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Corticosteroids Suppress In Vitro Tonsillar Proliferation in Children with Obstructive Sleep Apnea.

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Running Head : Corticosteroids and tonsil proliferation...

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Abstract

Introduction: Intranasal corticosteroids (CS) are potentially useful interventions for children with OSA, and may reduce lymphadenoid tissue size in the upper airway. We hypothesized that CS would reduce cellular proliferation and production of pro-inflammatory cytokines in a tonsil/adenoid mixed-cell culture system.

Methods: Dissociated tonsils or adenoids harvested intra-operatively from children with polysomnographically-diagnosed OSA were cultured in control medium (CO) or after stimulation with LPS and concanavalin A (STIM), and incubated with dexamethasone (DEXA; 10^{-5}-10^{-7}M), fluticasone (FLU; 10^{-5}-10^{-14}M), and budesonide (BUD; 10^{-4}-10^{-14} M). Proliferation and apoptosis were assessed, and supernatants were assayed for cytokines TNF-α, IL-6, and IL-8.

Results: STIM increased tonsillar and adenoidal proliferation compared to CO (1976 ± 133 cpm vs. 404±69 cpm; $p<0.00001$; n=54). DEX, FLU, and BUD reduced cellular proliferation rates, and exhibited dose-dependent effects, with the potency being FLU>BUD>DEX ($p<0.0001$; n=25/group). Conversely, CS increased cellular apoptosis (n=20/group; $p<0.0001$). Furthermore, TNF-α, IL-8 and IL-6 concentrations in the supernatant were increased by STIM, and markedly reduced by all CS (n=48/group).

Conclusions: Whole tissue cell cultures of adenoids and tonsils provide a useful approach for in vitro assessment of therapeutic efficacy of CS in the management of the lymphadenoid hypertrophy that underlies OSA in children.

KEY WORDS: Obstructive sleep apnea, tonsillar hypertrophy, children, proliferation, T lymphocytes, corticosteroids, inflammation, cytokines

Introduction

Obstructive sleep apnea (OSA) is a common condition in children, and is characterized by increased upper airway resistance, snoring, and partial or complete intermittent obstruction of the upper airway during sleep, leading to episodic oxyghemoglobin desaturations, hypercapnia, and repeated arousals (1). Adenotonsillar hypertrophy is by far the major contributor to the pathophysiology of OSAS in children (2,3), and although a combination of structural and neuromuscular abnormalities contributes to the occurrence of OSA in children, the severity of OSA correlates, albeit loosely, with adenoid and tonsillar size (4-6). Thus, surgical extirpation of these tissues (T&A) is usually the first line of treatment (7).

It has now become apparent that both nasal and oropharyngeal mucosal inflammation are present in children with OSA, and may contribute to increased adenotonsillar proliferation in children with OSA (8). The recently reported increase in the expression of glucocorticoid receptors in upper airway lymphoid tissues would globally predict favorable outcomes when using intranasal corticosteroids in pediatric OSA (9), and indeed intranasal corticosteroids (CS) have shown promising efficacy in reducing the size of the upper airway lymphoid tissues (10-14). However, the mechanisms underlying the favorable effects of topical CS in inducing the involution of hypertrophic adenoids and tonsils remain unclear.

We recently developed a novel method allowing for in vitro cell culture of tonsils and adenoids derived from children undergoing T&A (15). We hypothesized that CS would lead to dose-dependent reductions in cellular proliferation and increased apoptosis in

whole tonsillar and adenoid cell cultures obtained from children with OSA, and that these effects would be associated with decreased production of pro-inflammatory cytokines.

Methods

Subjects

The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained from the legal caregiver of each participant. Consecutive children who underwent tonsillectomy for OSA were identified before surgery and recruited into the study. Overnight polysomnography was performed using standard methods that have been published in detail elsewhere (16) The diagnosis of OSA was defined by the presence of an obstructive apnea-hypopnea index (AHI) ≥ 5 events/ hour of total sleep time in the presence of habitual snoring in otherwise healthy children without any disorders requiring treatment with medications, including inhaled or oral corticosteroids, leukotriene modifiers, antibiotics, or anti-histaminics, or with any known genetic or craniofacial syndromes.

