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Proteomic Evaluation of Avian Peripheral Blood Monocytes for Functional Proteins

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Abstract: Monocytes as well as other professional Antigen Presenting Cells (APC), Dendritic Cells (DC) and macrophages, play a critical role in adaptive and innate immune responses. A Differential Detergent Fractionation (DDF) analysis was conducted on avian monocytes to reveal proteins related to cell adhesion, uptake and antigen presentation to lymphocytes, receptor proteins, proteases and cytokines. We identified a total of 3,229 proteins with 46 of these involved in the functions of professional APC. Of these proteins, fourteen were receptor proteins, four were related to antigen presentation (including MHC Class I), six to antigen uptake, ten to cell adhesion, two Toll-like receptors (TLR 4 and 15) and nine protease proteins were identified. This research demonstrates that the DDF approach provides meaningful, interpretable, functional, information concerning protein expression profiles associated with monocyte activation and differentiation into macrophages and/or immature DC in avian species. This data will be instrumental in future experiments evaluating protein expression of monocytes in stressed broilers.

Key words: Broiler, monocyte, functional proteins

INTRODUCTION

Monocytes (CD14+cells) originate in bone marrow from a specialized progenitor and make up 5 to 10% of the circulating leukocytes in humans (Seta and Kuwana, 2007). These cells are important players in immune defense due to their ability to phagocytose foreign material, present antigens to immunocompetent cells and produce an array of cytokines after stimulation with bacterial products via Toll-Like Receptors (TLR) (Seta and Kuwana, 2007; Lee *et al*., 2006). Mammalian monocytes serve as professional antigen presenting cells, as they express high levels of both Major Histocompatibility Complex (MHC) class I and II molecules, co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86), endocytic and phagocytic specific receptors and adhesion molecules (Kruger *et al*., 2003). Monocytes typically circulate in the blood for 10-20 h before migrating from the capillaries to the tissues. Once in the tissue, under influence of cytokines, monocytes differentiate into macrophages or Dendritic Cells (DC) (Kruger *et al*., 2003). Recent studies suggest that monocytes are not only precursors for macrophages and DC, but also for osteoclasts, microglia of the central nervous system and Kupffer cells of the liver (Seta and Kuwana, 2007).

Research indicates that avian monocyte development, much like its mammalian counterparts, is influenced by colony stimulating factors (Qureshi, 2003). Approximately 3 d after their arrival into the bloodstream, avian monocytes seed various tissues and organs (Qureshi, 2003). Blood monocytes are progenitors for 30% of avian alveolar macrophages and nearly 100% of avian Kupffer cells of the liver (Qureshi, 2003). Unlike mammals, chickens have no residential macrophages present in their abdominal cavity. However, monocytes can be recruited into the tissue in response to an active inflammatory signal at the tissue site (Qureshi, 2003). Avian monocytes can migrate toward chemotactic signals that arise from bacterial products, synthetic peptides, complement or certain products of an immune reaction and factors released by damaged cells and extracellular matrix (Qureshi, 2003). Avian monocytes recognize pathogens through TLR and display antibacterial properties such as nitric oxide production (Bowen *et al*., 2006; He *et al*., 2006). Genetic origin and differences in MHC have been shown to play a role in avian monocytes' chemotactic potential and that avian monocytes typically have an active response to chemotactic signals (Qureshi *et al*., 1988; Puzzi *et al*., 1990; Golemboski *et al*., 1995).

Our long-term goal is to understand the effects of stress on the innate immunity in chickens. In the presence of some stress, monocyte numbers are typically increased (Landmann *et al*., 1984), however their function and cytokine release is often decreased (Joyce *et al*., 1997). To this end, we need a basic measure of proteins expressed in monocytes, in order to go forward with our interests in understanding the effects of adrenal hormones, like corticosterone, on influencing the proliferation and migration of monocytes during stress. At present there is little information concerning protein expression profiles in avian monocytes. Therefore, we

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used proteomics methodology to obtain interpretable and meaningful information on the proteins expressed in normal chicken monocytes.

MATERIALS AND METHODS

Experimental facility: The experiments were conducted in a facility containing floor pens on a concrete pad with side wall curtains. All floor pens measured 1.5 x 2.9 m. Each pen contained one tube feeder, one bell type drinker and used litter supplemented with about 5 cm of new shavings. The facility temperature was maintained at a low of 69°F to a high of 80°F using curtain ventilation. The lighting program consisted of 22 h of light throughout the trial period.

