

Cooperative Effects of Corticosteroids and Catecholamines upon Immune Deviation of the Type-1/Type-2 Cytokine Balance in Favor of Type-2 Expression in Human Peripheral Blood Mononuclear Cells

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Abstract

A growing number of studies show strong associations between stress and altered immune function. *In vivo* studies of chronic and acute stress have demonstrated that cognitive stressors are strongly correlated with high levels of catecholamines (CT) and corticosteroids (CS). Although both CS and CT individually can inhibit the production of T-helper 1 (TH1, type-1 like) cytokines and simultaneously promote the production of T-helper 2 (TH2, type-2 like) cytokines in antigen-specific and mitogen stimulated human leukocyte cultures *in vitro*, little attention has been focused on the effects of combination CT and CS in immune responses that may be more physiologically relevant. We therefore investigated the combined effects of *in vitro* CT and CS upon the type-1/type-2 cytokine balance of human peripheral blood mononuclear cells (PBMC) as a model to study the immunomodulatory effects of superimposed acute and chronic stress. Results demonstrated a significant decrease in type-1 cytokine production (IFN- γ) and a significant increase in type-2 cytokine production (IL-4, IL-10) in our CS+CT incubated cultures when compared to either CT or CS agents alone. Furthermore, variable enhancement of type-1/type-2 immune deviation occurred depending upon when the CT was added. The data suggest that CS can increase the sensitivity of PBMC to the immunomodulatory effects of CT and establishes an *in vitro* model to study the combined effects of *in vivo* type-1/type-2 cytokine alterations observed in acute and chronic stress.

Introduction

A growing body of literature recognizes the dynamic interactions between components of the nervous, endocrine, and immune systems as a cohesive unit responsible for protection of the host⁽¹⁻⁴⁾. In this model, products of the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic nervous system (SNS) shift immunity from an intracellular pathogen-specific (TH1, type-1 like) immune response to an extracellular pathogen-specific (TH2, type-2 like) response during times of high psychological stress^(1, 4). Although this model of immune deviation is an adaptive mechanism that protects the host from the sequelae of systemic inflammation⁽³⁾, maladaptive responses to stress-induced immune deviation may exacerbate and/or contribute to the pathogenesis of immune-based clinical disorders⁽⁵⁾.

In vivo studies of chronic and acute stress situations such as those modeled by the Trier Social Stress Test (TSST) and seen in the medical student exam stress model (MSESM) have demonstrated that cognitive stressors such as public speaking and medical student exams are strongly correlated with high levels of CT and/or CS^(6, 7). In addition, stressful events (particularly chronic) have been associated with adverse clinical effects such as viral reactivation and exacerbation of inflammatory disorders^(7, 8). It seems reasonable to speculate that acutely stressful events might have an even more adverse clinical effect in individuals already chronically stressed, but this has not been well documented.

It has long been suggested that these effects can be partially explained by alterations of the cytokine network responsible for an optimally protective adaptive immune response⁽⁷⁾. Studies by our group and others have shown that products of the HPA and SNS, namely CS and the CT epinephrine (EPI) and norepinephrine (NE) can alter the balanced expression of cytokines from TH1 in favor of TH2 *in vitro*⁽⁹⁻¹²⁾. Specifically, dexamethasone (DEX, a corticosteroid analog for cortisol used for its stable *in vitro* properties), EPI, and/or NE added as single agents at the initiation of cultures can alter the cytokine network responsible for the adaptive immune response by inhibiting the *in vitro* production of type-1 cytokines (IFN- γ , IL-12) while favoring the production of type-2 cytokines (IL-4, IL-10, IL-13)⁽¹⁰⁻¹²⁾.

While studies have largely investigated the individual effects of *in vitro* CS or CT in immune deviation, little attention has been focused upon the *in vitro* effects of combination CT and CS in immune responses that may be more physiologically relevant to the TRIER and medical student exam stress models. For example, CS can influence the long-term responses of various tissues to CT by increasing β -adrenergic receptor surface (β 2-AR) expression and intracellular cAMP activity in human smooth muscle cells⁽¹³⁾ and epithelial cells^(14, 15) lining the respiratory tract. More recent data suggest that β 2-AR expression on lymphocytes and APC may respond similarly to the long-term modulation of CS in murine and equine models⁽¹⁶⁻¹⁸⁾. *In vitro* exposure to CS can increase β 2-AR expression and cAMP activity in murine lymphocytes *in vitro*⁽¹⁶⁾ and murine and equine lymphocytes *in vivo*^(17, 18). Furthermore, the combination of CT and CS decreased the pro-inflammatory cytokines IL-8, IL-6, and TNF- α *in vitro* from synovial tissue isolated in patients with rheumatoid arthritis⁽¹⁹⁾. Although documented, the combined effects of endogenous CS and CT on the type-1/type-2 cytokine network in human leukocytes are limited in the literature and have not been well studied.

We therefore studied the combined physiological stress equivalents of endogenous CS and CT on antigen-specific stimulated human PBMC as an *in vitro* model of the immunomodulatory effects of superimposed chronic and acute stressors (i.e., neuroendocrine stress hormones). Our studies demonstrate that the combined effects of *in-vitro* CS and CT significantly decreases the production of type-1 cytokines and simultaneously increases the expression of type-2 cytokines in our antigen-specific stimulated PBMC cultures when compared to either CS or CT agents alone. These effects further shifted the balanced production of type-1/type-2 cytokines in favor of a type-2 response.

