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Macrophage effector responses of horses are influenced by expression of CD154

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Abstract

Reactive intermediates contribute to innate immunity by providing phagocytes with a mechanism of defense against bacteria, viruses and parasites. To better characterize the role of CD154 in the production of reactive intermediates, we cloned and expressed recombinant equine CD154 (reqCD154) in Chinese Hamster Ovary (CHO). In co-culture experiments, CHO cells ectopically expressing reqCD154 elicited superoxide production in monocyte-derived macrophages (MDM). Collectively, our results indicate that regulation of CD154 expression plays a role in innate host defenses.

Keywords

CD154; Reactive intermediates; Equine

Introduction

One mechanism by which macrophages and dendritic cells eliminate pathogens is through reactive oxygen and nitrogen species. These free radicals are important components of the innate immune response of these phagocytic cells and help limit infection by an array of pathogens, including bacteria, viruses, and parasites. Reactive oxygen species (ROS) mediate antibacterial activity primarily by DNA damage to bacteria via hydroxyl or ferryl radicals produced by the Fenton reaction (McCormick et al., 1998). Additional reactive

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intermediates of oxygen and nitrogen, some cooperatively derived, such as peroxynitrite, also contribute significant antimicrobial activity (Fang, 2004; Beckman and Koppenol, 1996). Given the importance of the CD40/CD154 interaction, we initiated studies to characterize equine CD154 in order to determine its role in activation of equine macrophages using a functional assay for evaluation of macrophage activation.

CD40L ligand (or CD154) is a 33 kD, Type II transmembrane protein with sequence homology to the tumor necrosis factor (TNF) superfamily of cell surface lymphocytes, natural killer cells, eosinophils, basophils, mast cells, dendritic cells, monocytes, macrophages, fibroblasts, platelets and endothelial cells (Lee et al., 2003; Noelle, 1996; Tuladhar et al., 2011; Henn et al., 1998). CD154 binds to CD40, a 50 kD Type I transmembrane receptor phosphorylated glycoprotein that is a member of the TNF receptor superfamily (Grewal and Flavell, 1998). The CD40 receptor is expressed on the surface of monocytes, macrophages, thymic epithelial cells, B lymphocytes, platelets and dendritic cells (Noelle, 1996; Grewal and Flavell, 1998). Binding of CD154 with CD40 contributes to the activation of antigen presenting cells (Lee et al., 2003; Banchereau et al., 1995; Caux et al., 1994a; Caux et al., 1994b; Cella et al., 1996). Without CD154, antigen presentation by both dendritic cells and macrophages is markedly impaired, as is macrophage-mediated killing of both intracellular and extracellular pathogens (Noelle, 1996; Grewal and Flavell, 1998; Murray et al., 2003). The notion that the CD154/CD40 interaction is necessary for optimal presentation of antigen is supported by *in vivo* studies demonstrating that ligation of CD40 on dendritic cells by CD154 on T cells plays an important role in dendritic cell longevity, T Cell Receptor (TCR) engagement, and clonal T cell expansion, all important aspects of up-regulation of sustained cell-mediated immune responses (Miga et al., 2001). Moreover, in the presence of IFN- γ , macrophages upregulate expression of CD40 which further enhances the effect of the CD40/CD154 interaction (Lee et al., 2003; van Essen et al., 1995). Ligation of CD40 by CD154 also leads to enhanced APC expression of B7 molecules and secretion of cytokines, such as IL-12, that promote T cell differentiation. In addition to activation of macrophages, antigen presentation by B cells is also increased by CD154/CD40 interaction. A feedback mechanism has been identified by which T cells up-regulate CD154 expression and enhance B cell activation via ligation of CD40 with a net effect of increased B cell division, antibody isotype class switching, and differentiation of B cells to plasma cells (Clark et al., 1996; Foy et al., 1993). Thus, for macrophages, dendritic cells, and B cells, ligation of CD40 by CD154 enhances activation, presentation of antigen, and other cell-specific effector functions, including production of reactive intermediates. In this report, we describe the cloning, sequence analysis, and expression of equine CD154 in Chinese hamster ovary (CHO) cells and characterization of the recombinant protein by immunofluorescence. In addition, we measure the production of superoxide by equine monocyte derived macrophages co-cultured with CHO cells expressing CD154 as a sensitive and functional means of measuring activation of equine macrophages by co-stimulation with CD154. Given the importance of the CD154/CD40 interaction in basic macrophage-lymphocyte “cross-talk”, we evaluated ectopic CD154-induced production of reactive intermediates by macrophages as an indicator of macrophage responsiveness to CD154 stimulation. Our studies indicate that ectopic expression of equine CD154 elicits robust

