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IL-4 induced CD23 (Fc_εRII) up-regulation in equine peripheral blood mononuclear cells and pulmonary alveolar macrophages

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Abstract

The objectives of this study were to quantify the induction of equine CD23 transcripts in equine peripheral blood mononuclear cells (PBMCs) and pulmonary alveolar macrophages cultured with recombinant equine IL-4 (rEq IL-4). PBMCs were isolated from blood drawn from four healthy horses. Bronchoalveolar lavage (BAL) fluid was collected from three healthy horses and alveolar macrophages were purified using adherence to plastic for 120 min. PBMCs and alveolar macrophages were cultured using four different conditions: rEq IL-4 and LPS, LPS alone, rEq IL-4 alone and a media control. Total RNA was isolated from cells cultured for 24 or 48 h. Reverse transcribed mRNA was amplified and quantified in real-time polymerase chain reaction (RT-PCR) using a fluorescein labeled internal TaqMan[®] probe for CD23 expression. Without exception, the relative value for CD23 mRNA transcripts from equine PBMCs and pulmonary alveolar macrophages cultured with rEq IL-4 for 24 and 48 h were higher than those cultured with LPS alone or the untreated control. Furthermore, morphologic changes were noted in alveolar macrophages cultured with rEq IL-4 prompting an investigation of cytokine expression levels. Alveolar macrophages cultured with LPS exhibited increased IL-8 and IL-12 p40 expression when compared to rEq IL-4, rEq IL-4 + LPS or the untreated control. These findings support two conclusions, (1) equine CD23 has a role in IL-4 mediated immune responses in the horse and (2) rEq IL-4 can modulate LPS-induced, pro-inflammatory cytokine production by equine pulmonary alveolar macrophages.

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1. Introduction

CD23 (Fc_εRII), the low-affinity receptor for IgE, is expressed on the surface of B-cells prior to isotype switching, as well as on monocytes, eosinophils and platelets. This type II transmembrane glycoprotein is a

member of the C-type lectin family. CD23 plays an important role in the differentiation and activation of B lymphocytes and is up-regulated, or induced, by interleukin 4 (IL-4) (Bettler et al., 1989; Conrad, 1990). Expression of CD23 is thought to play a central role in IgE-associated immune responses, including allergies (Fugiwara et al., 1994; Yu et al., 1994) and in B lymphocyte neoplasia (Fournier et al., 1994). CD23 can be induced on human alveolar macrophages and has been associated with induction of allergic asthma

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(Williams et al., 1992). Alveolar macrophages from humans with allergic asthma express CD23 at a high frequency when compared to non-asthmatics (Gagro and Rabatic, 1994).

Horses suffer from a number of allergic conditions in which CD23 has the potential to play a major role in the etiology, including culicoides hypersensitivity, recurrent urticaria, recurrent airway obstruction (RAO) and summer pasture-associated obstructive pulmonary disease (SPAOPD) (Evans et al., 1992; Lavoie et al., 2001; Wilson et al., 2001; Beadle et al., 2002). RAO is a disease of mature stabled horses and to date there are no early indicators or markers of this disease. If these horses could be identified at an early age, prior to the development of clinical signs, then, potentially the severity of their disease could be altered through therapeutic and environmental management strategies. We propose that the expression of CD23, in appropriate populations of equine cells, is an excellent candidate for an early marker of local IL-4 mediated immune responses and therefore, RAO and SPAOPD. The major objective of this study was to determine if equine CD23 mRNA transcripts were up-regulated in vitro in response to recombinant equine IL-4 (rEq IL-4).

While IL-4 is well known for its pivotal role in regulating T and B lymphocyte differentiation, where it is required for the development of type 2 immune responses, it has other immunomodulatory effects. In this study macrophages cultured with rEq IL-4 in vitro exhibited morphological changes that prompted further investigation. We hypothesized that the morphologic changes observed represented a change in activation status of the IL-4 treated cells. There are three cytokines known in other species to have strong macrophage-deactivating effects, transforming growth factor beta, IL-4 and IL-10 (Bogdan and Nathan, 1993). We present evidence in this study, down-regulation of mRNA transcripts for IL-8 and IL-12 p40 from IL-4 treated macrophages, that rEq IL-4 is a potent deactivator of equine pulmonary alveolar macrophages.

2. Materials and methods

2.1. Animals

Horses were healthy adults with no history of respiratory disease or signs of other health problems.