Materials and Methods

Reagents: EPI, NE, phorbol myristate acetate (PMA), ionomycin, and DEX were purchased from Sigma Chemicals (St. Louis, MO). Recombinant rhIL-2 was a gift from Dr. Jeffrey Rossio at the NIH-Frederick Cancer Research Center (Bethesda, MD). Tetanus toxoid (TT) was purchased from Massachusetts Biologic Laboratories (Boston, MA). ELISA kits are from Pharmingen (San Diego, CA).

Subjects: Fourteen healthy study subjects (evenly distributed between men and women) aged 18-50 were recruited for participation in the study (blood draws). Subjects were excluded for any past medical history of smoking, psychiatric illnesses, cardiovascular diseases, or recent medical use of CS, CT, and adrenergic antagonists. Blood samples were drawn in the morning between 0800 and 1200 hours to control for diurnal variation in immune responses. The Committee for the Protection of Human Subjects (CPHS) at the University of Texas Medical School at Houston approved protocols for the study. Informed consent was obtained from each subject prior to enrollment.

Isolation of PBMC: Venous blood was collected in heparinized Vacutainer tubes (Becton Dickinson, San Diego, CA) and PBMC were isolated via ficoll-hypaque density gradient centrifugation at 3000g for 20 minutes. Cells were washed twice with Ca²⁺/Mg²⁺ free HBSS (Sigma), and resuspended in RPMI 1640 supplemented with 90U/ml penicillin, 90 µg/ml streptomycin, 2mMol/L L-Glutamine (all from Sigma), and 10% human AB serum (Atlanta Biologicals).

PBMC Cultures: For incubation experiments, DEX at concentrations of 1x10⁻⁸ M (mimicking the stress-physiologic plasma levels⁽¹¹⁾ of endogenous glucocorticoids) was added on the first day of stimulation followed by the addition of EPI or NE at the stress physiologic concentrations (1x10⁻⁸ M, 1x10⁻⁷ M, respectively) on fifth and tenth days of stimulation. PBMC Cultures at 1x10⁶ cells/ml were stimulated with 0.5 µg/ml TT and 10 IU/ml rhIL-2 for 5 days at 37°C and 5% carbon dioxide for all experiments as previously described^(11, 12). On day 5, 0.5 ml of supernatant was removed and replaced with 20 IU/ml rhIL-2 for cellular expansion and 0.5 ml fresh media containing 10% human AB serum. On day 10, 0.5 ml of supernatant was removed and replaced with 0.5 ml media containing 10 µg/ml phytohemagglutinin (PHA) and 1 ng/ml phorbol myristate acetate (PMA) and incubated for 24 hours as previously described^(11, 12). Briefly, a clonal population of antigen specific T-lymphocytes predominates by the end of the 11 day culture period. These clonal cells are stimulated with PHA and PMA on the 10th day of culture for 24 hours to amplify the production of cytokines from phenotypically committed cells that otherwise produce cytokines at levels too low for detection by ELISA. The use of mixed-cell cultures such as PBMC is closer to physiological conditions than cultures using isolated CD4⁺ naïve T-cells for our set of experiments. For cultures coincubated with both CS and CT (simulating acute stress episodes superimposed upon chronic stress), EPI was added for convenience on the 5th and 10th days at the same time the media was scheduled for change as previously described^(11, 12). Supernatants were harvested on day 11 and stored at -70°C.

Cytokine analysis: Levels of type-1 and type-2 cytokines in culture supernatants were determined by enzyme linked immunosorbent assays (ELISA) using paired antibodies specific for IFN-γ, IL-10, and IL-4 (Pharmingen). Developed ELISA plates were read on an Emax plate reader (Molecular Devices) at 450nm. Sample concentrations were determined based on calculated standard curves using SOFTmax (Molecular Devices). The lower limit of detection of the IFN-γ, IL-10, and IL-4 kits was 4 pg/ml.

Statistical methods: Cytokine data (with the exception of Type-1/Type-2 ratio data) were transformed into a logarithmic scale (Log₁₀) to normalize the results, thus bringing

the data closer to a Gaussian distribution. Data are presented in graph form as mean \pm standard deviation (SD). Type-1/Type-2 cytokine ratios were calculated from untransformed cytokine values for each individual and culture condition before summarized in bar graph format. The data are presented as mean \pm SD. Comparisons between 3 or more groups utilized the repeated measures one-way analysis of variance (ANOVA) with post test analysis for group differences by Tukey's Multiple Comparisons Test. Statistical analyses were calculated with Prism (Version 4.0 Graphpad Software, San Diego, CA).