Experimental design, bird husbandry and diets: One strain of commercial broilers was obtained from a local hatchery after being set and hatched in a common incubator. The vaccination program consisted of Marek's vaccination *in ovo*. Fifty broiler chicks were distributed into five pens in the experimental facility, each containing ten birds. All birds were fed a common diet throughout the trial period.

Monocyte isolation: Peripheral blood was collected into an EDTA anti-coagulation tube from twenty-five broilers bled via cardiac puncture. Monocytes were isolated according to methods used by Lee *et al.* (2008) with minor revisions. Peripheral blood mononuclear cells were isolated using Percoll (1083) gradients and separation occurred in tubes rather than Petri dishes. The $CD14^+$ monocytes were positively selected by using magnetic cell separation methodology (Lee *et al*., 2008). The total monocyte cell count was 4.2×10^7.

Protein extraction, trypsin digestion and mass spectrometry analysis: Proteins were isolated using the Differential Detergent Fractionation (DDF) methodology as described by Lee *et al.* (2006). A series of detergents were used to extract proteins from cellular compartments. Repeated washes with digitonin buffer were used for cytosolic protein isolation. This is followed by isolation of the membrane, nuclear and cytoskeletal proteins by triton X-100 (TX), Deoxycholate (DOC), Tween 40 and SDS buffers. For each of the detergent fractions, equal amounts of protein were precipitated with 25% tricholoracetic acid to remove salts and detergents. Protein pellets were solubilized and then digested with 100 ng^{-1} trypsin (50:1 ratio of substrate to enzyme) overnight at 37°C. Peptides were desalted using a peptide microtrap (Michrom, BioResources, Inc.) and eluted by 0.1% triflouroacetic acid, 95% acetonitrile solution. Desalted peptides were dried and resuspended in 0.1% formic acid.

The Mass Spectrometry (MS) analysis was done as described by Lee *et al.* (2006). Liquid Chromatography (LC) analysis was accomplished by Strong Cation Exchange (SCX) followed by Reverse Phase (RP) LC coupled directly in-line with Electrospray (ESI) ion trap mass spectrometer. Samples were loaded into a LC gradient ion exchange system including a Thermo Separations P4000 quaternary gradient pump (ThermoElectron Corporation) coupled with a 0.32 x 100 mm BioBasic SCX column. A flow rate of 3µL/min was used for both SCX and RP columns. A salt gradient was applied in steps of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 57, 64, 71, 79, 90, 110, 300 and 700 mM^{-1} ammonium acetate in 5% acetonitrile, 0.1% formic acid and the resultant peptides were loaded directly into the sample loop of a BioBasic C18 RP LC column of a Proteome X workstation (ThermoElectron). The RP gradient used 0.1% formic acid in acetonitrile and increased the acetonitrile concentration in a linear gradient from 5-30% in 30 min and then 30-65% in 9 min followed by 95% for 5 min and 5% for 15 min. The spectrum collection time was 59 min for every SCX step. The LCQ Deca ion trap mass spectrometer was configured to optimize the duty cycle length with the quality of data acquired by alternating between a single full MS scan followed by three tandem MS scans on the three most intense precursor masses from full scan. The collision energy was normalized to 35%. Dynamic mass exclusion windows were set at 2 min and all of the spectra were measured with an overall mass/charge (m/z) ration range of 200-2000.

Protein identification and analysis: Mass spectra were analyzed as previously described (Lee *et al*., 2008). Briefly, the search term Gallus gallus was searched against the organism field of the National Center for Biotechnology Information to create a chicken-specific protein database. The mass spectra were compared to the chicken-specific protein database using the TurboSEQUEST (Bioworks Browser 3.1; Thermo Electron) at the MSU Life Sciences and Biotechnology Institute. Each peptide was considered genuine if Delta Cn values were >0.1 and the presence of a C-terminal lysine or arginine. Alignments of amino acid sequences were conducted with the Clustal W version 1.81 program (Thompson *et al*., 1994).