Cell Culture

Surgically removed tonsils and adenoids were placed in ice cold phosphate buffered saline (PBS) plus antibiotics and processing was started within 30 minutes after surgical excision under aseptic conditions. Briefly, tonsils were washed thoroughly with PBS, manually dissected into Petri dishes, and gently grounded with a syringe plunger through a 70 μ mesh screen to obtain a mixed cell suspension through mechanical dissociation. Red blood cells were removed by lysis buffer. Cells viability of all specimens was determined by trypan blue exclusion. Specimens with a viability of less than 70% were discarded. Cells cultures were established in standard medium RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics, which included

streptomycin, fungisone, gentamycin, and penicillin to prevent bacterial and fungal contamination. Mixed cell suspensions were transferred onto 96-round bottom-well plates at a concentration of $1x10^6$ cells/well. Cells were cultured in a 5 % CO_2 incubator at 37°C for 48 hours. Cells were also cultured using 24-well plates to determine pro-inflammatory cytokine levels. Cultures were either maintained as described (CO) or stimulated with 25 µg/ml lipopolysaccharide (LPS) plus 10 µg/ml concanavalin A (conA) to test proliferation under stimulated conditions (STIM). LPS from *Escherichia coli* 055:B5 and Concanavalin A from *Canavalia ensiformis* were purchased from Sigma Chemical Co. (St. Louis, MO).

Corticosteroids were added to the medium 24 hours after plating to achieve final concentrations ranging from 10^{-5} to10^{-7} M for dexamethasone (DEX) and 10^{-5} to 10^{-14} M for fluticasone (FLU) or budesonide (BUD), all of which were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Proliferation Assay:

Cells were incubated for the final 18 to 20 hours with 0.0185 MBq (0.5 µCi) ^{3}H-thymidine in complete medium (Amersham Biosciences, UK). Cells were then harvested onto glass-fiber filters with a cell harvester, and radioactivity was measured in a liquid scintillation counter. All experimental conditions were always performed in triplicate, and ^{3}H-thymidine uptake results were expressed as the average of the 3 wells in counts per minute (cpm).

BrdU Cell Proliferation and Apoptosis Annexin V Assays With Flow Cytometry

To detect T-cell and B-cell specific proliferation, we employed bromodeoxyuridine (BrdU) pulsed proliferation analysis using flow cytometry. All procedures were measured using the APC BrdU flow kit (BD Biosciences, San Diego, CA) as recommended by the manufacturer. In brief, at the end of 48 hours of cell culture in 24-well plates, cells were pulse-labeled with 1 mM BrdU for 4 hours. The cells were then washed with PBS, and BrdU labeled cells were stained with a 3-color antibody combination consisting of mouse anti-human CD45/PerCP Cy7, CD3/PE, and CD19/APC-Cy7 antibodies (BD Biosciences, San Diego, CA) in 50 µl staining buffer for 15 min on ice. Following binding, the cell-surface antibodies, cells were fixed and permeabilized with cytofix/cytoperm buffer. After this procedure, cells were suspended with DNase (300 µg/ml) for 1 hour at 37°C. The anti-BrdU APC antibody was added in perm/wash buffer and incubated for 20 min at room temperature. Isotype controls relevant for each antibodies were used to establish background fluorescence. Negative control was used as a sample which was untreated with BrdU and was not stained with specific fluorescence antibodies. Data were acquired on a FACS Aria flow cytometer using the FACS Diva 5.5 software (BD Biosciences, San Diego, CA). After gating of lymphocytes based on CD45+ cells, T-cell and B-cell numbers were calculated as CD3+/CD19- and CD3-/CD19+ cell populations, respectively. Moreover, proliferation of T-cells and B-cells was identified by counting CD3+/BrdU+ and CD19+/BrdU+ cell populations. A similar approach was undertaken using Annexin V mouse anti-human antibodies to quantify T-cell or B-cell specific apoptosis. The results were displayed as two color dot-plots and

analyzed by FlowJo software (Tree Star, San Carlos, CA). All data are expressed as the percentage of positive cell from the total cell population.

Apoptosis Assay: Cellular apoptosis was quantified using a Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN). This immunoassay specifically detects the histone region (H1, H2A, H2B, H3, and H4) of mono- and oligonucleosomes that are released during apoptosis. Apoptosis was measured in duplicate from each treatment group and expressed as absorbance ratios of the CS-treated cell lysates versus absorbances calculated from controls, the latter arbitrarily set at 1.0. The detection limit for this ELISA is 10^2 apoptotic cells.

Cytokine Assays:

Concentrations of TNF-α, IL-8 and IL-6 were measured using commercially available ELISA kits from the supernatants of either CO or STIM conditions. To determine cytokine production, cells were incubated in 24-well flat-bottom plates in complete RPMI medium supplemented with 10% FBS with or without addition of 25 μg/ml LPS plus 10 μg/ml concanavalin A. Supernatants were collected after 48 hour, and stored at -80ºC until assay. TNF-alpha levels were measured according to manufacturer's instructions, using a high-sensitivity ELISA assay able to detect concentrations as low as 0.09 pg/ml (BioSource Europe S.A., Belgium). IL-8 was evaluated using a commercial ELISA kit(R&D Systems; Minneapolis, MN.) with a detection range between 0 and 2000 pg/ml. To determine IL-6 concentrations was used the IL-6 EASIA assay (BioSource Europe S.A., Belgium). The concentrations of cytokines in the supernatants were normalized to

the number of cells plated, and expressed as pg/10^6 cells. For all assays, calibration curves were performed in duplicate for each experiment.