Results

Decreased Production of Type-1 Cytokines by Combined Physiological Stress Levels of Corticosteroids and Catecholamines

To determine the combined effects of adrenergic agents and CS, we used physiological stress concentrations of EPI⁽¹²⁾ and DEX (mimicking the stress-physiologic plasma levels of endogenous glucocorticoids)⁽¹¹⁾ together or as single agents. In these sets of experiments IFN- γ levels were determined in culture supernatants from TT stimulated cultures treated with combination DEX at 10^{-8} M and EPI at 10^{-8} M. Coincubation cultures with DEX and EPI were added either on day one of stimulation (DEX+EPI₁) or DEX on the 1st day followed by EPI added on 5th and 10th days of antigenic stimulation (DEX+EPI_{5,10}). In addition, DEX, EPI₁ (added as a single agent on the first day of stimulation) and EPI_{5, 10} (EPI added as a single agent on the 5th and 10th days of stimulation) groups were added as supplementary controls. The means from the treatment groups differed significantly by repeated measures ANOVA ($p < 0.0001$). Tukey's post-test analysis (TPTA) revealed significant inhibition of IFN- γ production from baseline control in the DEX, EPI₁, DEX+EPI₁, DEX+EPI_{5,10} groups. In addition, TPTA demonstrated significantly more inhibition of IFN- γ production from both the DEX and DEX+EPI₁ groups. The mean of the DEX+EPI₁ treated group did not differ significantly from the DEX treated group, suggesting that the inhibition of IFN- γ in the DEX+EPI₁ group was due to DEX and not to the additive effect of EPI in long-term antigen-specific stimulated cultures (Figure 1).

Increased Production of Type-2 Cytokines by Combined Stress Levels of Corticosteroids and Catecholamines

Production of IL-10 and IL-4 production was determined from the same culture supernatants described above. The means for both IL-4 and IL-10 among the treatment groups differed significantly by repeated measures ANOVA ($p < 0.0001$). TPTA revealed a significant increase of IL-10 production in the DEX, EPI₁, DEX+EPI₁, and DEX+EPI_{5,10} groups from baseline control (Figure 2). In addition, TPTA revealed a significant increase in IL-4 production in the EPI₁ and DEX+EPI_{5,10} groups but not the DEX, EPI_{5,10}, or DEX+EPI₁ groups when compared to control (Figure 3). Furthermore, TPTA revealed that the DEX+EPI_{5,10} group significantly increased the production of IL-4 and IL-10 over the DEX, DEX+EPI₁, and EPI₁ groups (Figures 2, 3).

A balanced production of cytokines is needed for an adaptive immune response that is appropriate for the inciting antigen⁽²⁰⁾. To further investigate the impact of CS and CT on this balance, type-1/type-2 cytokine ratios were calculated based upon cytokine expression levels for all treatment groups demonstrated in Figures 1-3. The group means

in the IFN- γ /IL-10 graph (Figure 4) and IFN- γ /IL-4 (Figure 5) differed significantly by repeated measures ANOVA ($p < 0.0001$). The IFN- γ /IL10 ratios were significantly decreased in the DEX, EPI₁, DEX+EPI₁, and DEX+EPI_{5,10} groups when compared to baseline control from TPTA (Figure 4). Similarly, the IFN- γ /IL-4 ratios were significantly decreased in the DEX, EPI₁, DEX+EPI₁, and DEX+EPI_{5,10} groups from baseline control (Figure 5) by TPTA. The type-1/type-2 ratio in the DEX+EPI_{5,10} group was significantly less than the DEX, EPI₁, and DEX+EPI₁ by TPTA. In both the type-1/type-2 graphs (Figures 4 and 5), demonstrating that the additive effects of EPI added several days later to long-term antigen-specific cultures significantly decreased the balanced production of cytokines further in favor of type-2 cytokines.

Propranolol Blocks the Type-2 Cytokine Shift from the Additive Effects of β 2-Agonists

We wished to investigate if the cooperative effects of CS and CT were mediated in part by the β 2-adrenergic receptor (β 2-AR). Previously, PRO, a non-selective β -adrenergic receptor antagonist, has been shown to mitigate the *in vitro* effects of catecholamines in antigen-specific PBMC cultures (X). The mean production of IFN- γ , IL-10, and IL-4 in the DEX+EPI_{5,10}+PRO group did not significantly differ from the DEX group (Figures 1 - 3) by TPTA. However, the mean production of IFN- γ in the DEX+EPI_{5,10} group was significantly less than the DEX+EPI_{5,10}+PRO group by TPTA. In addition, the mean production of type-2 cytokines (IL-10 and IL-4) were significantly higher in the DEX+EPI_{5,10} group than the DEX+EPI_{5,10}+PRO group by TPTA suggesting that the observed differences were mediated by β -agonists (Figures 1 - 3). In addition, the type-1/type-2 ratios between the DEX+EPI_{5,10}+PRO and the DEX groups (Figure 4, 5) did not significantly differ by TPTA suggesting that the significant differences between the DEX+EPI_{5,10} and DEX groups were mediated by β -agonists (Figures 4, 5).

Discussion

Recent studies have shown that CT amplify the anti-inflammatory effects of CS in fibroblasts and smooth muscle cells⁽²¹⁻²⁴⁾, thus attenuating the effects of CT-induced tachyphylaxis of the β 2-AR (β 2-AR desensitization) in human respiratory smooth muscle cells^(22, 25). In addition, the combination of CT and CS has a cooperative anti-inflammatory effect on synovial tissue by inhibiting type-1 cytokines *in vitro* and *in vivo*⁽¹⁹⁾. Furthermore, the combined effects of pharmacologically available adrenergic agonists with CS suppress T-cell proliferation and type-1 cytokine production in short-term mitogen-stimulated human PBMC cultures *in vitro*, although variable effects were observed with type-2 cytokines⁽²⁶⁾. However, these observations do not address physiologic parameters such as the use of stress doses of endogenous CT with CS on antigen-specific immune responses that would model *in vivo* stress-induced immune dysfunction.