RESULTS AND DISCUSSION

A total of 3229 proteins have been identified. The majority of these proteins (1,265 or 39%) were found in the nuclear compartment of the cells. The membrane compartment contained 30% (978), with 26% (831) identified in the cytosolic and 5% (155) found in the cytoskeleton (Table 1). Results indicate that of the 3,229 total proteins identified, 46 were involved in immune

Table 1: Sub-cellular location of identified proteins

Cell compartment	Number of identified proteins	% of total protein profile
Cytosolic	831	26%
Membrane	978	30%
Nuclear	1265	39%
Cytoskeleton	155	5%

Table 2: Proteins organized by immune function

Protein	DDF	Accession number	Peptide
Toll-like receptors			
TLR4	T	XP_415518	NKHSMICHTPAYMK
TLR15	TD	XP_419294	TEENKTSPPAATLR
Cell adhesion proteins			
αV integrin (CD51)	T	NP_990770	SHQWFGASVR
	TD		VNAALEVNPTILNPENK
			EPVGTCYLFDGSK
			ILACAPLYHWR
			ACSLADVKVSCFKVK
			GKLPNSLNFQVELLLDK
α 11 integrin	TD	XP_413930	WSTSSCKGPFR
ß1 (CD29)	D	XP_418572	RVLEDREVTNR
ß5 integrin	TD	NP_989814	DSKNIIELIVK
CD47	T	XP_416623	IYRHESVPSANFLSK
Contactin 6	TD	XP_425170	RGMPHFER
Cell-adhesion	T, TD	XP_422207	TLPYHNKYYWIGIRK
Protocadherin 18	T, D, TD	XP_420404	FRAMQRGNSPLLVVR
cHz cadherin	T, S, TD	XP_418340	RWHNIKIK
Hyaluronan receptor	T, S, TD	XP_414495	KMSSLCMELMKLR
Proteases			
ADAM 8	D	XP_421552	GDCCQDCKVKAAGVLCR
ADAM 12	T, TD	XP_423549	KPLPADPLNK
ADAM 17 (TACE)	TD	XP_419944	MLLEQFSFDIAEK
ADAM 20	TD	XP_428276	GGSCLYQAPALGSYYTL
ADAMTS-5	TD	XP_425541	EKGLEVNR
ADAMTSL-3	TD	XP_413844	LIGNDNRLIEPPNLR
MMP3	D	XP_417175	KIDAAVHDQNTK
MMP27	D, TD	NP_990331	EVVDKAIQK
Heparanase	S	NP_989498	FGGTSTDFLIFNPNK
Antigen uptake			
ced-12/ELMO	T	XP_417479	AFEELFAICIQLLNK
MR CD206	D	XP_001235095	QNAKWENQACNQR
MR CD280	TD	XP_418071	MCSDYGSTLVTITNR
			WSDGLGFFYHNFDR
CD36	TD	XP_415975	NNFIQLLLNTWIK
Clathrin heavy chain	TD	XP_415878	IHEGCEEPATHNALAK
Antigen presentation			
MHC class I	T, TD	AF013493	AHGFYPRPIVVSWLK
B-G	D	AAA48627	ILASKLMKQMEK
	TD	AAA48618	HFQNMYLSAGK
Rfp-Y	TD	XP_415354	ATLKRSVQPEVR
		AAP33136	WKHELGTVCVQNLR
Calnexin	TD	XP_414608	CGEDYKLHFIFR
Receptors			
CD40	T	NP_989996	VKGTNTSDVICESSRR
IGF	T	NP_990363	MCWQYNPKMR
Leptin	T	NP_989654	MLIPSEMSISASQER
Notch	D	XP_415420	CEGDVNECLSNPCDAR
C-delta	S, TD	NP_990304	NEDSVKEEHGK
RORß	D	XP_425885	QRNCLIDRTNR
Opioid growth factor	D	XP_425708	KSEDACAAQAALLLSAGR
IL-20R α	TD	XP_419723	LKMADTVDELLGKGR
Oncostatin M (ß-subunit)	T, TD	XP_425020	DAELVMSFEIQVRR
M-CSF (CD115)	TD	XP_414597	EDSVLKVAVKMLK
Megalin	TD, S	XP_422014	QDLIKTK
Somatostatin	T	XP_426102	MRAVAQRVGWQQR
FAS	TD	XP_421659	LIHIDVDLTHHVPDIVR
Osteoprotegerin	TD	XP_418394	QVMCNQCPPGSYVK