Animals were housed at the University of California Davis equine research facility. Four horses were used for PBL collection and three horses were used for the alveolar macrophage collection.

2.2. Alveolar macrophage isolation

Bronchoalveolar lavage (BAL) was performed as previously described and BAL fluid was kept on wet ice until processed (Watson et al., 1997). Cells were harvested from BAL fluid by centrifugation for 20 min at $400 \times g$ and washed twice in cold Hank's Balanced Salt Solution (HBSS) without calcium and magnesium (Invitrogen Carlsbad, CA). BAL cells were resuspended in RPMI medium (RPMI 1640 (Invitrogen Carlsbad, CA)) at $2 \times 10^5 \text{ ml}^{-1}$ and transferred to tissue culture flasks (75 cm^2). Cells were incubated at 37°C and 5% CO_2 for 2 h and non-adherent cells were rinsed out with two washes of sterile HBSS.

2.3. Peripheral blood mononuclear cell isolation

PBMCs were isolated from whole blood using histopaque (Sigma–Aldrich, St. Louis, MO) density gradient. Five milliliters calcium and magnesium free phosphate buffered saline (PBS) were added to 5 ml of whole blood and the mixture was gently layered over 5 ml of room temperature histopaque-1077 using a pasteur pipet in a 15 ml centrifuge tube. Tubes were then centrifuged for 20 min at $400 \times g$ and the mononuclear cell layer was removed with a pasteur pipet and washed twice with HBSS. Cells were resuspended in RPMI 1640 at $2 \times 10^5 \text{ ml}^{-1}$ and transferred to tissue culture flasks (75 cm^2). Cells were incubated at 37°C and 5% CO_2 .

2.4. Cell culture

Four culture conditions were used: media alone, supplementation with LPS at $10 \mu\text{g/ml}$, recombinant equine IL4 at 150 U/ml (Hammond et al., 1999), or both. Cells were cultured in RPMI 1640 containing 10% fetal calf serum, 50 U/ml penicillin G, and $50 \mu\text{g/ml}$ streptomycin sulfate at 37°C and 5% CO_2 for 24 and 48 h. Harvested cell pellets were frozen immediately at -80°C . Cell morphology in flasks was examined and photomicrographs were taken using a

phase-contrast inverted microscope (Zeiss ICM-405, Oberkochen, Germany).

2.5. Total RNA isolation and cDNA synthesis

Total RNA was isolated from each cell pellet using the Qiagen RNAeasy mini kit (Qiagen Inc., Valencia, CA). All RNA extractions were treated with 1 μ l (0.5 U) RNase-free Dnase I (Roche Diagnostics, Indianapolis, IN) for 30 min and then heated to 75 °C for 5 min. First strand synthesis was performed according to the manufacturer's directions in a 20 μ l volume containing 50 units Superscript II (Invitrogen, Carlsbad, CA), 1 μ l random hexamer primer and 1 nM dNTP mix. The reaction proceeded for 50 min at 42 °C and was terminated at 70 °C for 15 min. Reaction mixes were diluted to 100 μ l for use in the real time quantitative PCR reactions.

2.6. Real-time polymerase chain reaction (RT PCR) assay

A real-time quantitative (TaqMan[®]) PCR system was designed for equine CD23 using the cDNA sequence (Heid et al., 1996; Watson et al., 2000). The Primer Express software package (PE Biosystems, Foster City, CA) was used to select two primers and an internal oligonucleotide probe. The probe was labeled at the 5'-end with 6-carboxyfluorescein (FAM) reporter dye and at the 3'-end with 6-carboxytetramethyl-rhodamine (TAMRA) quencher dye. The PCR primers were designed to amplify a small (124 bp) product in order to eliminate the need for an extension step. To prevent amplification of genomic DNA, the primers were placed in consecutive exons with the probe spanning the junction between the exons. Primer and probe sequences for GAPDH were based on published sequences (Leutenegger et al., 1999). IL-8 and IL-12 p40 sequences were generously provided by C. Leutenegger and U.C. Davis. All primer/probe sequences are shown in Table 1. The PCR reactions contained 400 nM of each primer, 80 nM of each probe, commercially available PCR Mastermix (TaqMan[®] Universal PCR Mastermix, Applied Biosystems) and 5 μ l of diluted cDNA sample in a final volume of 25 μ l. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI 7700 Sequence Detection System, Applied Biosys-

