory signaling effectively enhanced CIK cell proliferation and potentiated their cytotoxic activity against tumor cells.

Allergic asthma is a chronic inflammatory disease of the airways, one of the major public health concerns now a day. Its pathophysiology is driven mainly by T helper type2 (Th2) immune responses. In response to allergen inhalation, the Th2 cells proliferate and secrete cytokines (Interleukin (IL)-4, IL-5, IL-9 and IL-13). The role of IL-4 is activating plasma cells to produce immunoglobulin (Ig) E, while IL-5 regulates eosinophil proliferation, differentiation and histamine releasing. Moreover, IL-13 production by Th2 cells effects on induction of goblet cells and airway hyper responsiveness. Th2 cytokines action was causing eosinophilia airway inflammation, goblet cell hyperplasia, mucus secretion, airway hyperresponsiveness (AHR) and when chronic, airway remodeling (19-21). At present, using

bronchodilators and corticosteroids is a major option for treatment of allergic asthma. These drugs can cause varies adverse effects, such as osteoporosis, metabolic imbalances, glaucoma, and immunosuppression (22-24). Accordingly, it is very beneficial to fine a new therapeutic approach for millions of asthmatic patients.

The Th1/Th2 balance is very important and may define whether the immune response is appropriate or reach to severe immunopathologies. Overexpression of Th1 cytokines are involved in delay-type hypersensitivity reactions and autoimmune diseases. Conversely, allergic disorders dependent on regulation of Th2 phenotypes (25, 26). One of the novel treatments allergic asthma is cell-based immunotherapy. Previous preclinical studies with adoptive transfer of IFN-γ-producing cells into antigen-sensitized recipient mice protected from airway eosinophilia after allergen challenge. Certainly, when transferred $CD4^+$ Th1 cells into recipient mice, these cells inhibited Th2-induced eosinophilia and mucus production through secretion of IFN-γ, whereas IFN-γ knockout mice provided eosinophilia (27). Anyway, other studies showed that adoptively transferred Th1 cells might motivate an inflammatory response due to the proinflammatory properties of IFN-γ (28, 29). However, application of Th1 cell to treat allergic asthma is still a matter of debates. Recently, natural killer (NK) cells are reported to play a role in resolution of airway inflammation by clearing eosinophil and antigen-specific T cells (30, 31). Since, there are various subtypes of NK cells, it is still in controversy how these different subtypes and their respective cytokines/chemokines play role in promotion/cessation of allergic asthma (32, 33).

Studies of cytokine-producing profile of CIK cells support the hypothesis that CIK cells have the ability to treat allergic asthma. Indeed, patient severe asthma display significantly reduced production of IFN-γ in response to antigen when compare to normal individuals (34, 35). The allergy resolution not to be related with a reduction cytokines produce by Th2 cells, but with normalization of IFN-γ levels (36).

In the present study, to investigate the potential of CIK cells as a treatment in allergen-induced asthma mouse model by examining the total inflammatory cells and eosinophil in bronchoalveolar lavage fluid (BALF), Th2 cytokines and IgE levels in serum, inflammatory cells infiltration and goblet cells hyperplasia in lung. Altogether, this study can also exhibit whether application of CIK cells could be beneficial for treatment of allergic asthma.

CHAPTER 2

OBJECTIVES

2.1 To generate the effective and reproducible protocol for murine CIK cells generation and function.

2.2 To ascertain a novel effective immunotherapy of allergic asthma using the OVA-induced asthma mouse model.

2.3 To examine effectiveness and adverse effects of applying CIK cells to treat allergic asthma.

2.4 To compare effectiveness of CIK cells with corticosteroids in treating asthma in mice.

CHAPTER 3
MATERIALS AND METHODS

3.1 Animals and ethic statement

5-6 weeks old male mice on the C57BL/6 background were purchased from National Laboratory Animal Center (Mahidol University, Bangkok, Thailand). The mice were housed in a temperature-controlled environment on 12 hours (h) light–dark cycles with unrestricted access to food and water. All experimental were performed in accordance with the guidelines of Mahidol University and the Office of the National Research Council of Thailand (NRCT) and approved by the Committee on Animal Care and Use of Siriraj hospital (SiACUC).

3.2 Reagents

The following reagents were used in this study: Roswell Park Memorial Institute medium (RPMI 1640) and fetal bovine serum (FBS) (Gibco Inc., Life Technologies, Carlsbad, CA, USA); mouse rIFN-γ (eBioscience, San Diego, CA, USA); anti-mouse CD3e mAb, anti-CD28 mAb and mouse rIL-2 (BioLegend, San Diego, CA, USA); Phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich, St. Louis, MO, USA); BrefeldinA solution (Catalogue no 347688, BD Biosciences, San jose ,CA, USA), BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA); fluorescein isothiocynate (FITC)-labeled anti-mouse Thy1.2 mAb, phycoerythrin (PE)-labeled anti-mouse IFN-γ mAb, PE-labeled anti-mouse TNF-α mAb, PE-labeled anti-mouse granzyme-B mAb and PE-labeled anti-mouse perforin mAb (eBioscience); FITC-labeled anti-mouse CD8 mAb and PE-labeled anti-mouse NK1.1 mAb (BioLegend).

3.3 Cell line culture

The mouse T lymphoma cell line (YAC-1) was a kind gift from Dr. A. Wongkajornsilp (Mahidol University, Bangkok,Thailand). YAC-1 cells were cultured in RPMI 1640 supplemented with 10% FBS and antibiotics (100 µg/mL streptomycin and penicillin G 100 IU/mL) at 37°C in a humidified atmosphere with 5% CO_2.

3.4 Generation of CIK cells

CIK cells were generated as previously described1 with slight modifications. Briefly, single cell suspensions were prepared from the thymus (3 mice per experiment). The cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 100 µg/mL

streptomycin, 100 IU/mL penicillin G and 50 μM 2-mercaptoethanol in the presence of 1000 IU/mL mouse rIFN-γ at 37°C in a humidified atmosphere with 5% CO_2 for 24 h. Next, the cells were transferred to a dish pre-coated with 50 ng/mL anti-mouse CD3e mAb, and cultured with 300 IU/mL mouse rIL-2 in the presence or absence of 2 μg/mL anti-mouse CD28 mAb. The concentration of anti-CD28 mAb was chosen based on previous reports (19). These cells were maintained at a density of 1×106 cells/mL, and medium (supplemented with 50 IU/mL mouse rIL-2) renewal was done every 2 to 3 days to ensure availability of nutrients for cell cell culture. After 3 weeks of culture, the cells were harvested and monitored by trypan blue staining. Moreover, thymocytes at day 0 were used as control cells (Figure 1).

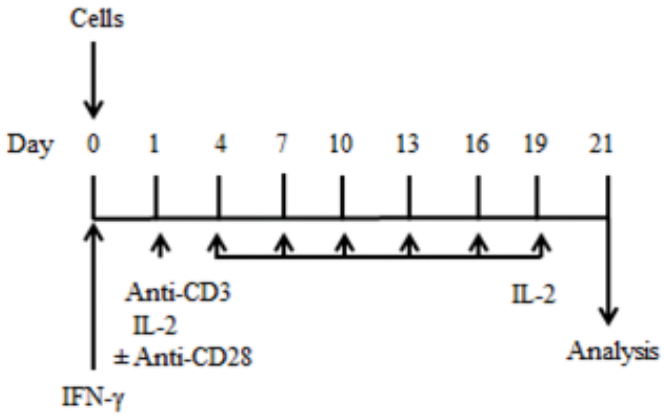

Figure1. Schematic protocol for generation of thymocytes-derived murine CIK cells. Thymocytes from C57BL/6 mice were suspended at a density of $1x10^6$ cells/ml in a complete RPMI culture medium supplemented with 1000 U/mL recombinant mouse IFN-γ on the first day of culture and incubated at 37°C in a 5% CO_2 incubator. After 24 hours, cells were transferred to dish coated with 50 ng/mL of anti-CD3 MAb and 300 IU/mL recombinant mouse IL-2 was added. Every 3 days, complete RPMI was added supplemented with IL-2 (50 IU/mL). On day 21, cells were collected and used for further assays.