Statistical Analysis

All data were expressed by mean ±SE, unless stated otherwise. Statistical analyses were performed using SPSS software (version 16.0; SPPS Inc., Chicago, Ill.). For normally distributed data, analysis of variance, followed by *post-hoc* tests were used which included Bonferroni correction. Cytokine data were not normally distributed, and were therefore log-transformed and compared using non parametric tests (Mann Whitney). To determine whether significant differences in potency occurred among the 3 CS, multivariate analyses of variance (MANOVA) with Scheffe post hoc tests were used. All p-values reported are 2-tailed with statistical significance set at <0.05.

Results

Study population. A total of 86 children out of 94 suitable candidates with a clinical and polysomnographic diagnosis of OSA undergoing T&A agreed to participate and completed the study. The demographic characteristics and major overnight polysomnographic findings for the group are shown in Table 1. Of note, due to tissue availability constraints, only a limited number of experimental conditions were possible for every recruited patient, such that the number of subjects included in each experiment varied, and is indicated as appropriate.

Cell Proliferation Assay:

Basal proliferative rates for mixed cell cultures derived from either tonsils or adenoids were significantly higher after stimulation with LPS and conA (Figure 1; basal conditions: 404.2±69.0 cpm vs. STIM: 1976.3±133.1 cpm; n=54; $p<0.00001$). Because both the pattern and magnitude or responses were similar between tonsils and adenoids, only combined findings are reported.

To assess the effect of CS on proliferation, initial experiments were conducted using dexamethasone (DEX) in both control (CO) and STIM conditions. Figure 1 shows the dose-response to incremental doses of DEX, whereby a significant reduction (~50% reduction) in proliferation was already apparent at 10^{-7} M compared to control conditions, either without (panel A) or following STIM (panel B). Reductions in proliferation also occurred with either BUD (n=18; Figure 1, panels C and D) or FLU (n=18; Figure 1, panels E and F), except that the concentrations required to achieve ~50% reduction in proliferation were markedly lower for FLU (10^{-14} M) and BUD (10^{-12} M) when compared

to DEX (Figure 1; $p<0.0001$ for both vs. DEX). In addition, FLU was more potent than BUD in reducing cellular proliferation by on average 1 logarithmic order of magnitude (Figure 1). Indeed, MANOVA revealed dose-dependent effects, with significant differences in potency as follows: FLU>BUD>DEX ($p<0.0001$; n=25/group).

To assess whether T and B cells are lymphocyte cell subtypes that may be affected by CS, we performed a limited number experiments (n=4) in which cell cultures were pulsed with BrDU, after which flow cytometry was employed using either T cell or B cell markers. These experiments confirmed that both CD3 (+) cells (Figure 2 A left panel) and CD 19 (+) cells (Figure 3 A left panel) incorporated BrDU, thereby indicating the presence of cellular proliferation. Furthermore, FLU reduced BrDU incorporation in both T cells (Figure 2) and B cells (Figure 3), thereby illustrating the anti-proliferative effect of FLU on lymphocyte proliferation.

Apoptosis Assays: Cellular apoptosis was assessed only in control, unstimulated cell culture conditions. Evidence for increased apoptosis occurred in the presence of all CS at both the maximal dose and at the dose associated with ~50% reduction in proliferation. Depending on the CS, the mean magnitude of apoptosis was 3.8-6.7 fold that of control conditions at the maximal dose (10^{-5} M for DEX and 10^{-8} M for FLU and BUD; n=20/group; Figure 4), and around 2.5-3.9 fold at the dose associated with ~50% reduction in proliferation (10^{-7} M for DEX, 10^{-12} M for FLU, and 10^{-13} M for BUD; n=20/group; Figure 4).

Using annexin V antibodies and the flow cytometric approach described above, we further found that FLU induced increased apoptosis in both T cells (Figure 2B) and B

cells (Figure 3B) from a mixed cell culture system of tonsils derived from pediatric OSA patients. These findings were consistently observed in 3 different subjects.