Legend: DDF; Differential Detergent Fractionation, D; Digitonin, T; Triton X-100, TD; Tween 40-deoxycholate, S; Sodium dodecyl sulfate

functions of the professional APC. In Table 2, proteins were categorized into seven groups according to immune function. Of the proteins, two TLR, ten cell adhesion, nine proteases, six antigen uptake, four antigen presentation and fourteen receptor proteins were identified and compared to the database. The following discussion is based on the comparison of the chicken monocyte proteins to their mammalian counterparts in order to create a basis for understanding the function of normal avian monocytes.

Toll-like receptor proteins: TLR are the key component of a signaling pathway which serves as the frontline subsystem against invasive microorganisms for both innate and adaptive immune responses (Pinchuk *et al*., 2007). These receptors, which are widely expressed by leukocytes and epithelial cells, function through recognition and interaction with conserved motifs expressed on the surface of pathogen associated molecular patterns, such as lipopolysaccharides (Higgs *et al*., 2006). This TLR-LPS interaction allows the receptor to discriminate between microbial pathogens or their products and to initiate trans-membrane signaling (Laflamme and Rivest, 2001). There are over eleven identified TLR in mammals, which recognize a variety of ligands from pathogens to trigger immune responses (Oda and Kitano, 2006). Some TLR (1, 2, 6, 4, 5) are located on the surface of the plasma membrane, while others are cytoplasmic or intracellular (TLR 3, 7, 9) (Akira and Takeda, 2004).

In the chicken, TLR 1, 2, 3, 4, 5, 7 have been shown to be expressed by heterophils (Kogut *et al*., 2005). Toll-like receptor 4 (Table 2), one of two TLR identified herein plays a key role in the response to Gram-negative bacteria during pathogen invasion (Laflamme and Rivest, 2001) through recognition of the LPS associated outer membrane (Poltorak *et al*., 1998). Expression of TLR-4 is upregulated by Gram negative bacteria, especially in granulocytes and monocytes. The other TLR identified, TLR-15 (Table 2), appears to be avian specific (Higgs *et al*., 2006). This TLR has been found on chromosome 15 and is characterized by an archetypal toll-interleukin receptor, transmembrane domains and a distinctive arrangement of extracellular leucine rich regions. Researchers believe that TLR-15 plays an important role in the avian defense against bacteria, as it has been shown to be upregulated in the presence of *Salmonella enterica* (Higgs *et al*., 2006).

Cell adhesion proteins: Integrins are receptors for different Extracellular Matrix (ECM) components depending on different combinations of alpha and beta chains. When binding to the ECM, integrins transmit outside - in signals resulting in cytoskeletal rearrangements which facilitate cell motility.

We found the expression of the αV and α11 subunits and the ß1 and 5 subunits (Table 2), which predict at least three integrin heterodimers, αß1, αVß5 and α11ß1 with specificity for the ECM components collagen, fibronectin and osteopontin (Humphreys *et al*., 2006).

Protease proteins: Chicken monocytes express a number of surface and secreted proteases that may facilitate entry into the tissues from the bloodstream. Several members of the protease family A Disintegrin and Metalloprotease (ADAM) along with the Membrane Metalloproteases (MMP) are surface expressed on leukocytes and recognize ECM components as substrates (Stefanidakis and Koivunen, 2006). The binding of monocyte integrins to ECM proteins induce outside-in signaling through integrins resulting in upregulation in expression of proteases for movement through the endothelial basement membrane (Sudhakaran *et al*., 2007).

Antigen uptake proteins: Chicken monocytes express a number of receptors which may be important for antigen uptake and clearance upon differentiation to macrophages. Peptides derived from the chicken homologue of the CD36 scavenger-type receptor typical of the myeloid lineage were identified (Table 2). CD36 is important in clearance of apoptotic cells (Bottcher *et al*., 2006) and for oxidized lipoproteins (De Villiers and Smart, 1999). The peptides for two members of the mannose receptor family were identified in the digitonin and Tween 40-deoxycholate detergent fractions (Table 2). Mannose receptors function as phagocytic pattern-recognition receptors in innate immunity with specificity for mannose and fucose residues on prokaryotic glycoproteins and glycolipids (McGreal *et al*., 2005). The predicted CD206 protein showed 48% amino acid homology in Clustal alignments with human C-type 1 mannose receptor (accession number NP_001009567). The predicted CD280 protein showed 72% amino acid homology with human (accession number NP_006030) C-type 2 collagen receptor.