The goal of the study was to develop an *in vitro* antigen-specific model to examine the immune effects of endogenous CT stress hormones that appear acutely in the context of chronic stress CS hormones. These experiments demonstrated that the combination of CS and CT significantly decreased the production of IFN- γ and simultaneously increased the production of IL-10 and IL-4 when compared to either agent alone (Figures 1-5). Furthermore, the balanced production of type-1/type-2 cytokines significantly shifted towards a type-2 cytokine profile in the DEX+EPI_{5,10} group over the DEX+EPI₁, DEX, and EPI₁ groups (figures 4, 5). The EPI-mediated cytokine alterations were blocked by PRO indicating that the cooperative effects were mediated through the β 2-AR. Our results are novel in that both type-2 cytokines in our study (IL-4 and IL-10) were significantly increased and IFN- γ was significantly decreased using long-term antigen-specific stimulated human PBMC cultures. In addition, these results were obtained using physiologic stress concentrations of endogenous CT (EPI, NE) in contrast to therapeutic doses of pharmacologically available long-acting β -agonists in short-term mitogen-stimulated cultures.

Of note, we observed variations in cytokine expression levels from different study subjects. For example, the DEX+EPI_{5,10} group had significantly lower type-1 cytokine production and increased type-2 cytokines, although some individuals were more sensitive than others to the cooperative effects of CS and CT. These within group differences may be at least partially explained by subject heterogeneity such as functional polymorphisms of the β 2-AR⁽²⁷⁻²⁹⁾, glucocorticoid receptor^(30, 31), and cytokine receptors^(32, 33). In addition to polymorphisms, different isoforms of the GCR due to transcript alternative splicing and alternative translation initiation⁽³⁴⁻³⁶⁾ may also contribute to variation of cytokine expression and potentially immune deviation of the type-1/type-2 cytokine balance. These possibilities await future investigation.

Kinetic differences were observed between our DEX+EPI₁ and DEX+EPI_{5,10} cultures (Figures 1-5). In particular, these two treatment groups demonstrated that the cooperative effects of combination CS and endogenous CT in antigen-specific cultures appear at later time points (DEX+EPI_{5,10} cultures) rather than earlier exposure to adrenergic agents (DEX+EPI₁ cultures) in our antigen-specific cultures. Combined with the adrenergic

antagonist data with PRO, the data suggest that the mechanism responsible for these observations is time-dependent and may therefore require modification or *de novo* synthesis of the AR or CS signaling apparatus after antigenic-stimulation. Possible mechanisms may include increased nuclear translocation of the GCRC⁽²⁶⁾, increased cell surface AR density and function (from *de novo* synthesis or redistribution of internalized receptors between the internal and external cellular compartments), and/or increased secondary signaling from the AR.

Although our DEX+EPI₁ data differed from those previously reported by Goleva et al⁽²⁶⁾, several differences in the experimental design may explain these discrepancies. Goleva et al used short-term mitogen stimulation (PMA and Ionomycin) vs. long-term antigen-specific cultures (TT) used in our study. Mitogen stimulation requires shorter incubation periods than antigen-specific stimulation due to bypassing antigen presentation, costimulation by the B7 receptor family (i.e., B7.1, B7.2), and differentiation of naïve T-cells (TH0) into TH1 and TH2 clones. Moreover, the physical and biological differences between endogenous CT and long-acting synthetic β -agonists such as salmeterol include elimination half-lives of 2-3 minutes for EPI compared to 5.5 hours^(37, 38) for salmeterol due in part to the lipophilic nature of salmeterol, reduced ability of salmeterol to induce tachyphylaxis of β 2-AR⁽³⁸⁾, alternate routes of metabolism, and the relative instability of endogenous CT (EPI and NE) at culture pH and temperature. In effect, these differences may allow for the *in vitro* manifestations of long-acting β -agonists on PBMC to appear more readily in short term cultures than the additive effects of EPI or NE manifested in long-term antigen-specific cultures until later time points.

Based upon preliminary experiments, the likely sources of expressed cytokines in our long-term antigen-specific cultures were clonal populations of tetanus-specific CD4+ T-lymphocytes. Greater than 90% of the viable cells remaining in culture at day 11 were CD4+ T-lymphocytes, 5-9% were CD8+ T-lymphocytes, and less than 1% were CD19+ B-lymphocytes (data not shown). CD14+ APC, also a source of IL-10, were not detected. This suggests that immune deviation of the type-1/type-2 paradigm had occurred primarily at the level of the T-cell in our *in vitro* model.

In summary, our study was designed to establish an *in vitro* human model of stress hormone-induced immune changes. This model demonstrated that the combination of CS and CT enhanced immune deviation of the cytokine network towards a type-2 adaptive immune response to recall antigen (TT) *in vitro*. Future research will explore possible mechanisms for the cooperative effects of combination CS and CT on immune function such as changes in the function and expression of β 2-AR on specific leukocyte populations (lymphocytes and APC) as a complementary mechanism to increased nuclear translocation of the GCRC. Additionally, our *in vitro* model can serve as a guide to developing future clinical studies that will investigate specific immune dysfunction after acutely stressful situations in patients who are already chronically stressed. Understanding these effects should allow development of interventional strategies to mitigate or prevent the adverse immunological effects of acute and chronic stress in patients with various inflammatory diseases.