Cytokine Assays:

Basal release of TNF-α IL-8, and IL-6 to the supernatants was increased in tonsillar cultures from children with OSA after STIM (Figure 5; n=48/group). Addition of DEX at 10^{-7} M (or BUD at 10^{-12} M – data not shown) induced significant reductions in pro-inflammatory cytokine production ($p<0.001$; Figure 5)

Discussion

The present study shows that stimulation of tonsils in a dissociated mixed cell culture system induces increased proliferative responses and release of pro-inflammatory cytokines. More importantly, treatment with any of the CS not only markedly reduces proliferation in a dose-dependent fashion under both control and stimulated conditions, but also reveals striking differences in the respective potencies of DEX, FLU, and BUD. The marked decreases in proliferative rates with CS treatment are also accompanied by increased apoptotic cell death, as well as by reductions in the production and release of the pro-inflammatory cytokines TNF-α, IL-8 and IL-6. Taken together, we would surmise that the beneficial effects of intranasal and topical CS to reduce the severity of sleep-disordered breathing in children may be due to CS-induced involution of the tonsils and adenoids, and that this novel in vitro approach may provide new insights into therapeutic approaches aiming to resolve frequent pediatric diseases associated with adenotonsillar hypertrophy.

Before we discuss the potential implications of our findings, two technical and methodological comments are needed. Firstly, the pediatric population included here was not specifically and objectively screened for the presence of allergic rhinitis, even though subjects were not receiving any topical or systemic medications at the time of the surgical removal of their adenoids and tonsils. Previous studies have reported a high prevalence of allergic sensitization in children with OSA (17-19), even if the role of allergic rhinitis in the pathophysiology of OSA remains unclear (20), and despite the similar responses to intranasal CS in mild pediatric OSA, irrespective of allergic history (14). Notwithstanding such debate, the mixed culture system used herein should allow for

exploration of differential responses to established interventions in the presence or absence of an allergic component, as well as in the context of obese children, who traditionally have fared less well after T&A for OSA (21). Secondly, the selection of LPS and ConA as the combined stimulus for the proliferation assays reported herein was based on the lymphocyte populations that reside in tonsillar tissues (22-25). Indeed, these 2 substances have marked effects on the predominant populations of immune cell types represented in tonsillar tissues.

The use of topical steroid therapy in pediatric OSA has been the subject of renewed interest, particularly when considering that surgical extirpation of hypertrophic adenoids and tonsils for OSA is accompanied not only by an increased risk for potential postoperative complications and emotional distress for the patient and family, but is also associated with increased health-related costs associated with the surgical procedure. Therefore, studies providing critical assessment of nasal corticosteroids in the management pediatric OSA have been conducted, and have globally produced very encouraging results (10-14). These studies have shown improved respiratory patterns during sleep across the spectrum of OSA severity. Interestingly, work from our laboratory and also by Alexopoulos and colleagues followed the patient cohorts after cessation of treatment with topical CS for up to 9 months, and found evidence for a sustained effect, without rebound increases in symptoms or re-growth of the adenoids and tonsils (12, 14). However, the optimal dosage and duration of therapy remains undefined, and whether particular groups of patients, such as younger age vs. older age, or non-obese vs. obese would be more likely to favorably respond also remains unknown. Additionally, it remains unclear whether addition of other anti-inflammatory agents, such

as leukotriene receptor antagonists (26, 27) may yield additive or synergistic effects. The in vitro adenotonsillar mixed culture system used in this study provides the opportunity to examine these issues in great detail, and therefore should allow for delineation and identification of particular sub-groups of patients with OSA, for whom intranasal therapy with CS, for example, would be anticipated to elicit optimal outcomes.

The biological potency of the 3 CS employed in this study differed, with FLU having the highest potency in our mixed cell system proliferation assay. These findings were somewhat anticipated, considering the reported differences in bioaffinity and activity among these compounds (28, 29). Indeed, FLU is 300-fold more lipophilic than BUD, has a 3-fold higher relative affinity for the glucocorticoid receptor than BUD, and the half-life of the FLU active steroid-receptor complex is >10 hours, compared with approximately 5 hours for BUD (30). Similarly, FLU has been found to have increased potency compared to BUD in inhibiting human T-cell migration and proliferation, and inhibiting CD4+ T-cell cytokine release, and in stimulating inflammatory cell apoptosis (30). Considering the increased abundance of T cells, particularly CD4+ T cells, in the tonsillar tissues of children with OSA (31), and the important roles that these cell populations may have in local regulation of immune responses and proliferation (32-34), the use of CS as therapeutic tools seems a logical approach in an attempt to reduce T-cell proliferation and promote apoptosis, while reducing pro-inflammatory cytokine production (35-37). In the present study, we present preliminary evidence that both T and B lymphocytes proliferate in tonsillar cultures, and that such proliferation can be abrogated by CS, and further elicit increased lymphocyte apoptosis.