Antigen presentation proteins: The B-complex encodes the MHC class I (BF) and class II (BL) antigen-presenting glycoproteins (Pink *et al*., 1977; Vainio *et al*., 1984) that are linked to the highly polymorphic *BG* proteins (Kaufman *et al*., 1990; Miller *et al*., 1991). Also on the same chromosome are the Y-complex genes (Briles *et al*., 1993) which function similar to minor MHC proteins (Thoraval *et al*., 2003).

Evidence for the expression of MHC class I in monocytes was obtained with the observation of a BF-specific peptide which is found in the α3 domain of the BF2 gene product (Guillemot *et al*., 1988; Hunt and Fulton, 1998), which is the predominantly expressed BF gene (Hunt and Fulton, 1998; Fulton *et al*., 1995).

Two BG-specific peptides were found in the data set. The peptide identified from proteins in the digitonin detergent fraction is encoded in the cytoplasmic region of the bg17 transcript from the B21 haplotype (Miller *et al*., 1991). The peptide identified from proteins in the Tween 40-deoxycholate could have come from the product of the bg3 or bg14/8 genes. Both genes are expressed at the protein level in the B21 haplotype (Miller *et al*., 1991).

Previous studies have identified the expression of YF genes in various chicken tissues at the RNA and protein level (Afanassieff *et al*., 2001; Hunt *et al*., 2006). Two YF-specific peptides were identified from proteins isolated with the Tween 40-deoxycholate detergent. The peptide "ATLKRSVQPEVR" spans the border of the α2 / α3

domains in the predicted amino acid sequence from the jungle fowl YF gene (accession number XP_415352). The other YF-specific peptide is found within the α3 domain of the YF genes reported by Thoraval *et al.* (2003).

Receptor proteins: The tryptic peptides from 14 receptor type proteins were found in the different detergent fractions analyzed by mass spectrometry in this study (Table 2). These proteins have been shown to enhance the immunological functionality of mammalian monocytes.

The TNF family member CD40 and its ligand have been identified and characterized in the chicken (Tregaskes *et al.*, 2005; Kothlow *et al.*, 2008). Evidence shows that expression of CD40 by activated monocytes and dendritic cells or differentiated monocytes is involved in enhanced cytokine production (Banchereau *et al.*, 1994). Chicken monocytes express CD40 (Kothlow *et al.*, 2008) and produce nitric oxide when stimulated with a fusion protein consisting of chicken CD40L, mimicking the interaction of monocytes with TH1 helper T-cells (Tregaskes *et al.*, 2005).

In chickens, the receptor for leptin has been well characterized (Niv-Spector *et al.*, 2005). The role of leptin in regulating feed intake in chickens has been well established (Denbow *et al.*, 2000). In the immune system, the leptin receptor expressed in human peripheral blood mononuclear cells is responsible for mediating leptin activation of monocytes leading to production of pro-inflammatory cytokines (Zarkesh-Esfahani *et al.*, 2001). Leptin can also stimulate production of reactive oxygen species as a result of monocyte activation (Sanchez-Pozo *et al.*, 2003).

The role of the Notch family of proteins in the chicken hematopoietic system has only been described for B-cell development in the bursa of Fabricius (Morimura *et al.*, 2001). It has been shown that mammalian monocytes express relatively high amounts of Notch-1 and Notch-2 and through the immobilized extracellular domain of the Notch ligand, Delta-1, may induce apoptosis in peripheral blood monocytes cultured with macrophage colony-stimulating factor (M-CSF), but not granulocyte-macrophage CSF (GM-CSF). Data indicates a key role for Notch signaling is in regulating cell fate decisions by bi-potent macrophage/dendritic precursors (Ohishi *et al.*, 2001).