Table 1
Primer and probe combinations for RT PCR

GAPDH.74f	AAGTGGATATTGTCGCCATCAAT
GAPDH.161r	AACTTGCCATGGGTGGAATC
GAPDH.108p	TGACCTCAACTACATGG- TCTACATGTTTTCA
CD23.495f	CCAGAACGTCTCTCAGGTTTCC
CD23.619r	CATCTGTTCTGCTCATCTTGGA
CD23.554p	AGGAATCCCAGGCTGCCAAGATGTC
IL8.160f	TGATTGAGAGTGGGCCACT
IL8.263r	ATAATCTGCACCCACTTTGTATGG
IL8.183p	CGAAAACCTCAGAAATCAT- TGTAAGCTCGTCAAC
IL12.40.638f	GGATGCTGTTTACAAGCTCAAG
IL12.40.739r	ATGGCTTCAGCTGCAGGTTT
IL12.40.687p	CATCAGGGACATCATCAAACCAGACCCA

Sense primers are designated by (f), antisense primers by an (r), and the probe is designated by a (p). All sequences are listed 5' to 3' orientation. Shaded areas on probe indicate the junction between neighboring exons. All probes labeled with 5' FAM and 3' TAMRA.

tems). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Relative quantification of the target was done using the signal for GAPDH, a housekeeping gene, in each sample. Raw data was analyzed using the $\Delta\Delta C_T$ method as follows: the value for each sample was normalized using the corresponding GAPDH value (sample value – GAPDH value) and all values for each set of conditions were calibrated using the value for the untreated control. Transcript quantification is reported as the *n*-fold difference relative to a calibrator cDNA (transcription in un-treated cells).

2.7. Statistical analysis

Due to the non-normal distribution of the data in this study the non-parametric method of Friedman analysis of variance by ranks was used.

3. Results

3.1. Up-regulation of CD23 in PBMCs

CD23 transcription increased more than 10-fold in PBMCs treated with rEq IL-4 when compared to PBMCs treated with LPS or untreated controls.

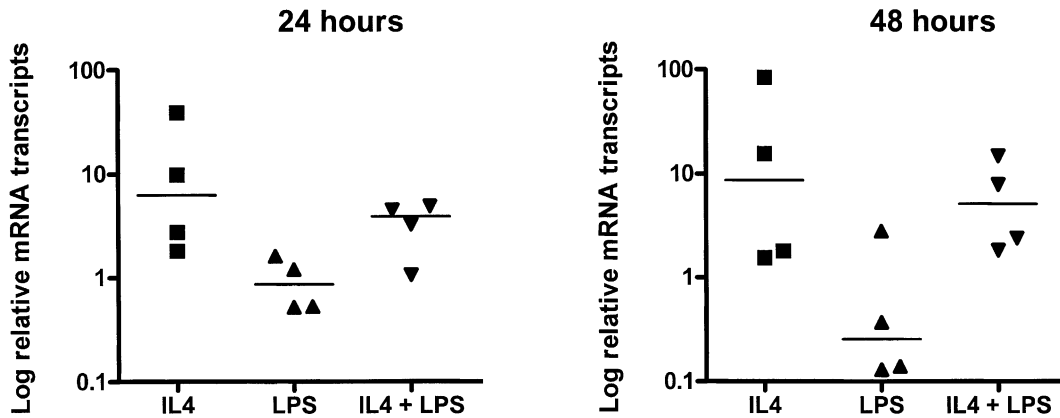


Fig. 1. Relative CD23 mRNA transcription levels in PBMCs cultured for 24 and 48 h with rEq IL-4, rEq IL-4 + LPS and LPS alone as indicated ($n = 4$). All relative transcript values were calibrated to the value for PBMCs in media alone. $P = 0.0062$.

These results were significantly different at the 24 and 48 h time points at the level of $P = 0.0062$ (Fig. 1).

3.2. Up-regulation of CD23 in pulmonary alveolar macrophages

When alveolar macrophages were cultured with rEq IL-4 or both rEq IL-4 and LPS for 24 or 48 h, the relative value for CD23 transcripts was increased 100–10,000-fold when compared to LPS alone or untreated controls. These results were significantly different at the 24 h time point at the level of $P = 0.0417$ and at the 48 h time point at the level of $P = 0.0017$ (Fig. 2).