In summary, tonsils and adenoids obtained from children with OSA undergoing T&A display increased proliferative rates and pro-inflammatory cytokine production when stimulated with LPS and conA. Furthermore, treatment with CS resulted in marked dose-dependent reductions in proliferative rates, increased cellular apoptosis, and diminished cytokine release. The relative potency of the 3 CS used in the study was highest for FLU and lowest for DEX. These findings support the use of tonsillar or adenoidal tissue cell cultures as a potentially useful approach for *in vitro* assessment of therapeutic efficacy of CS and other candidate drugs in the treatment of the lymphadenoid hypertrophy that underlies OSA in children.

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Table 1. Demographic and polysomnographic characteristics in 86 children with obstructive sleep apnea undergoing T&A.

	OSA (n=86)
Age (years)	6.3±0.4
Gender (% female)	50
African American (%)	33
BMI z score	1.23±0.33
AHI (/hTST)	10.7±1.9
Nadir SaO2 (%)	83.1±3.6
Arousal Index (/hTST)	17.9±4.6

BMI - body mass inde; AHI – obstructive apnea hypopnea index; TST – total sleep time;

Figure Legends

Figure 1. Effects of various concentrations of DEX (panels A and B), BUD (panels C and D), and FLU (panels E and F) on tonsillar cellular proliferation in children with OSA in a mixed cell culture system under basal conditions (panels A, C, and E) or after stimulation with LPS and conA (STIM; panels B, D, and F).

Figure 2. Flow cytometry assessment of proliferation of T lymphocytes (CD3+ cells) in tonsils from a child with OSA in a mixed cell culture system in the presence of vehicle (A left panel) or FLU (A right panel). Reduced T cell proliferation after FLU is apparent. Conversely, T lymphocyte apoptosis, as assessed by annexin V, was increased by FLU (panel B).

Figure 3. Flow cytometry assessment of proliferation of B lymphocytes (CD19+ cells) in tonsils from a child with OSA in a mixed cell culture system in the presence of vehicle (A left panel) or FLU (A right panel). Reduction in B cell proliferation after FLU is clearly detected. Conversely, B lymphocyte apoptosis, as assessed by annexin V, was increased by FLU (panel B).

Figure 4. Effects of the DEX, BUD, and FLU maximal concentrations and the concentrations leading to 50% reduction in proliferation on tonsillar cellular apoptosis in children with OSA in a mixed cell culture system. Results are shown as fold change compared to control conditions (arbitrary value of 1).

Figure 5. Boxplots of concentrations of TNF-α (panel A), IL-8 (panel B), and IL-6 (panel C) in the supernatants of tonsillar cell cultures in basal conditions (CO) or after stimulation with LPS and conA (STIM) in children with OSA, and the effects of DEX on the production of these cytokines.

The y axis in all graphs is displayed in a logarithmic scale.

Figure 1

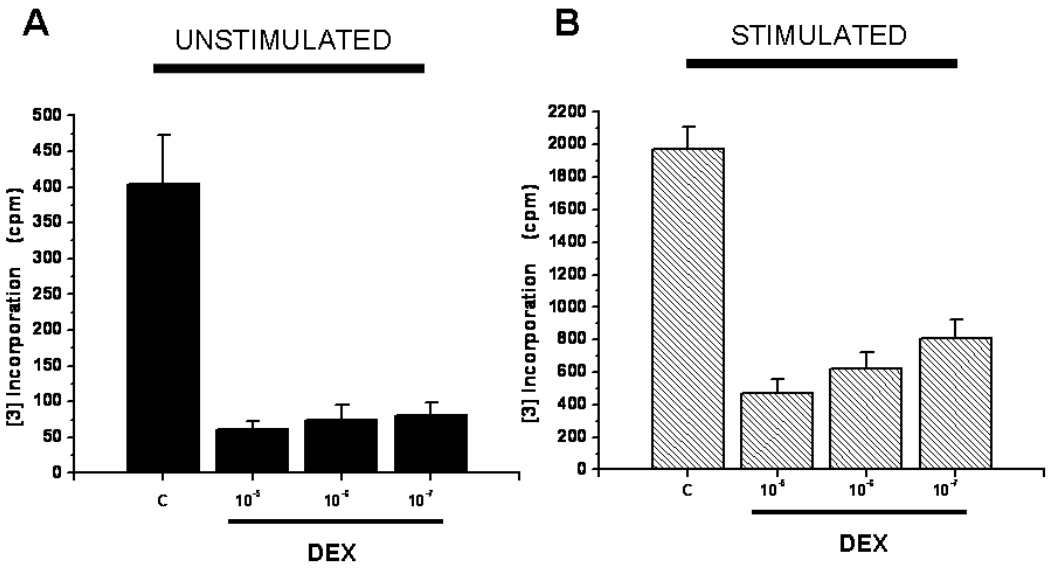
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Figure 1(continued)