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Figure 1

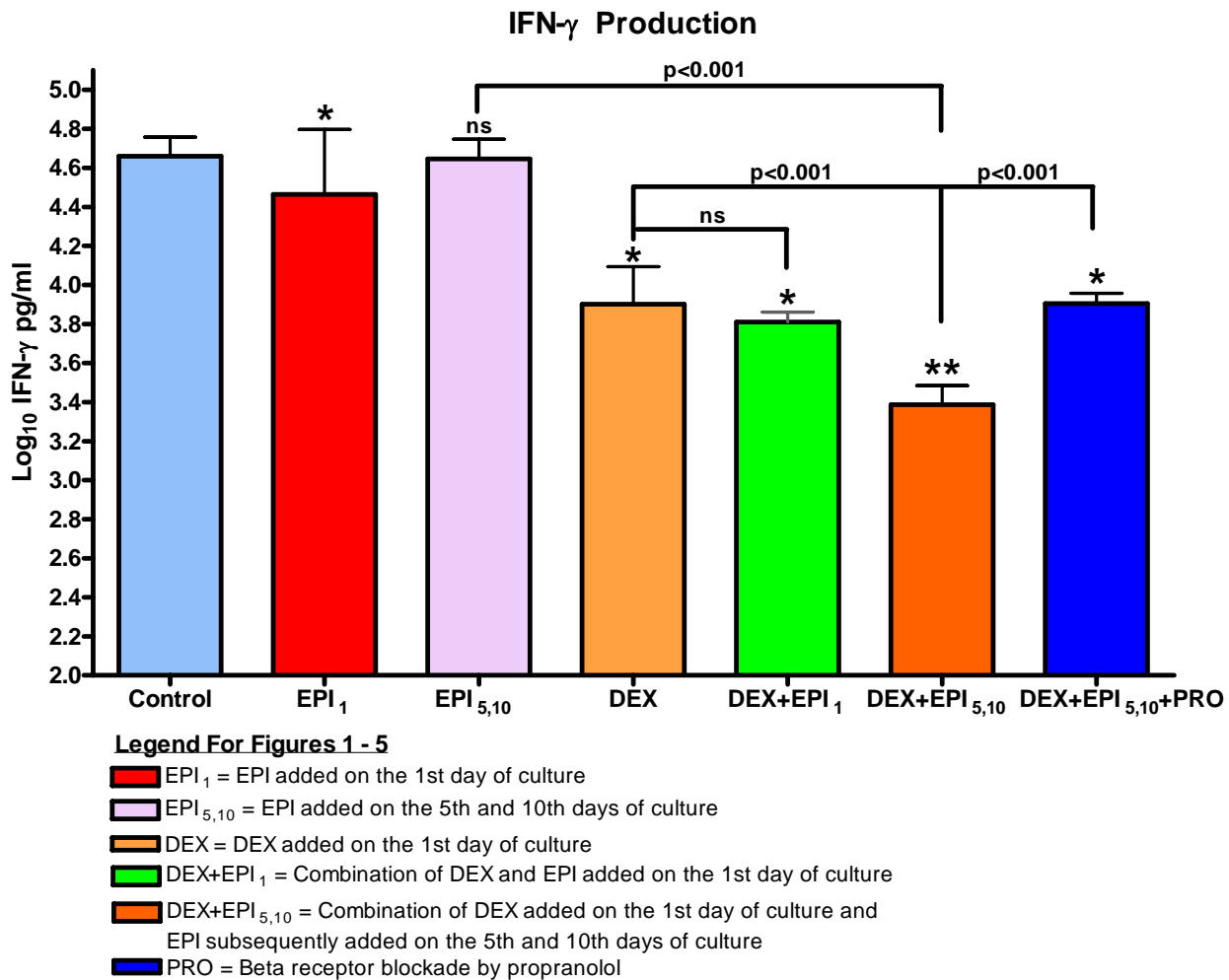


Figure 1: Detection of IFN- γ production in 11-day Tetanus-Toxoid (TT) stimulated cultures by ELISA. The data represent the effects of physiologic-stress doses of CT added at different time points (EPI₁, EPI_{5,10}), CS (DEX), and the combination of CT+CS (DEX+EPI₁, DEX+EPI_{5,10}) in human PBMC from a sample size of 14 healthy adults. Data are represented as mean \pm standard deviation. * = p < 0.5, ** = p < 0.001, and represented p values calculated by Tukey's Multiple Comparisons test.