3.5 Flow cytometry analysis

The phenotypes of cells were determined by staining for both cell surface and intracellular markers on day 21. For cell surface markers, FITC-conjugated antibodies against CD8 or Thy1.2, and PE-conjugated antibody against NK1.1 were used. Briefly,one million cells (Control cells, or CIK cells) were washed once with phosphate buffered saline (PBS) and re-suspended in 100 μL PBS containing 1% FBS (FACS buffer). Then, the cell suspension was incubated with specific antibodies for 30 min in the dark on ice or at 4°C,

washed twice with PBS and re-suspended in 100 µL FACS buffer. Analysis was performed using a flow cytometer (FACSCalibur, BD Biosciences). Data were analyzed using Flowing Software 2.5 (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland).

For intracellular cytokine (IFN-γ and TNF-α) staining, cells were stimulated for 24 h with 50 ng/mL PMA and 500 ng/mL ionomycin. Brefeldin A solution at final concentration of 10 µg/mL was added 4-6 hour prior to harvesting the cells. Subsequently, the harvested cells were monitored for cell death by trypan blue staining. The cells were fixed and permeabilized with BD Cytofix/Cytoperm according to the manufacturer's instructions, and then stained with PE-conjugated mAb against IFN-γ or TNF-α. For the intracellular cytolytic granule (granzyme B and perforin) stainings, the cells were only permeabilized using BD Cytofix/Cytoperm. These cells were then stained with PE-conjugated mAb against granzyme B or perforin. Cells were finally washed with PBS, resuspended in FACS buffer and immediately analyzed by flow cytometry. In parallel, thymocytes (day 0) stimulated with PMA/Ionomycin (24 hours) were similarly stained and analyzed (Control).

3.6 Assessment of cytotoxicity of CIK cells

The method for assessment of the cytotoxicity of CIK cells was adapted from Wongkajornsilp *et al*., 2013. Briefly, the tumor-killing ability of control cells (thymocytes cultured in media only) and CIK (day 21) effector cells was assessed against YAC-1 target cells. YAC-1 cells (2×104 cells/well) prepared in serum-free RPMI were seeded (40 µL/well) in a 96-black well/clear bottom plate, and were co-cultured with the effector cells (40 µL/well) at the desired effector to target (E:T) ratios of 3.125:1, 6.25:1, 12.5:1 and 25:1 for 4 h at 37°C in a humidified atmosphere with 5% CO2. Propidium iodide (PI) solution (20 µL/well of 10µg/mL PI in PBS) was added. After incubation for 1 h, fluorescence intensity was assessed at 482 nm excitation wavelength and 630 nm emission wavelengths using the Spectra Max M5 microplate reader (Molecular Device, Sunnyvale, CA, USA). The % cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = 100\ (Fl_x - Fl_0) / (Fl_{100} - Fl_0)$$

where, Fl_x represents the fluorescence intensity emitted from the wells containing the target cells co-cultured with the effector cells at various E:T ratios (3.125:1, 6.25:1, 12.5:1 and 25:1). Fl_0 represents the fluorescence intensity emitted from the wells containing the target cells only. Fl_{100} represents the fluorescence intensity emitted from the wells containing the target cells incubated with 20 µL/well of 0.04 N isopropanol.

3.7 Asthmatic mouse model

Mice were arbitrarily divided into four groups, including normal control (mice were not sensitized); Asthma group (mice were sensitized with ovalbumin), asthma + CIK cell group (asthmatic mice treated with CIK cells) and asthma + dexamethasone (Dex) group (asthmatic mice treated with Dex). As shown in Fig 2, asthmatic group mice and other treatment groups were sensitized with an intraperitoneal (i.p.) injection of 0.3 milliliter (mL) phosphate buffer saline (PBS) containing 50 microgram (μg) ovalbumin (OVA; Sigma-Aldrich, USA) and 25 μg Inject Alum (Thermo Scientific, USA) on days 0 and 12. On days 22, 26 and 30, asthmatic group mice and other treatment groups were challenged with OVA aerosol (2% (w/v) in saline) using a nebulizer (APEX, Taiwan) for 30 minutes (min). Mice in normal control were both sensitized and challenged with PBS. In asthmatic group mice treated with CIK cells, CIK cells ($1x10^7$ CIK cells in 300 microliter (μL) of PBS) were transferred via their tail vein on day 21 while in asthmatic group mice treated with Dex, 2mg/kg/day dexamethasone was given i.p. on day 21 to 30. Mice were then evaluated within 24 h after last challenge (Figure 2).

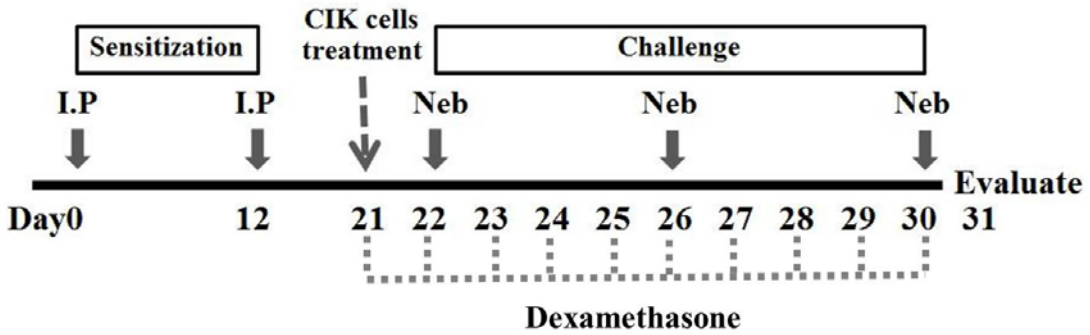

Figure2. Diagram of the ovalbumin-induced allergic asthma and CIK cells transfer strategy. C57BL/6 mice were sensitized with an intraperitoneal (i.p.) injection of 50 μg ovalbumin and 25 μg Alum on days 0 and 12. On days 22, 26 and 30, mice were challenged with OVA aerosol (2% (w/v) in saline) for 30 min. In treatment group, CIK cells ($1x10^7$ cells) were transferred via tail vein on day 21 while in asthmatic group mice treated with 2mg/kg/day dexamethasone was given i.p. on day 21 to 30. Mice were then evaluated within 24 h after last challenge.

3.8 Airway inflammation measurements

Twenty four hours after the final exposure to OVA challenge, mice were anesthetized. Lungs were lavaged with 1 mL PBS for three times and cells in the Broncho alveolar Lavage Fluid (BALF) were counted using hematocytometer. The differential cell counts are prepared by using Cytospin (Thermo Fisher Scientific, USA). Slide preparations were stained with methylene blue and eosin, and BAL cell differential percentages were determined based on light microscopic evaluation (>300 cells/slide).

3.9 Th2 cytokines and IgE levels measurements in serum

On day 31 of the asthmatic mouse model, mice were anesthetized to collect the blood. Then, blood was centrifuged, and aliquots of the serum stored at –80° Celsius until analyzed by ELISA. The concentrations of IL-4 (R&D Systems, USA), IL-5 (eBioscience, USA), IL- 13 (eBioscience, USA) and OVA specific IgE (BioLegend, CA) were measured according to the procedures recommended by the manufacturer. The minimum detectable concentrations were 2 picogram (pg)/mL for IL-4, 3.3 pg/mL for IL-5, 2.8 pg/mL for IL-13 and 20.7 pg/mL for IgE.

3.10 Lung histological analysis

Following bronchoalveolar lavage, the left lung was removed and fixed with 4% paraformaldehyde, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Semiquantitative scoring systems were used to grade the extent of lung inflammation and goblet cell hyperplasia. Briefly, to determine the severity of inflammatory cell infiltration, cell counts were performed blindly based on a five-point scoring system: 0, no cell; 1, a few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells layer deep; and 4, a ring of inflammatory cells >4 cells layer deep. To determine the extent of mucus production, quantified goblet cell hyperplasia in the airway epithelium using a five-point grading system: 0, no goblet cells; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Scoring of inflammatory cells and goblet cells was performed in at least 5 different fields for each lung section and mean score was calculated from 3 animals per group.