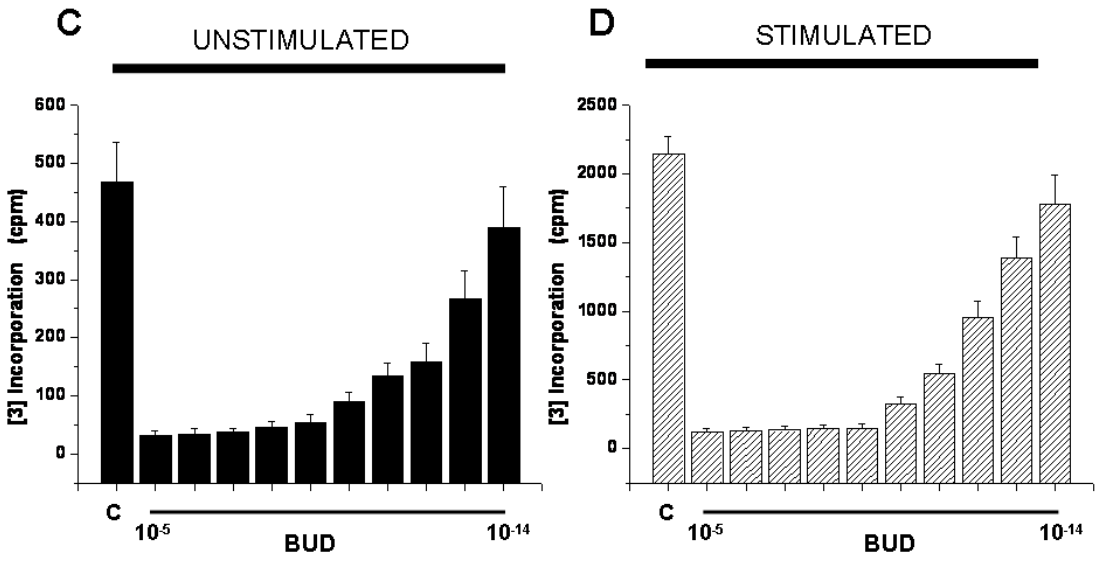


Figure 1 (continued)