Figure 2

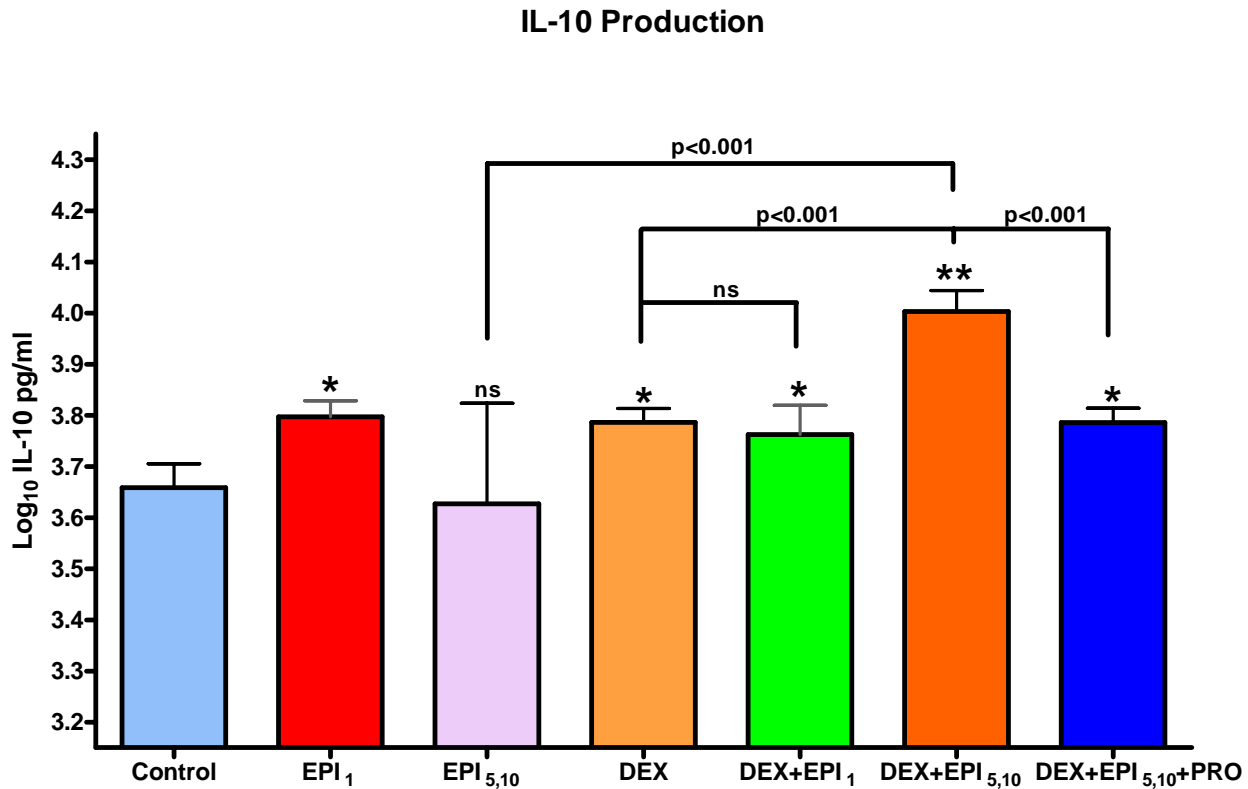


Figure 2: Detection of IL-10 production in 11-day TT (antigen-specific) stimulated cultures by ELISA. The data represent the effects of physiologic-stress doses of CT added at different time points (EPI₁, EPI_{5,10}), CS (DEX), and the combination of CT+CS (DEX+EPI₁, DEX+EPI_{5,10}) in human PBMC from a sample size of 14 healthy adults. Data are represented as mean ± standard deviation. * = p<0.5, ** = p<0.001, and represented p values calculated by Tukey's Multiple Comparisons test.