3.11 Statistical analysis

Data were analyzed using Graphpad Prism 5 statistics program. Statistical analysis was performed using Student’s t-tests with error bars representing the mean ± standard error of mean (SEM). Differences between means were considered significant when $P < 0.05$

CHAPTER 4
RESULTS

4.1 CD28 costimulation effectively increases total cell numbers

We additionally administered anti-CD28 mAb in the well-established CIK cell culture conditions and analyzed the viability and proliferation of the cultured cells at the indicated times. As such, cultures at day 21 demonstrated viability more than 90% which is in agreement with that observed previously (37). In comparison to the medium alone condition, the standard CIK cell culture conditions allowed cells to expand significantly at all-time points tested (7, 14 and 21 days) after the start of the culture. Furthermore, cells cultured under the CIK cell conditions in which anti-CD28 mAb had been included showed markedly higher cell expansion than without the addition of anti-CD28 mAb (Figure 3A). Finally, by day 21, cultures reached a mean peak of 2-fold expansion in the group receiving both antibodies,and this expansion was significantly higher as compared with the group receiving anti-CD3 mAb alone (Figure 3B). Moreover, addition of anti-CD28 mAb at day 1 showed pronounced cell expansion when compared with those added at other time points (data not shown). Thus, we could affirm that earlier addition of anti-CD28 mAb to the well-established CIK cell culture condition enhances cell proliferation. Hence, further characterization of the cells was carried out using the cells obtained after 21 days of culture.

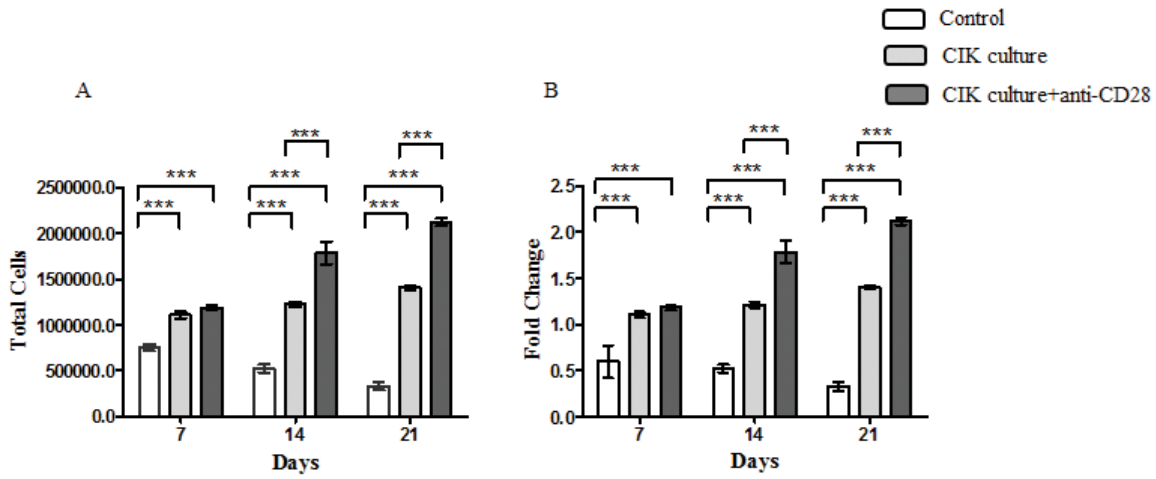

Figure3. Increase in total cell numbers by the addition of anti-CD28 mAb to the CIK cell culture. A) Total cell numbers and B) fold change in total cells from thymocytes cultured in medium only (Control), standard CIK cell culture conditions (CIK culture), and the CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) on days 7, 14

and 21. Data shown are the mean absolute number of cells ± SEM from three separate experiments (*** $p < 0.001$).

4.2 CD28 costimulation increases the percentage of CIK cells in culture

As murine CIK cells are positive for Thy1.2 and NK1.1, we identified the double positive population as CIK cells by flow cytometry. In the freshly isolated thymus, the majority of cells were T cells (99.86 ± 0.03% $Thy1.2^+$ $NK1.1^-$ cells) whereas double positive cells were rarely found (0.11 ± 0.02% $Thy1.2^+$ $NK1.1^+$). As expected, CIK cells expanded significantly underthe CIK cell culture conditions (24.32 ± 1.85%). With the addition of anti-CD28 mAb, a higher proportion of CIK cells were obtained (40.60 ± 2.87%) (Figure 4A). Previous reports, found that the majority of the CIK cell population ($Thy1.2^+$ $NK1.1^+$) is $CD8^+$. The addition of anti-CD28 mAb resulted in a higher proportion of $CD8^+$ $NK1.1^+$ cells (32.13 ± 0.53%) when compared with the CIK culture conditions without anti-CD28 mAb (16.70 ± 1.08%) (Figure 4B). Morphologically, the expanded CIK cells (day 21) growing in aggregates were larger in size and more irregular in shape when compared with control cells or fresh thymocytes (data not shown).

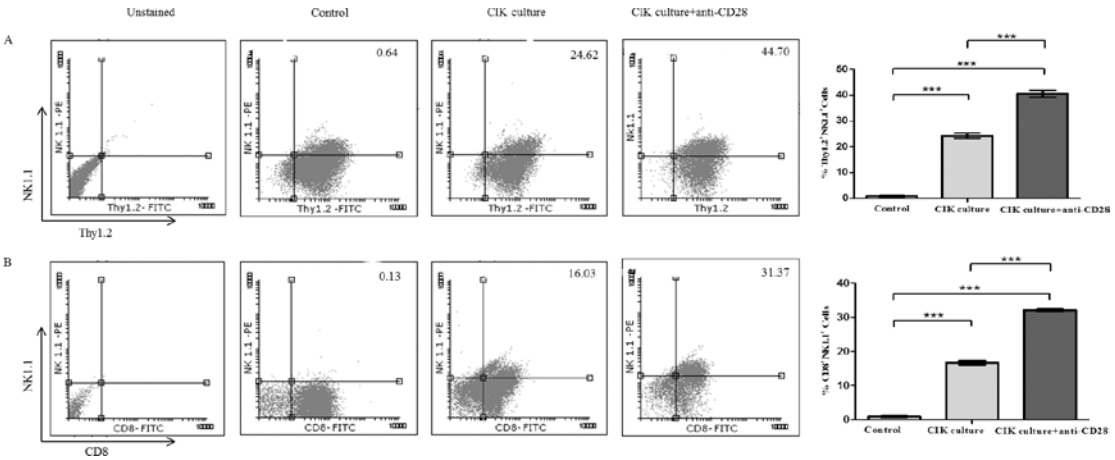

Figure4. Increase in the major effector population of CIK cells by the addition of anti-CD28 mAb to the CIK cell culture. Flow cytometry analysis of thymocytes cultured in medium only (Control) and CIK cells (day 21) cultured under the standard CIK cell culture conditions (CIK culture), and CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) were stained with A) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-NK1.1, and B) FITC-conjugated anti-CD8 and PE-conjugated anti-NK1.1. Unstained cells were used to define the negative populations. Dot plots showing the percentage of $Thy1.2^+$ $NK1.1^+$ and $CD8^+$ $NK1.1^+$ cells are representative of three experiments. The histogram shows the mean absolute percentages of $Thy1.2^+$ $NK1.1^+$ and

$CD8^+$ $NK1.1^+$ cells ± SEM, respectively, from three independent experiments. Statistical significance was determined using one-way *ANOVA* to compare between multiple groups (*** $p < 0.001$).

4.3 CD28 costimulation can potentiate cytotoxicity of CIK cells against tumor cells

As our previous results demonstrated that addition of anti-CD28 mAb to the standard CIK culture conditions enhances the proliferation of CIK cells, we next examined whether thus potentiated the CIK cell cytotoxicity against tumor cells. YAC-1 cells, a murine lymphoma cell line, were used as target cells to check CIK cell cytotoxicity. As expected,control cells had no cytolytic activity, but thymocytes grown in the CIK culture conditions showed significantly higher cytotoxicity against YAC-1 cells at all the effector-to-target (E:T) ratios tested (3.125:1, 6.25:1, 12.5:1 and 25:1). Notably, CD28 costimulation significantly augmented the tumor cytotoxicity of CIK cells (Figure 5).