3.3. IL-4 induced morphological changes in cultured pulmonary alveolar macrophages

Equine alveolar macrophages cultured with rEq IL-4 exhibited unique morphological characteristics compared to cells cultured with LPS alone or untreated controls (Fig. 3). This phenomenon was observed repeatedly during the course of our experiments. In general the macrophages cultured with rEq IL-4 appeared more stellate and fibroblast-like with long cellular projections. When equine monocyte progenitors are cultured with rEq IL-4 and rHu GM-CSF they develop extensive cellular projections, or dendrites and become only loosely adherent to plastic as they

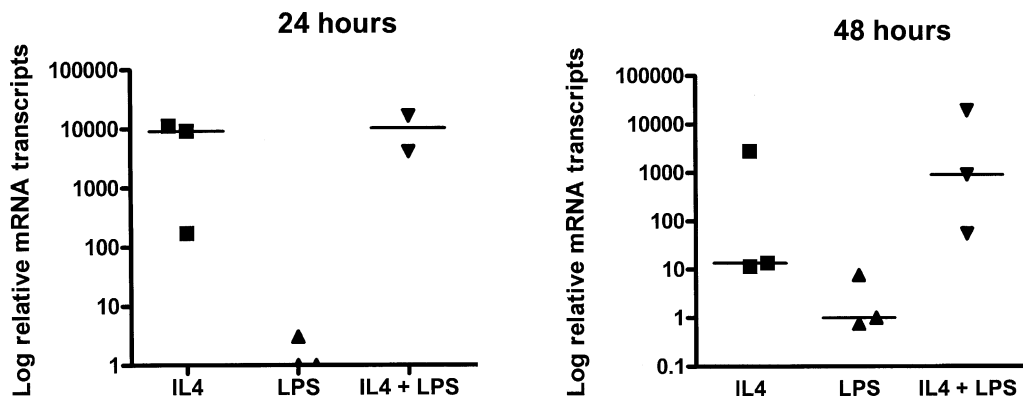
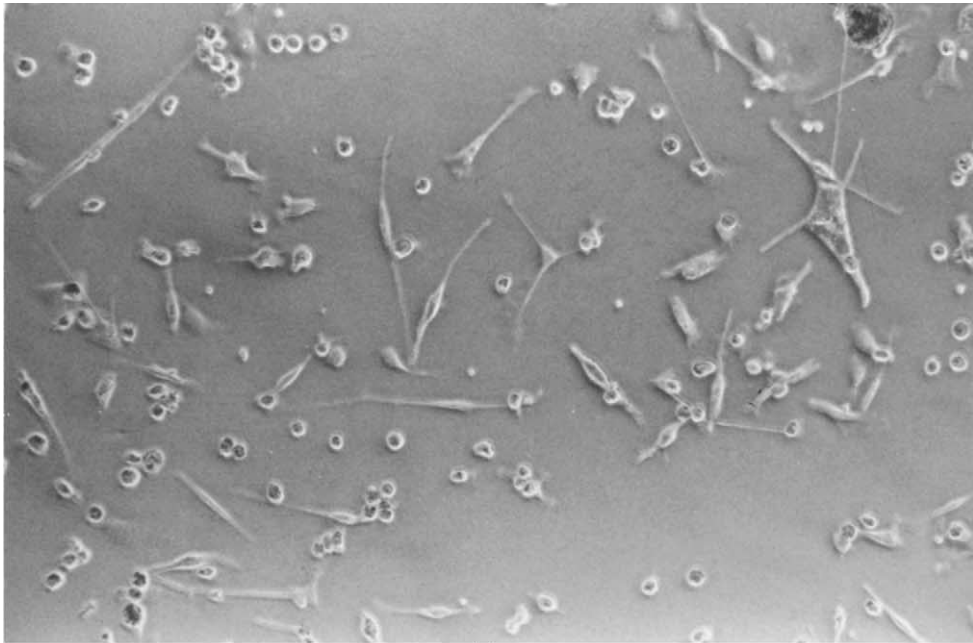
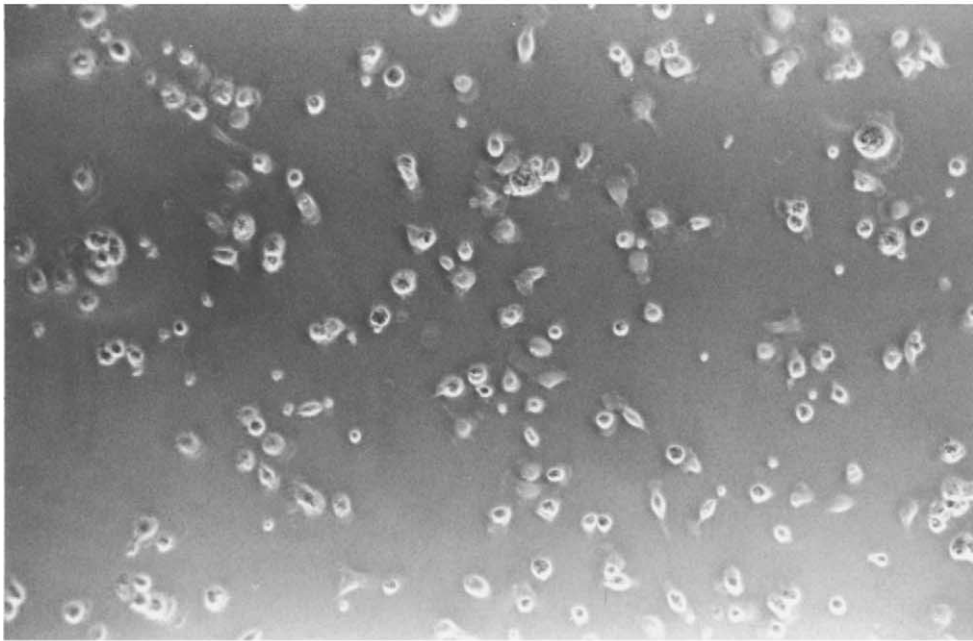