Figure 3

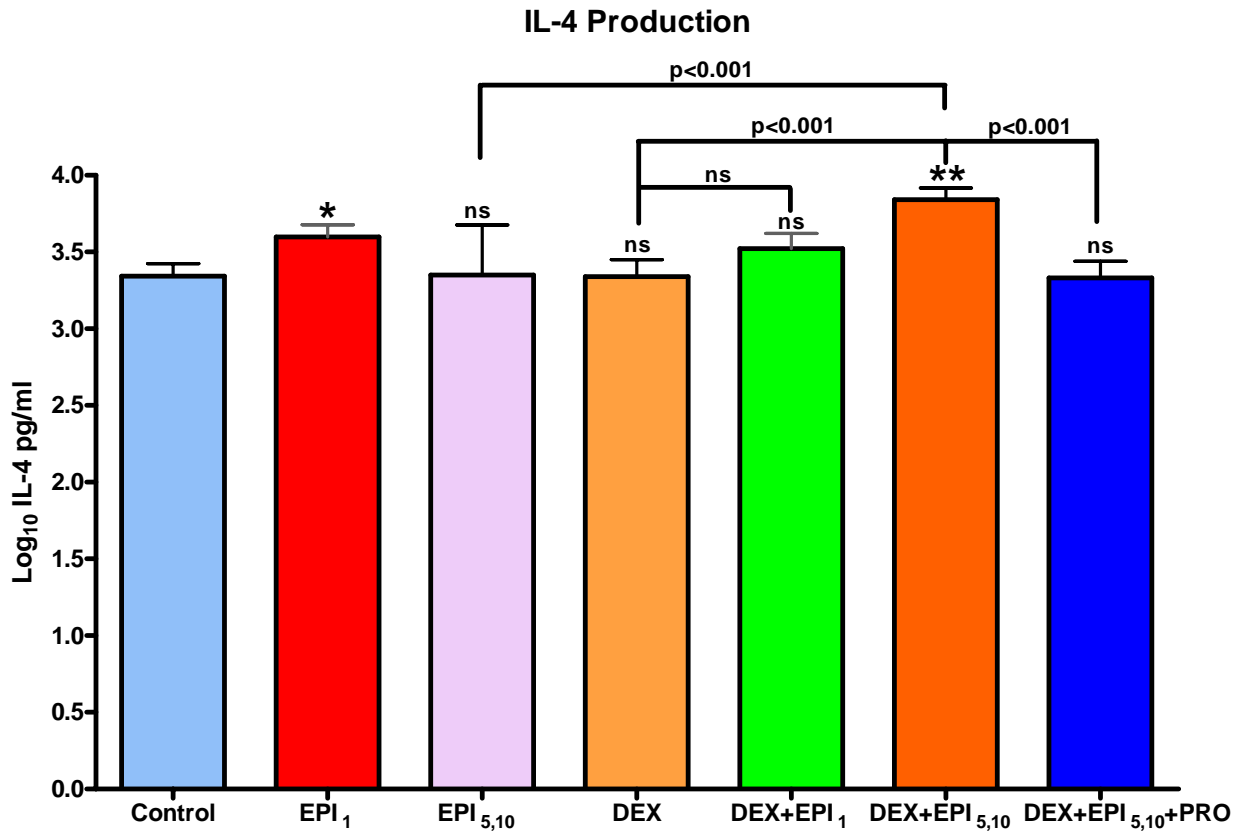


Figure 3: Detection of IL-4 production in 11-day TT (antigen-specific) stimulated cultures by ELISA. The data represent the effects of physiologic-stress doses of CT added at different time points (EPI₁, EPI_{5,10}), CS (DEX), and the combination of CT+CS (DEX+EPI₁, DEX+EPI_{5,10}) in human PBMC from a sample size of 14 healthy adults. Data are represented as mean \pm standard deviation. * = p<0.5, ** = p<0.001, and represented p values calculated by Tukey's Multiple Comparisons test.

Figure 4

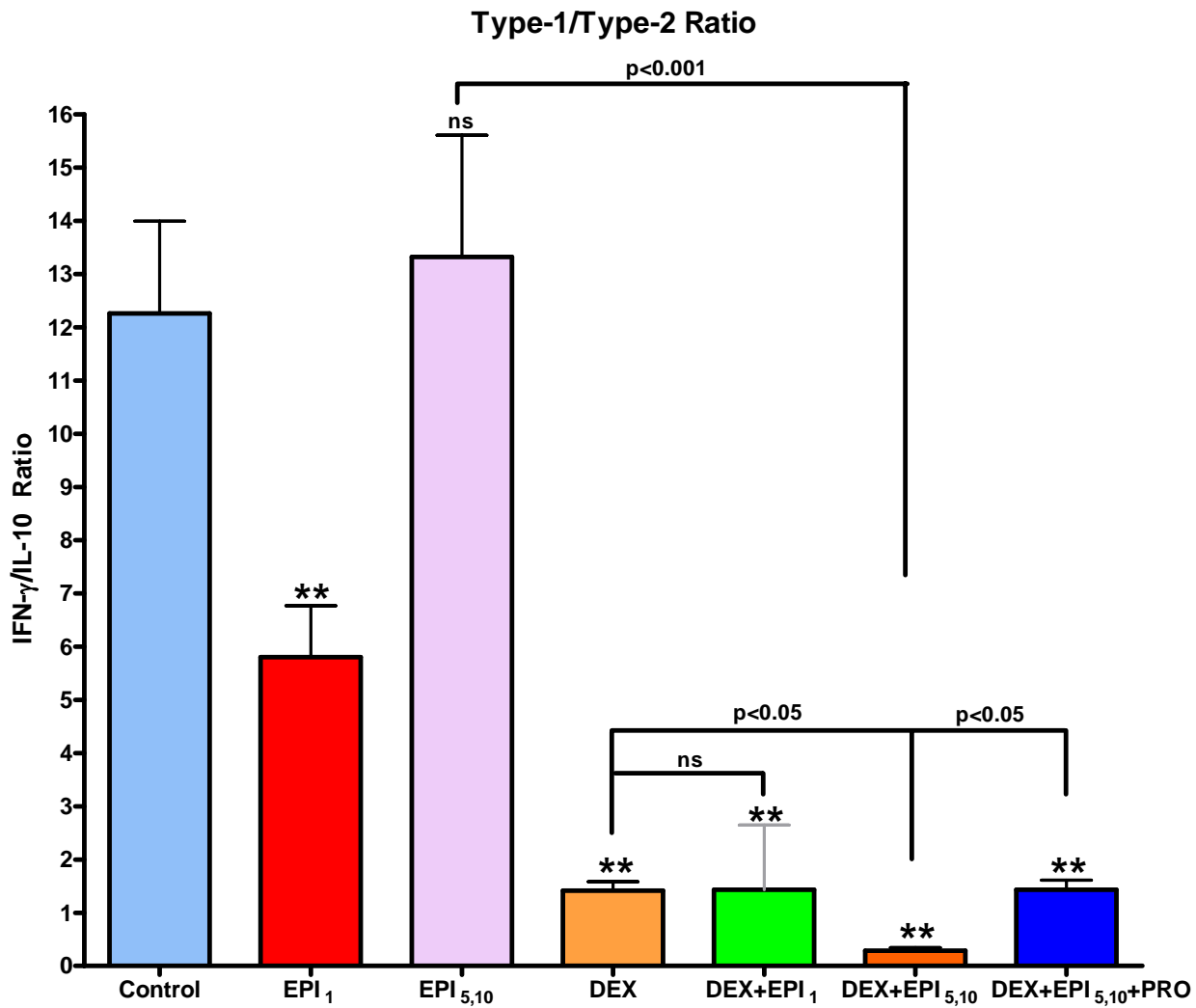


Figure 4: The balanced production of cytokines determines the adaptive immune response. Type-1/Type-2 (IFN- γ /IL-10) cytokine ratios were calculated from ELISA data of 11-day TT (antigen-specific) stimulated cultures. The balanced production of cytokines determines the adaptive immune response. Type-1/Type-2 (IFN- γ /IL-10) cytokine ratios were calculated from ELISA data of 11-day TT (antigen-specific) stimulated cultures. The data represent the effects of physiologic-stress doses of CT added at different time points (EPI₁, EPI_{5,10}), CS (DEX), the combination of CT+CS (DEX+EPI₁, DEX+EPI_{5,10}), or CT+CS+propranolol (DEX+EPI_{5,10}+PRO) in human PBMC from a sample size of 14 healthy adults. Data are represented as mean \pm standard deviation. ** = p < 0.001 and represented p values were calculated by Tukey's Multiple Comparisons test.