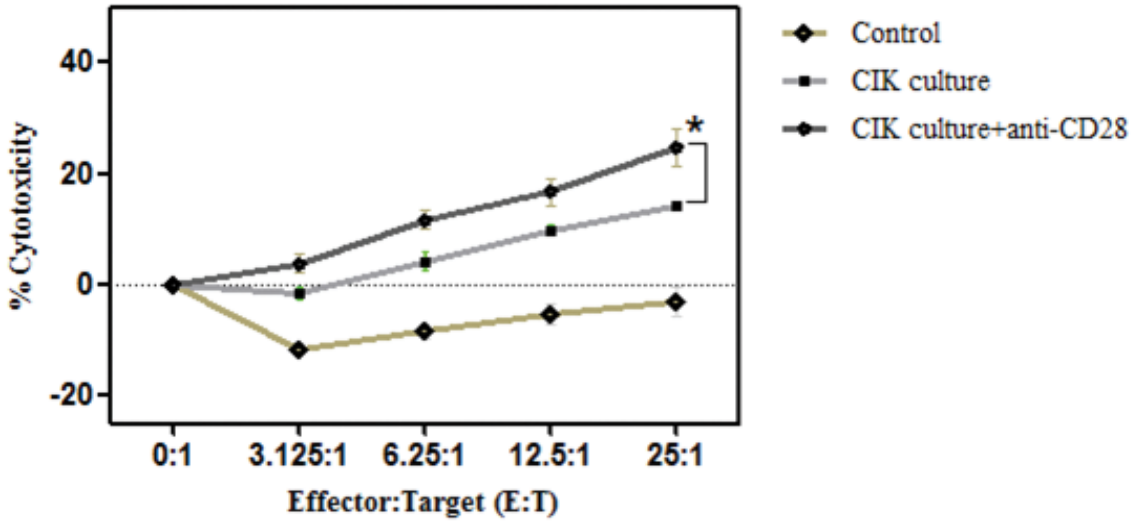

Figure5. Enhancement of CIK cell cytotoxicity against YAC-1 cells by addition of anti-CD28 mAb to the CIK cell culture. Cytotoxicity of thymocytes cultured in medium only (Control), CIK cells (day 21) cultured under the standard conditions (CIK culture), and CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) were assessed against YAC-1 cells at different effector-to-target ratios, ranging from 3.125:1 to 25:1. Data are the mean ± SEM of three independent experiments (* $p < 0.05$).

4.4 CD28 costimulation increases the production of TH1 cytokines and cytolytic granules

CIK cells are known for their ability to abundantly produce the Th1 cytokines IFN-γ and TNF-α (38). Therefore, we examined whether the potentiation of CIK cell cytotoxicity against tumor cells could be attributed for the upregulation of cytotoxic granules and cytokines. Hence, the production of these cytokines by CIK cells was measured using flow cytometry. Thymocytes were expanded under the conditions described above, and harvested at day 21. The harvested cells were re-stimulated *in vitro* with PMA and ionomycin for 24 h. The viability of re-stimulated cells was more than 90% as checked by trypan blue dye staining (Table 1). Subsequent flow cytometry analysis revealed that the number of cells producing cytokinesunder standard CIK cell culture conditions was increased significantly when compared control cells (IFN-γ: 23.40 ± 1.40% vs. 4.36 ± 2.44%, respectively, and TNF-α: 25.81 ± 6.88% vs. 4.82 ± 2.59%, respectively). Notably, the addition of anti-CD28 to the CIK culture conditions tended to increase the number of cells producing both cytokines (IFN-γ: 26.09 ± 1.40%) and TNF-α (34.3 ± 5.88%) (Figure 6A and 6B).

Along with the production of Th1 cytokines, CIK cells also release cytolytic granules (39). We next addressed whether the addition of anti-CD28 mAb to the CIK culture condition enhances the production of cytolytic granules, as defined by the number of granzyme B^+ and $perforin^+$ cells by flow cytometry analysis. We confirmed that the CIK cells culturedunder the standard CIK cell culture conditions had a significantly increased proportion of cells expressing cytolyticgranules (granzyme B and perforin) when compared with thymocytes cultured in media only (granzyme B: 20.50 ± 1.99% vs. 2.02 ± 1.63%, respectively, and perforin: 18.88 ± 6.17 % vs. 3.72 ± 2.60%, respectively). Additionally, the inclusion of anti-CD28 mAb in the CIK culture conditions further enhanced the number of cells producing these granules (granzyme B: 24.38 ± 0.86% and perforin: 25.55 ± 8.64%) (Figure 6C and 6D).

Culture Conditions	Percentage of live cells (mean ± SD)	
	Unstimulated	PMA/I stimulated (24 hours)
Thymocytes (day 0)	99 ± 0.82	95.6 ± 2.83
CIK (day 21)	95 ± 2.45	93.6 ± 2.17
CIK + anti-CD28 (day 21)	97 ± 1.63	94.7 ± 2.05

SD, Standard Deviations; PMA, Phorbol 12-myristate 13-acetate; I, Ionomycin

Table1 Cell viability of control thymocytes, CIK culture, and CIK culture + anti-CD28

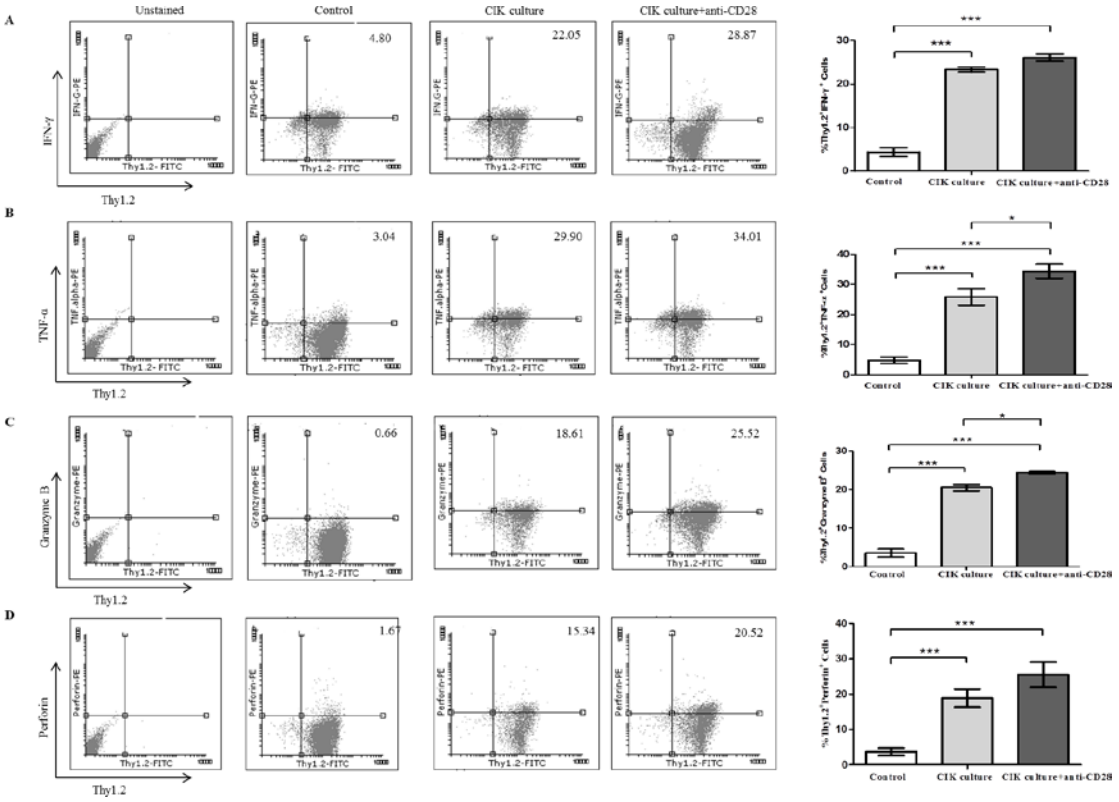

Figure6. Increase in the major effector population of CIK cells producing T_H1 cytokines and cytolytic granules by the addition of anti-CD28 mAb to the CIK cell culture. Thymocytes cultured in medium only (Control) and CIK cells (day 21) cultured under the standard CIK cell culture conditions (CIK culture), and the CIK cell culture conditions with the addition of anti-CD28 mAb (CIK culture + anti-CD28) were stained for intracellular cytokines, A) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-IFN-γ, B) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-TNF-α and for intracellular cytolytic granules, C) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-granzyme B and D) FITC-conjugated anti-Thy1.2 and PE-conjugated anti-perforin. Unstained cells were used to define the negative populations. Dot plots along with the percentage in A, B, C and D are representatives of three experiments for intracellular staining to determine the expression of the indicated cytokines and cytolytic granules. The histogram shows the mean absolute percentages of $Thy1.2^+$ IFN-γ^+, $Thy1.2^+$ TNF-α^+, $Thy1.2^+$ granzyme B^+ and $Thy1.2^+$ perforin+ cells ± SEM, respectively, from three independent experiments. Statistical significance was determined using one-way *ANOVA* (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