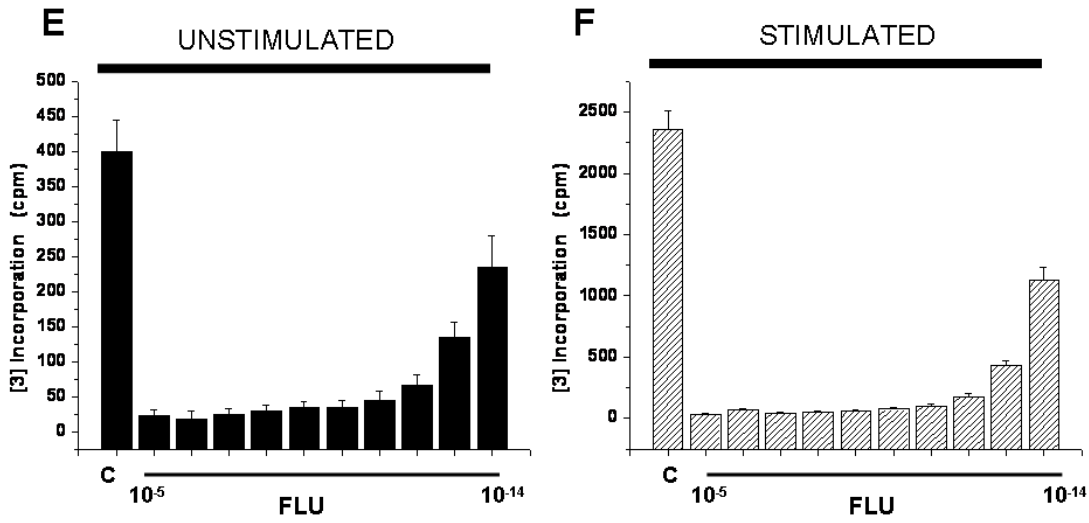


Figure 2

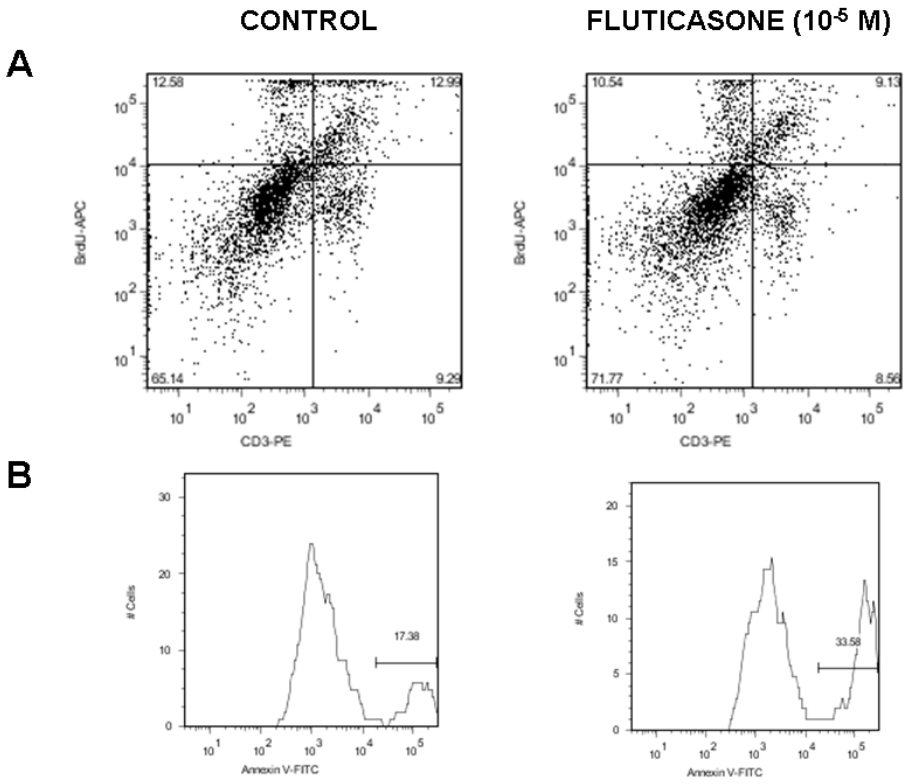

CONTROL
FLUTICASONE (10^{-5} M)
A
B
12.58
12.99
65.14
9.29
10.54
9.13
71.77
8.56
BrdU-APC
CD3-PE
Cells
Annexin V-FITC
17.38
33.58

Figure 3

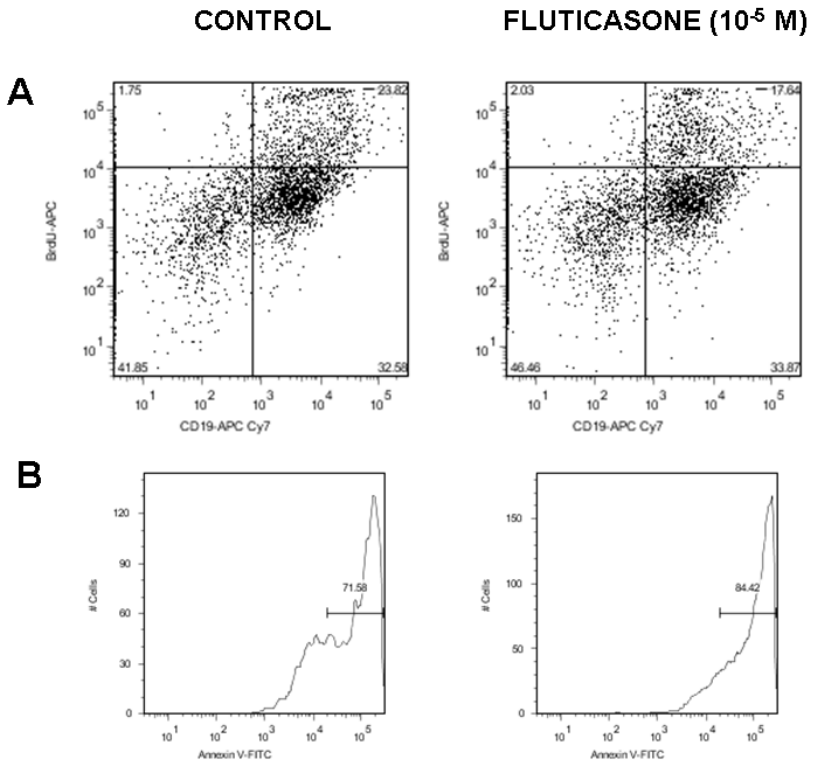

CONTROL
FLUTICASONE (10-5 M)
A
B
BrdU-APC
CD19-APC Cy7
1.75
23.82
41.85
32.58
2.03
17.64
46.46
33.87
Cells
Annexin V-FITC
71.58
84.42