Fig. 2. Relative CD23 mRNA transcription levels in alveolar macrophages cultured for 24 and 48 h with rEq IL-4, rEq IL-4 + LPS, and LPS alone as indicated ($n = 3$). All relative transcript values were calibrated to the value for alveolar macrophages in media alone. 24 h: $P = 0.0417$, 48 h: $P = 0.0017$.



(a)



(b)

Fig. 3. Morphology of equine alveolar macrophages cultured with (a) rEQ IL-4 and (b) media alone for 48 h (400 \times). Photomicrographs were taken using a phase-contrast inverted microscope (Zeiss ICM-405, Oberkochen, Germany).

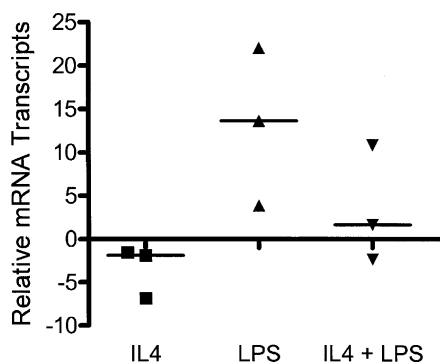


Fig. 4. Relative IL-8 mRNA transcription levels in alveolar macrophages cultured for 48 h with rEq IL-4, rEq IL-4 + LPS, and LPS alone as indicated ($n = 3$). All relative transcript values were calibrated to the value for alveolar macrophages in media alone. $P = 0.0174$.

mature into equine dendritic cells (Hammond et al., 1999). In contrast, these stellate macrophages were tightly adherent to plastic.

3.4. Down-regulation of IL-8 and IL-12 p40 transcripts in response to LPS

To investigate the activation state of macrophages in culture, pro-inflammatory cytokine transcripts produced in response to LPS stimulation were quantified. In alveolar macrophages cultured for 48 h, the relative value for IL-8 transcripts was at least four-fold lower in cells treated with rEq IL-4 and LPS compared to

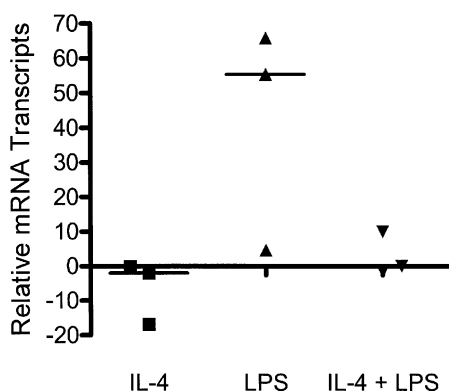


Fig. 5. Relative IL-12 p40 mRNA transcription levels in alveolar macrophages cultured for 48 h with rEq IL-4, rEq IL-4 + LPS, and LPS alone as indicated ($n = 3$). All relative transcript values were calibrated to the value for alveolar macrophages in media alone. $P = 0.0174$.