4.5 CIK cells could effectively reduce eosinophilic inflammation in the asthmatic lungs

To study the effectiveness of applying CIK cells to treat asthmatic mice, broncho alveolar lavage fluid (BALF) was collected from different groups of mice for total cell count. The total cell count was significantly raised by 15-fold in the mice with OVA challenge (OVA/PBS) compared to normal group of mice (PBS/PBS). Treatment of these asthmatic mice with CIK cells (OVA/CIK) or dexamethasone (OVA/Dex) significantly reduced the total cell counts in BAL fluid to the level below 50% (Figure 7). The differential cell counts are prepared by Cytospin and determine the specific inflammatory cell populations including monocytes, lymphocytes and eosinophils. The proportion and number of monocytes are significantly increased in the treatment group. In contrast, the total lymphocytes and eosinophils are significantly decreased in the treatment group (Figure 8-10). Interestingly, there are no differences in the BALF cell count, monocytes, lymphocytes and eosinophil numbers between mice treated with dexamethasone and CIK cells (Figure 7-10). Taken together, these results indicate that CIK cells can relieve airway eosinophilic inflammation induced by ovalbumin sensitization and challenge.

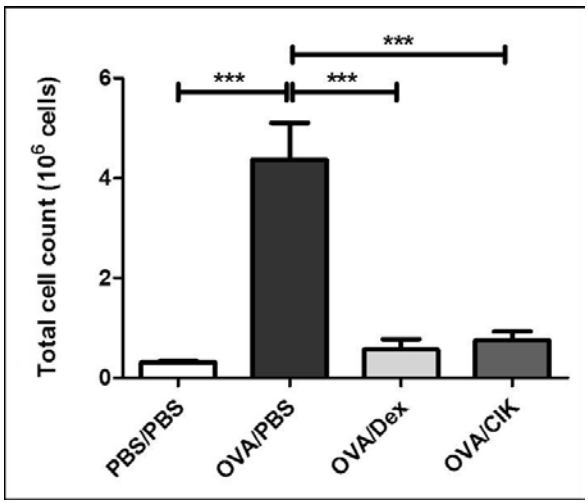

Figure7. Total cell counts were determined in BAL fluid.

Mice were sensitized and challenged with OVA and treated with PBS (OVA/PBS), dexamethasone (OVA/Dex) or CIK cells (OVA/CIK). Data are means ± SEM of 3 independent experiments including 6-8 mice/group are shown (*** $p < 0.001$).

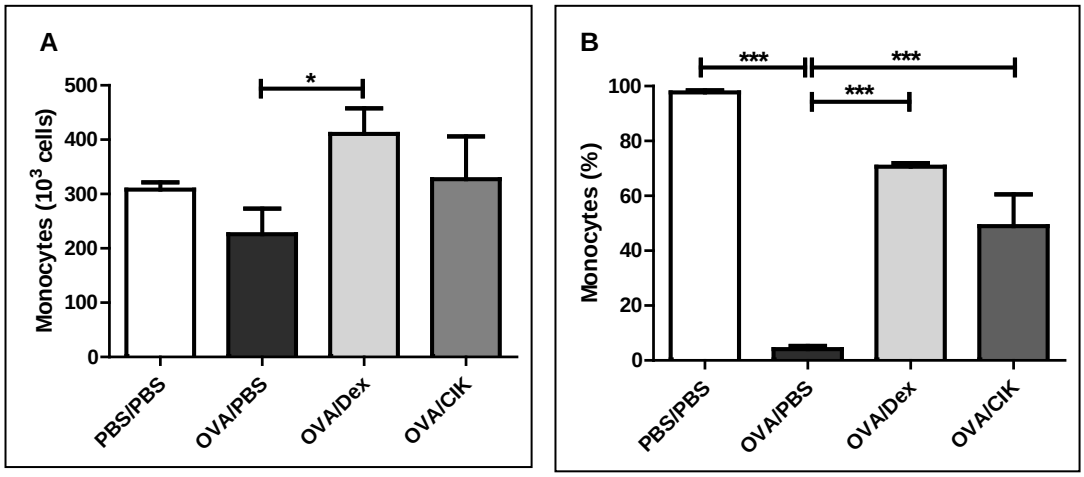

Figure8. The total numbers (A) and percentage (B) of monocytes were determined in BAL fluid. Mice were sensitized and challenged with OVA and treated with PBS (OVA/PBS), dexamethasone (OVA/Dex) or CIK cells (OVA/CIK). Data are means ± SEM of 3 independent experiments including 6-8 mice per group are shown (*** p <0.001).

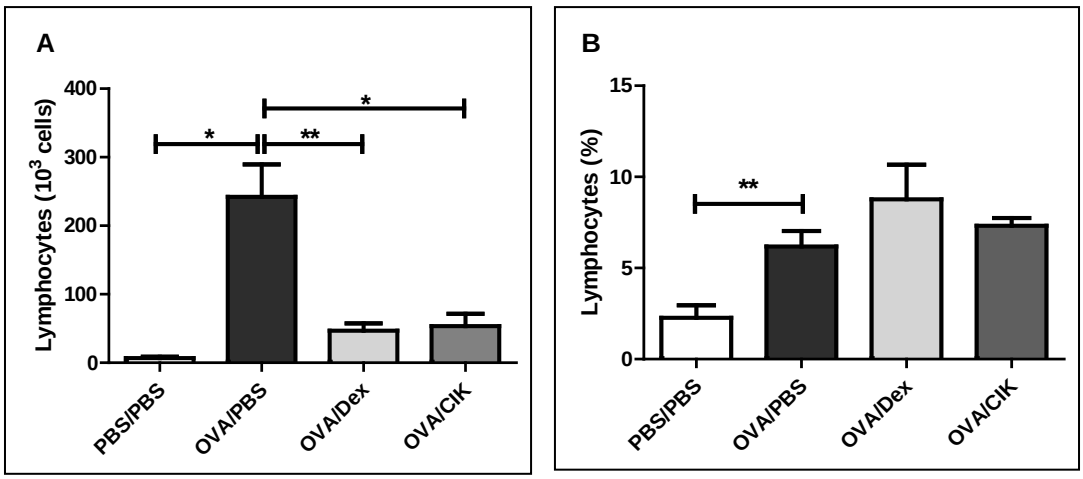

Figure9. The total numbers (A) and percentage (B) of lymphocytes were determined in BAL fluid. Mice were sensitized and challenged with OVA and treated with PBS (OVA/PBS), dexamethasone (OVA/Dex) or CIK cells (OVA/CIK). Data are means ± SEM of 3 independent experiments including 6-8 mice per group are shown (** p < 0.005).