Figure 5

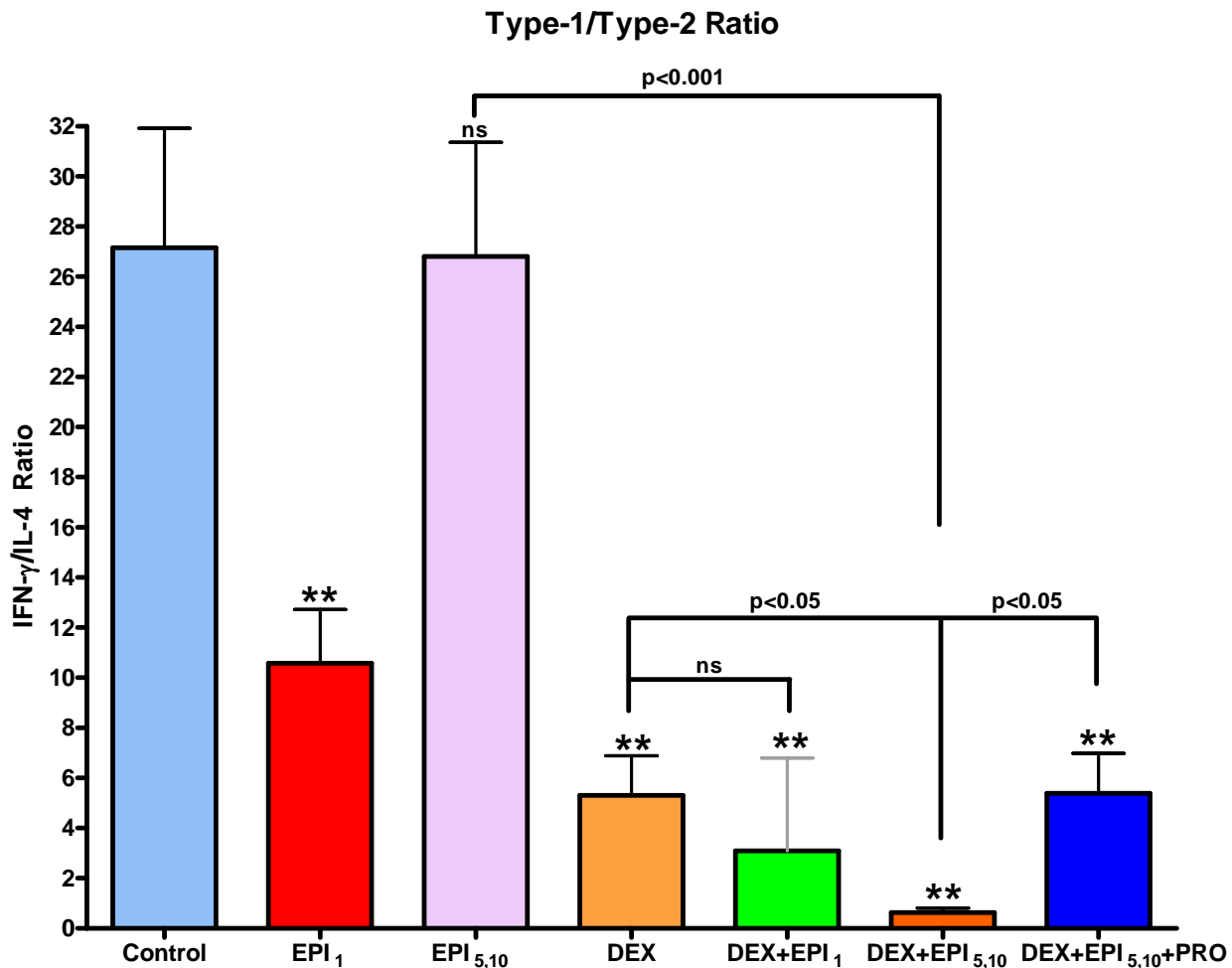


Figure 5: The balanced production of cytokines determines the adaptive immune response. Type-1/Type-2 (IFN- γ /IL-4) cytokine ratios were calculated from ELISA data of 11-day TT (antigen-specific) stimulated cultures. The balanced production of cytokines determines the adaptive immune response. Type-1/Type-2 (IFN- γ /IL-10) cytokine ratios were calculated from ELISA data of 11-day TT (antigen-specific) stimulated cultures. The data represent the effects of physiologic-stress doses of CT added at different time points (EPI₁, EPI_{5,10}), CS (DEX), the combination of CT+CS (DEX+EPI₁, DEX+EPI_{5,10}), or CT+CS+propranolol (DEX+EPI_{5,10}+PRO) in human PBMC from a sample size of 14 healthy adults. Data are represented as mean \pm standard deviation. ** = p<0.001 and represented p values were calculated by Tukey's Multiple Comparisons test.