LPS alone (Fig. 4). These results were significantly different at the level of $P = 0.0174$. Similarly, the relative value for IL-12 p40 transcripts was at least 10-fold lower in cells treated with rEq IL-4 and LPS compared to LPS alone (Fig. 5). These results were significantly different at the level of $P = 0.0174$. While we were not able to investigate these cytokines at the level of protein, others have shown that the relative fold increase in cytokine transcript level closely reflects the fold increase in cytokine as measured by ELISA (Collins et al., 1999; Fehniger et al., 1999).

4. Discussion

IL-4 was first recognized as a B cell growth factor, but is now known to influence T lymphocytes, NK cells, and monocytes as well (Ohmori and Hamilton, 1994; Kaplan et al., 1996). IL-4 has been shown to modulate the expression of several cell surface proteins including CD23. IL-4 has been shown to induce transient expression of CD23 on human PBMCs (Vercelli et al., 1988; Yokota et al., 1988), enhance the antigen-presenting ability of human bone marrow derived macrophages (Zlotnik et al., 1987), and human CD23 expression is increased in a number of allergic disorders both on B cells and monocytes (Pforte et al., 1990; Gagro and Rabatic, 1994; Jung et al., 1995).

This study provides evidence that CD23 gene expression can be induced in equine alveolar macrophages and in peripheral blood mononuclear cells (PBMCs) by rEq IL-4. While the relative increase in CD23 transcripts was much larger in the rEq IL-4 treated alveolar macrophages, it should be noted that control PBMC samples were routinely positive for CD23 transcripts at a threshold cycle (C_T) between 22 and 26, 10 cycles earlier than control macrophages. This finding fits the model of two forms of CD23, a constitutively expressed form and an inducible form. In other species CD23 is constitutively expressed on eosinophils and a subset of B cells, and is inducible on monocytes (Bettler et al., 1989; Conrad, 1990; Williams et al., 1992). We have not addressed the expression of the CD23 protein on the cell surface and it is possible that post-transcriptional or post-translational regulation could affect the actual receptor density induced on the cell. There are currently no antibodies

available for equine CD23, nor are there any cross-reactive human or mouse monoclonal antibodies for CD23. Work in our laboratory continues to focus on the production of a monoclonal antibody against equine CD23.

Morphological changes were induced by rEq IL-4 in cultured equine alveolar macrophages that cannot be explained on the basis of CD23 up-regulation alone. Equine alveolar macrophages cultured with rEq IL-4 down-regulate IL-8 and IL-12 p40 gene expression in response. We were initially surprised to find morphologic changes in the IL-4 treated equine pulmonary alveolar macrophages but, after demonstrating the down-regulation of IL-8 and IL-12 p40 in these same cells, we were convinced that together these findings represented deactivation of macrophages by IL-4. IL-4 has been shown to suppress a broad range of inducible pro-inflammatory genes in monocytes and macrophages including TNF- α and IL-12 p40 in the mouse (Levings and Schrader, 1999). Deactivators of macrophages, including IL-4, IL-13, IL-10, and glucocorticoids, are important for the maintenance of a balanced Th1- versus Th2-type immune response to various infectious disease agents (Bogdan and Nathan, 1993; Barnes, 1998). In most cases the inhibitory effects of IL-4 have been shown to target the transcription of pro-inflammatory gene products, however, it has also been shown that IL-4 down-regulates the pattern recognition receptors TLR2 and TLR4 on human monocytes (Staege et al., 2000). Change in the activation state of equine macrophages, induced by IL-4, could have a significant impact on the outcome of equine infectious diseases which require a significant Th1-type response and/or early recognition of bacterial pathogens.

5. Conclusions

We demonstrate for the first time that rEq IL-4 will induce equine CD23 mRNA transcription in equine pulmonary alveolar macrophages and peripheral blood mononuclear cells. This finding supports the hypothesis that CD23 expression is a good indicator of local IL-4 activity and that an antibody to this equine cell surface antigen would be extremely useful in the study of IL-4 mediated immune responses. We also present evidence to support rEq IL-4 induced deacti-

vation of equine pulmonary alveolar macrophages. Future studies will attempt to further define this response through the use of equine IL-4 receptor antagonists or neutralizing anti-IL-4 antibodies.

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