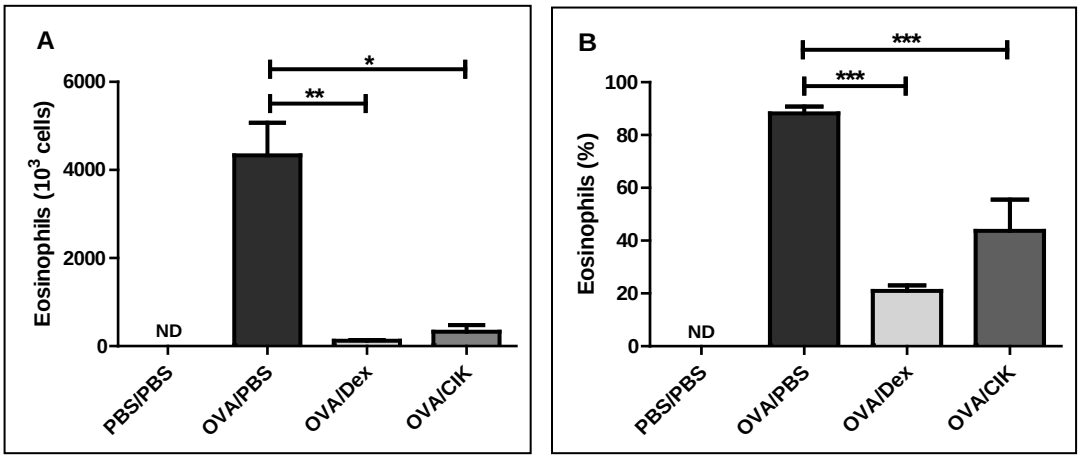

Figure10. The total numbers (A) and percentage (B) of eosinophils were determined in BAL fluid. Mice were sensitized and challenged with OVA and treated with PBS (OVA/PBS), dexamethasone (OVA/Dex) or CIK cells (OVA/CIK). Data are means ± SEM of 3 independent experiments including 6-8 mice per group are shown. ND: not detected (*** $p <$ 0.001).

4.6 Th2-based activity was suppressed in asthmatic mice treated with CIK cells

Next, to further examine therapeutic effects of CIK cells, blood was collected after final OVA-challenge and Th2 cytokines production, namely IL-4, IL-5 and IL-13 were detected by ELISA. IL-5 and IL-13 levels in the mice sensitize/challenge with OVA were significantly increased when compared to the normal mice. Treatment with CIK cells significantly lowered the productions of these cytokines (Figure 11). Moreover, the effects shown by the CIK treatment was similar to dexamethasone treatment. However, there were no changes in the levels of IL-4 and OVA-specific in all groups of mice.

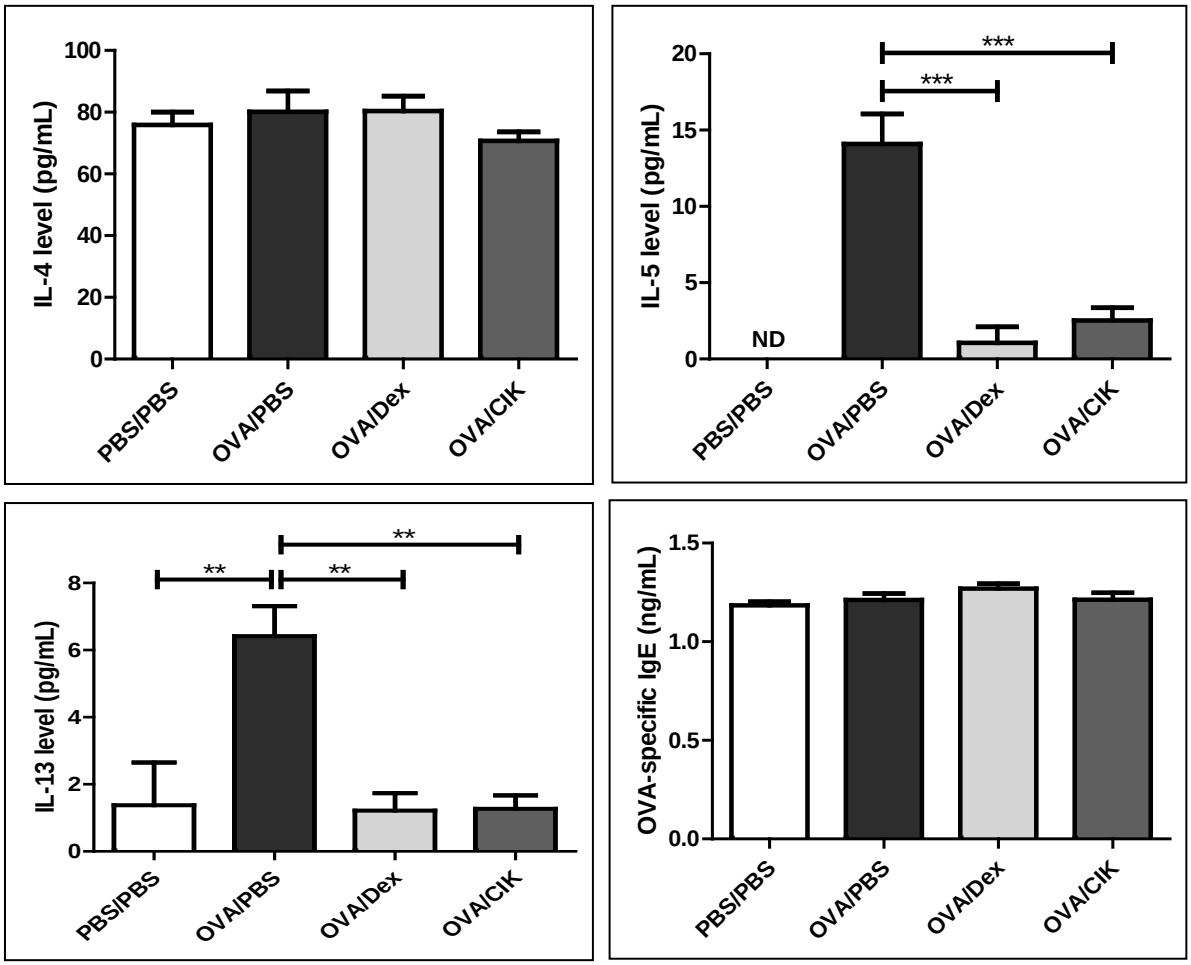

Figure11. The levels of IL-4, IL-5, IL-13 and OVA-specific IgE in serum were measured in normal mice group (PBS/PBS), asthmatic mice treated with PBS (OVA/PBS), dexamethasone (OVA/Dex) or CIK cells (OVA/CIK). The results shown are from 3 independent experiments and each bar represents mean ± SEM of 4-8 mice per group.

4.7 Treatment with CIK cells inhibited inflammatory cell infiltration and goblet cell hyperplasia in the asthmatic lungs

As asthmatic mice show increase in the infiltration of inflammatory cells and mucus producing goblet cells, we examined whether CIK cells could reduce these pathologies. Compared to the normal mice, the asthmatic mice exhibited an increase in both inflammatory cell infiltration and mucus-producing goblet cells in the lungs. Whereas, CIK cell treated-asthmatic mice exhibited less infiltration and goblet cells hyperplasia (Figure 12-13). The observations were confirmed by semi quantitative scoring of the inflammatory cells infiltration and PAS positive cells showing significant reduction in the airway inflammation and goblet cell hyperplasia in asthmatic mice treated with CIK cells, as compared to diseased

animals without treatment (Figure 14). Again, CIK cell treatment significantly reduced the cell infiltration and mucus-producing goblet cells in similar fashion to dexamethasone treatment.