Figure 4

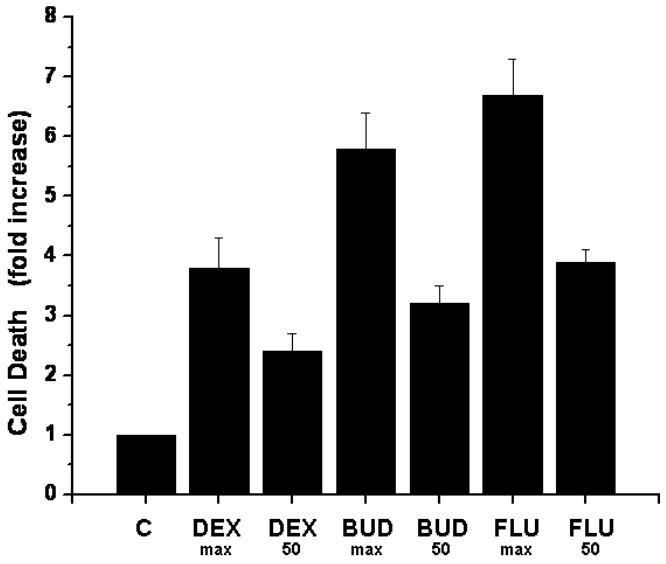
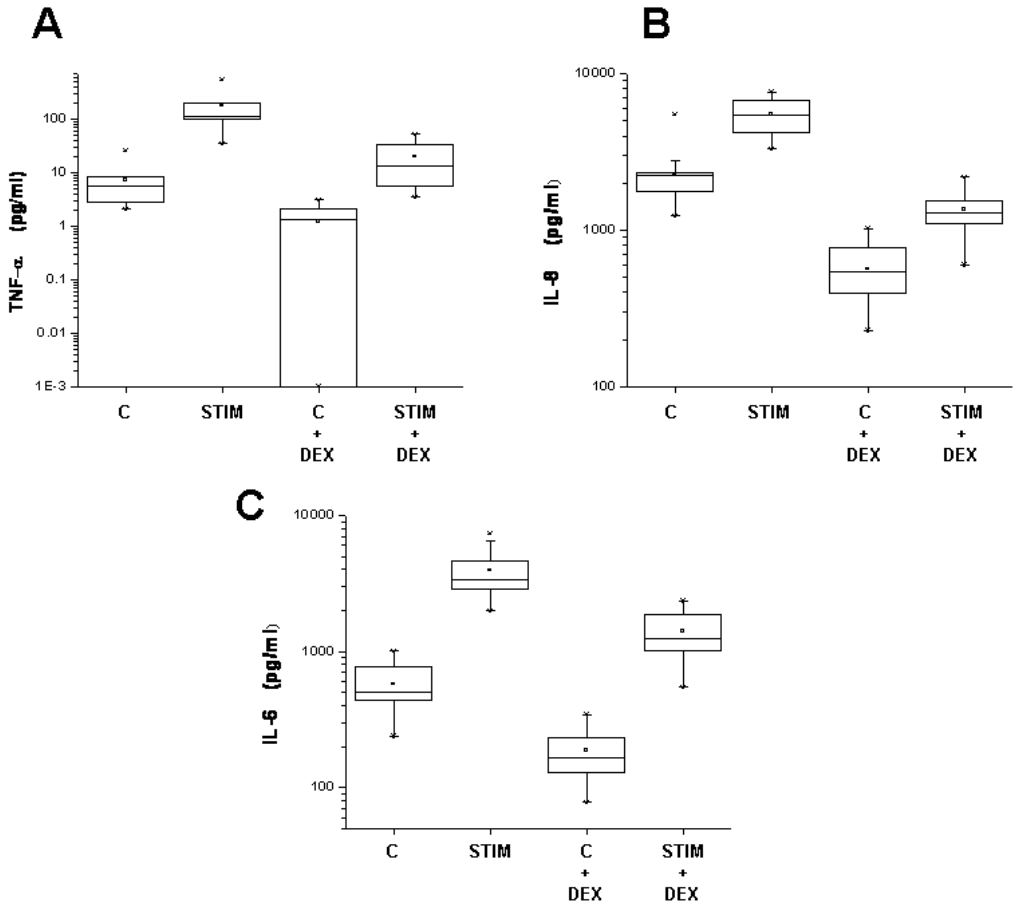

Cell Death (fold increase)
0
1
2
3
4
5
6
7
8
C
DEX max
DEX 50
BUD max
BUD 50
FLU max
FLU 50

Figure 5


A
TNF-α (pg/ml)
100
10
1
0.1
0.01
1E-3
C
STIM
C + DEX
STIM + DEX
B
IL-8 (pg/ml)
10000
1000
100
C
STIM
C + DEX
STIM + DEX
C
IL-6 (pg/ml)
10000
1000
100
C
STIM
C + DEX
STIM + DEX