The chicken IL-20Rα receptor protein identified by the peptide in the dataset (Table 2) shows 39% amino acid identity with the human IL-20Rα chain protein, termed IL-20R1 (accession number Q9UHF4). In mammals the IL-20 receptor consists of the IL-20R1 ligand-binding chain and the IL-20R2 signal transduction chain (Nagalakshmi *et al.*, 2004). The IL-20 receptor belongs to the class II cytokine receptor family and is responsible for binding IL-20 which has been shown to be produced by monocytes during incubation with TNF-α or LPS (Wang *et al.*, 2006; Wolk *et al.*, 2002). Research shows that IL-20 functions as a key regulator of keratinocytes proliferation during skin inflammatory responses (Wang *et al.*, 2006).

The predicted protein for the ß-chain of the chicken Oncostatin M (OSM) receptor showed 35% homology with the human OSM ß-chain protein (accession number AAC50946). Oncostatin M (OSM), a 28-kd cytokine in the IL-6 family, has been shown to be produced by macrophages and activated T-cells at sites of major inflammation. One study revealed enhanced levels of OSM, in synovial fluid from rheumatoid arthritis lesions. Oncostatin M played a key role in chronic joint inflammation through regulation of TIMP-1, MMP-1, as well as monocyte chemotactic protein-1 in synovial fibroblasts in vitro (Langdon *et al.*, 2000).

The predicted protein for chicken Monocyte Colony Stimulating Factor (M-CSF) showed 53% identity with the human M-CSF protein (accession number P07333) in Clustal alignments. In mammals M-CSF is responsible for differentiation of mononuclear phagocytes to macrophages. Activated M-CSF receptor associates with phosphatidylinositol 3-kinase (PI 3-kinase) and induces direct interaction of PI 3-kinase with SH2/SH3 adaptor protein Grb2. Saleem *et al.* (1995) show that M-CSF induces the formation of a PI 3-kinase-Grb2-Sos complex, supporting a potential role of PI 3-kinase in Ras signaling pathways in monocytes.

Somatostatin functions in the control of pituitary hormone release in the chicken (Geris *et al.*, 2000). Bhathena *et al.* (1981) demonstrated binding sites for somatostatin on lymphocytes and monocytes suggesting a role for somatostatin receptors in these cells of the human immune system. Dalm *et al.* (2003) found in one study that during differentiation of monocytes to macrophages or dendritic cells, time-dependent, significantly increased mRNA levels of somatostatin receptor-2 and cortistatin, a somatostatin-like peptide.

Park *et al.* (2003) revealed that FAS (CD95) can activate proinflammatory cytokine responses in normal human monocytes and macrophages. Following Fas ligation, both monocytes and monocyte-derived macrophages released TNF-α and IL-8 and conditioned medium from Fas-activated monocytes and macrophages induced the directed migration of neutrophils in a chemotaxis assay. Fas-induced monocyte cytokine responses were associated with monocyte apoptosis, nuclear translocation of NF κ - B and cytokine protein expression and were blocked by caspase inhibition but not by inhibition of IL-1ß signaling (Park *et al.*, 2003).

In chickens the receptor for osteoprotegerin has only been observed in ovarian tissues, primarily the postovulatory follicle (Bridgham and Johnson, 2003). In mammals osteoprotegerin is also an important

regulator of differentiation and activation of osteoclasts that also affects different cells of the immune system. It has been shown to significantly stimulate monocyte chemotaxis; however preincubation of monocytes with osteoprotegerin was shown to inhibit monocyte migration toward optimal concentrations of T-cell secreted monocyte chemotactic protein 1 and procalcitonin (Mosheimer *et al*., 2005). Seshasayee *et al*. (2004) found that osteoprotegerin ligand will up-regulate receptor activator of NF- κ B (RANK) receptor expression on monocytes, regulate their effector function by inducing cytokine and chemokine secretion, activate antigen presentation through up-regulation of co-stimulatory molecule expression and promote monocyte survival.

Conclusion: Our data suggest that chicken monocytes express proteins required for migration into vascularized tissues and differentiation into macrophages or immature dendritic cells. Future studies will be required to confirm the expression of the products identified by mass spectrometry.

In the presence of stress, monocyte numbers are typically increased (Landmann *et al*., 1984), however monocyte function and cytokine release by monocytes is often decreased (Joyce *et al*., 1997). Future studies with protein expression may be beneficial in determining why monocytic function is suppressed during stress.

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