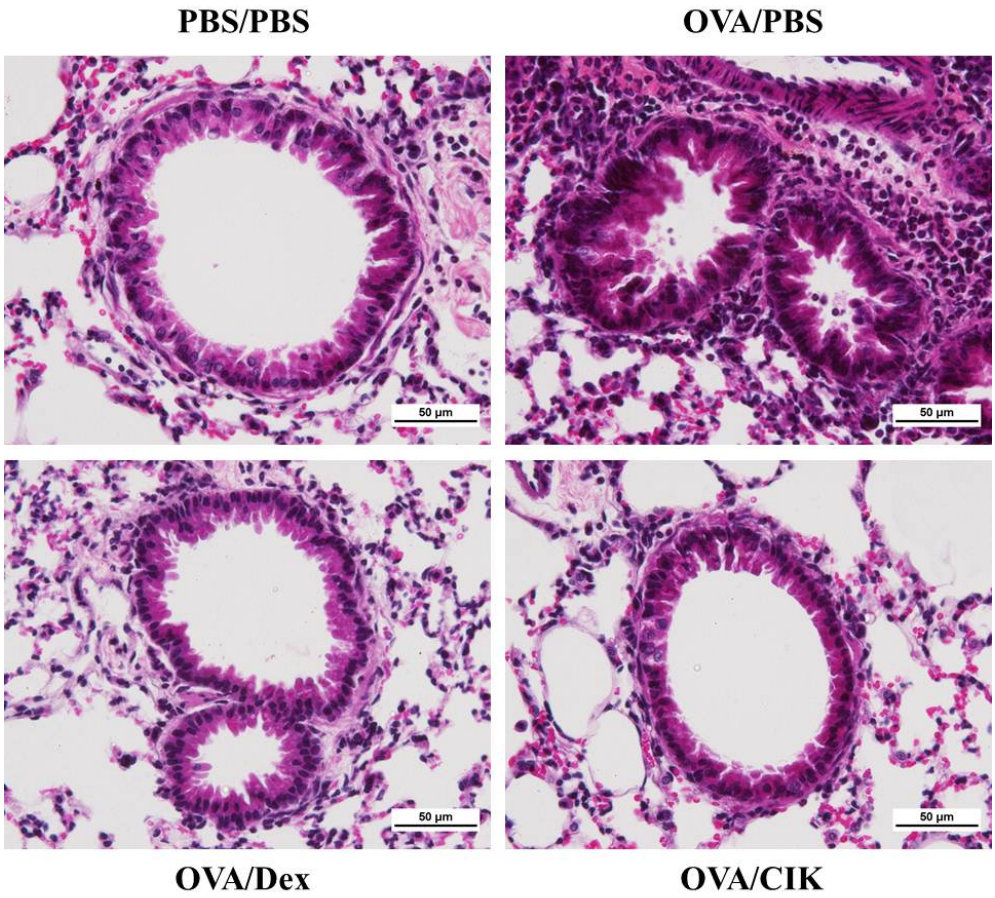

Figure12. Representative histological images of lung sections for the assessment of cellular inflammation (haematoxylin and eosin staining) from normal mice group (PBS/PBS), asthmatic mice treated with PBS (OVA/PBS), dexamethasone (OVA/Dex) or CIK cells (OVA/CIK.)

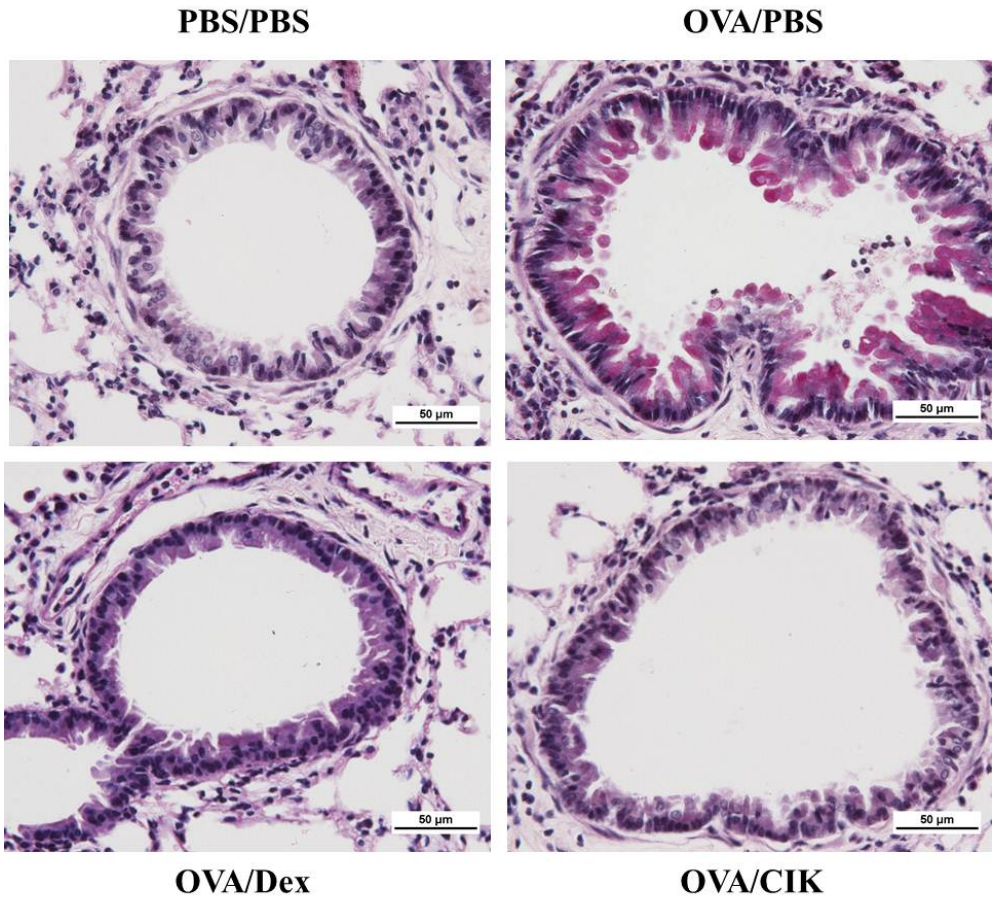

Figure13. Representative histological images of lung sections stained with periodic acid-Schiff (PAS) for the quantification of goblet cells from normal mice group (PBS/PBS), asthmatic mice treated with PBS (OVA/PBS), dexamethasone (OVA/Dex) or CIK cells (OVA/CIK).

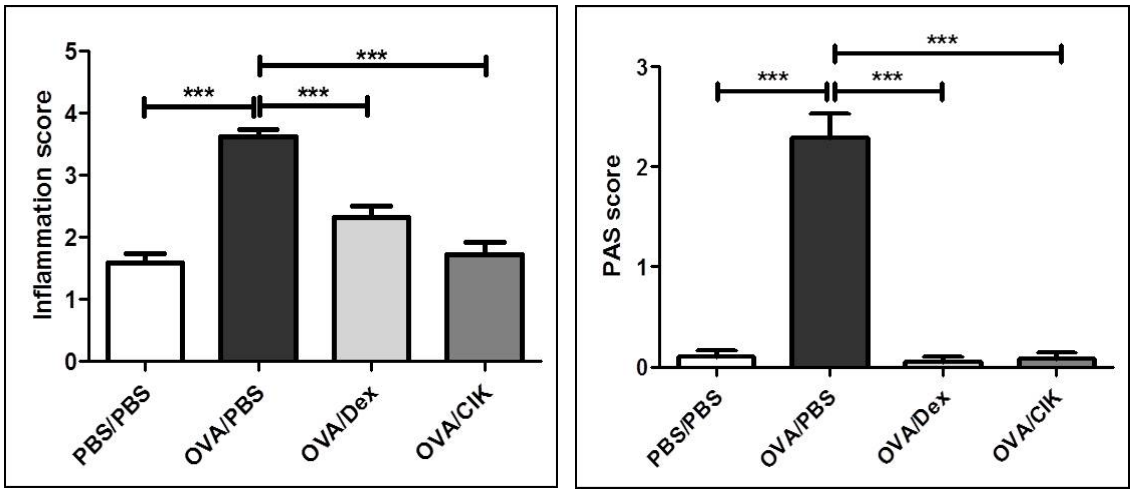

Figure14. Bar graph represents semi quantitative scoring of inflammatory cell infiltration (left) and PAS positive cells (right) (mean ± SEM). Scores were obtained from 3 animals/group (*** $p < 0.0001$

CHAPTER 5
DISCUSSION

Over the past several years, CIK cells have been recognized as the primary candidate for adoptive immunotherapy. Their dual Th1 and NK properties mediating effective tumor killing activity and resistance to Fas-mediated apoptosis promoted these CIK cells as a potential immunotherapeutic approach (12, 40, 41). Peviously, there are numerous research groups proving the efficacy and safety of CIK cells through clinical studies (2, 7, 41). Moreover, the functional properties of CIK cells, especially the production of IFN-γ, TNF-α, perforin and granzyme B, have been used to study the antiviral activity of human CIK cells against human immunodeficiency virus and Epstein–Barr virus (42). At present, there are a wide variety of mouse-based disease models that mimic human pathologies (1, 6). These mouse models can broaden the application of CIK cell therapy to diseases other than cancers, such as allergic asthma. Therefore, optimal generation of murine CIK cells and their use in preclinical studies will be highly beneficial when exploring other applications of CIK cells. Considering that the most potent CIK population consists of $CD8^+$ NKT-like cells, which are believed to be differentiated from T cells (11), we cultured T cells from mouse thymus to generate CIK cells.

The standard protocol to generate and expand human CIK cells is the incubation of PBMC in medium containing IFN-γ for 1 day, followed by stimulation with CD3 mAb and IL-2 for 21 days (43). Immobilization with CD3 mAb is used to trigger T cell proliferation (44). The addition of IFN-γ before IL-2 increases cellular cytotoxicity. Similarly, IL-2 is required as absence of this cytokine not only limits T cell expansion, but also results in the death of cells in culture (1). Adopting the well-established protocol for culturing human CIK cells to obtain murine CIK cells defined as $Thy1.2^+$ $NK1.1^+$ cells, we could expand this rarely found population from the thymus as many as 24-fold. As this well-established CIK cell generation method lacks a costimulatory signal, we examined whether the incorporation of this signal could increase the expansion of CIK cells and enhance their functional properties. We found that addition of anti-CD28 mAb to the culture increases the CIK cell population up to 40-fold. Furthermore, it has also been reported that CIK cells are $CD8^+$ cells (9). and we confirmed that CD8+ $NK1.1^+$ CIK cells were expanded up to16-fold in culture.Moreover, the addition of anti-CD28 mAb to CIK culture conditions increased this $CD8^+$ $NK1.1^+$ population even higherto 32-fold. Our results suggest that CD28 signaling acts synergistically with other stimuli. The reason behind enhanced proliferation of CIK cells mediated by CD28 signaling needs to be explored. Since, CD28 signaling was shown to

enhance T cell survival by enhancing the anti-apoptotic factor Bcl-2 (45); whether this holds true for CIK cells needs to be justified. Moreover, addition of CD28 at times other than day 1 decreased the number of CIK cells, and the anti-proliferative effect of CD28 signaling at later time needs to be elucidated.

In addition to gaining higher numbers of CIK cells, it is also important to generate CIK cells that have potent cytotoxicity against tumor cells. To assess this we determined the *in vitro* cytotoxic activity of murine CIK cells towards YAC-1 cells. As expected, the additional CD28 costimulatory signal also enhanced the tumor killing activity of CIK cells. The antitumor cytotoxicity of CIK cells is determined by their ability to produce Th1 cytokines (IFN-γ and TNF-α) and cytolytic granules (perforin and granzyme B). Furthermore, cytolytic granules are reported to be important both *in vitro* and *in vivo* for the anticancer activity of CIK cells (13, 46). Therefore, we further tested whether enhanced antitumor cytotoxicity of CIK cells generated in presence of anti-CD28 mAb is due to an increased production of cytolytic mediator.Our results demonstrated that the additional CD28 costimulation augmented the production of all these mediators(IFN-γ, TNF-α, perforin and granzyme B) compared with those in absence of CD28 signaling. Thus, the inclusion of anti-CD28 mAb to the culture not only increased CIK differentiation and proliferation, but also potentiated the function of CIK cells.

Nevertheless, cytotoxicity of CIK cells has been improved by eliminating the suppressive factors like removing or downregulating regulatory T (Treg) cells (47). Different strategies like addition of IL-15 (48), or IL-6 (49) to the CIK cell culture were shown to inhibit Treg cells which subsequently enhanced the cytotoxicity. Moreover, the observation that CD28 costimulation suppresses induction of Treg cells from naïve precursors (50) might be responsible for the enhanced cytotoxicity of the CIK cells cultured in presence of anti-CD28 mAb; however, further studies is warranted.

Allergic asthma is characterized by an increase in total number of inflammatory cells in BALF, goblet cells hyperplasia, high mucus production and high numbers of eosinophils infiltrating into the lungs (51-53). Corticosteroids are a standard therapy for allergic asthma, but their long term use is associated with several side effects and complications. This highlights the alternative strategy for treatment of allergic asthma. Previously, allergen specific Th1 lymphocytes have been found to suppress allergic airway inflammation in IFN-γ dependent manner (27, 54).

In this study, CIK cells were infused intravenously to treat allergic asthma in mice. The number of total cells, monocytes, lymphocytes and eosinophils were measured in the BALF of normal mice, asthmatic mice without treatment, or those treated with either CIK cells or dexamethasone. The result showed that CIK cells treatment significantly decreased numbers of

total inflammatory cells and eosinophils in BALF of asthmatic mice. This could be attributed to the secretion of IFN-γ by CIK cells. Previously, IFN-γ was shown to modulate activation, differentiation and recruitment of eosinophils (55, 56). In particular, productions of IFN-γ suppressed the expression of eotaxin receptor (CCR3) which was shown to be an important inducer of eosinophil differentiation (55). Moreover, OVA-sensitized mice were treated with recombinant IFN-γ, and subsequently challenged with aerosolized OVA exhibited reduced eosinophil infiltration into the airway

Allergic asthma is driven by Th2 cytokines (IL-4, IL-5 and IL-13), and Th2 cells also control the regulation of B cell class-switching to IgE (57), (58). So, we measured serum Th2 cytokines (IL-4, IL-5 and IL-13) and IgE levels after CIK cells treatment and found reduced production of IL-5 and IL-13. Our result suggests that CIK cell attenuate IL-5 or IL-13 pathways. Also, serum IL-5 and IL-13 are the best predictors for the blood eosinophilia phenotypes in asthma (59). However, we did not find any difference in the amount of IL-4 production between the groups. Given the information that IL-4 was identified as B cells growth factor and reported to promote IgE synthesis by B cells (60), IL-4 may not play an important role in our asthma model, which corresponds with those reported previously (61) that eosinophils derived IL-13, but not IL-4, was responsible for allergic respiratory inflammation in mice. Also, Rodrigues-Machado, *et al*. (62) reported that IL-4 may not play a significant role in chronic airway inflammation. As our results showed the similar levels of OVA-specific serum IgE in all groups collected after 31 days, this may be due collection timing (63).

Regarding infiltration of inflammatory cells and mucus producing goblet cells in the lungs, asthmatic mice treated with CIK cells showed markedly low inflammation scores and reduction in goblet cell hyperplasia. It was suggested that AHR directly correlated with increased inflammation of the inflammatory cells and mucus production in the lung (64, 65). Although AHR was not examined in this study, such findings suggested that CIK cells treatment can suppress the development of airway inflammation.

In summary, asthmatic mice treated with CIK cells showed significant reductions in all asthmatic parameters; eosinophilic inflammation of the lungs (BALF cellularity), serum Th2 cytokines (IL-5, IL-13) levels, inflammatory cell infiltration around pulmonary arterioles, and mucus secreting cells in the airways. Moreover, the efficacy of CIK cells treatment in OVA–induced asthmatic mice was comparable to dexamethasone. Further studies are required to clearly demonstrate the therapeutic mechanism of CIK cells in the treatment allergic asthma.

CHAPTER 6

CONCLUSION

The CD28 costimulatory signal enhances murine CIK cell differentiation and upregulation of cytotoxic mediator production, leading to greater cytotoxicity against tumor cells. It remains to be delineated how CD28 signaling enhances proliferation and cytotoxicity of the CIK cells. Moreover, whether CIK cell can be used as an adoptive therapy for treatment of viral diseases and other human pathological conditions using disease mouse models is of our interest. Hopefully, CD28 costimulatory signal proved beneficial for generating murine CIK cells from pure T cells. This will in turn help not only for the development of more effective cancer immunotherapy, but also for exploring novel clinical applications of CIK cells in the future.

Regarding application of CIK cells in treatment of allergic asthma, as the standard treatment of allergic asthma pose many serious side effects, we reported for the first time that CIK cells can be used to treat allergic asthma. CIK cells treatment in OVA-induced asthmatic mice significantly reduced the total inflammatory cell count and the eosinophil count in BALF. Th2 cytokines (IL-5 and IL-13) production in serum were also decreased to normal levels. Additionally, histological studies demonstrated that CIK cells potentially inhibited lung inflammatory cell infiltration and goblet cells hyperplasia. Thus, our study provides a first proof of applying CIK cells in the treatment of allergic asthma. Further studies are required to confirm this application in clinical practice. We hope that CIK cells will be an effective immunotherapy for allergic asthma in the future.

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