production of reactive intermediates by equine macrophages and that the CD154/CD40 interaction could play an important role in equine microbicidal macrophage host defenses.

2. Materials and methods

2.1 Horses:

Three Thoroughbred mares > 6 years of age were used as donors of peripheral blood mononuclear cells (PBMC). An adult male, castrated horse was used as a donor of PBMC for the cloning of CD154. Samples were collected in accordance with an IACUC approved protocol.

2.2 Lymphocyte culture

One of two methods based on density centrifugation was used to obtain equine peripheral blood mononuclear cells (PBMC) for isolation of lymphocytes. In both instances, whole blood was obtained by jugular venipuncture. For a large volume of cells, mononuclear leukocytes were banded by centrifugation at $750 \times g$ on a discontinuous double histopaque gradient; for a small volume, BD Vacutainer® CPT™ Cell Preparation Tubes (Becton, Dickinson and Company) were used. PBMC were collected and resuspended in Dulbecco's MEM (DMEM) containing 20% fetal bovine serum, and seeded in cell culture plates. Cells were maintained in phenol free DMEM containing 20% horse serum, 10% NCS and antibiotics

2.3 Monocyte derived macrophages

Blood samples.—180 mls of whole blood were obtained by jugular venipuncture from each foal. 450 mls of blood were obtained from the adults. Buffy coats were collected by centrifugation of whole blood in 50 ml conical tubes at 800 g for 30 min at 25°C. The resultant buffy coats for each animal sampled were aliquoted into two 50 ml conical tubes, diluted with 50 ml autologous plasma, and centrifuged at 800 g for 30 min at 25°C. Buffy coat cells were layered onto a double gradient of Histopaque-1077 and - 1119 (1.077 g/mL and 1.119 g/mL, respectively) (Sigma-Aldrich, St. Louis, MO) and the mononuclear cell and granulocyte fractions were obtained using a previously described protocol (Raabe et al., 1998; Smith et al., 1998; Sponseller et al., 2007). To remove platelets from the mononuclear cell fraction, a rinse with Hank's Balanced Salt Solution (HBSS) was performed. Monocyte enrichment and culture of macrophages were performed using a technique previously described (Sponseller et al., 2007). Briefly, 1×10^8 buffy coat cells were subjected to AutoMACs separation by first resuspending the cells in 1 ml of cold MACs buffer (PBS with 0.5% bovine serum albumin and 2 mM EDTA). Enrichment of monocytes was performed with a primary antibody (25 µg/ml VMRD DH59B/equine CD172a) and 100 µl/ml goat anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, CA) according to the company's protocol. Monocytes were allowed to adhere overnight at 37 °C and cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Corporation, Carlsbad, CA) containing 20% NCS and antibiotics. Adherent cells were incubated for 24 hours prior to co-culture experiments.

2.4 Cloning of equine CD154

A 780 nucleotide region of the CD154 gene was amplified from RNA using primers designed to amplify conserved regions as determined from sequence data available from CD154 GenBank. Primers had the following sequence: forward primer: 5'-GTTATGATAGAAACATACAGCCAACCTT-3' and reverse primer: 5'-TCAGAGTTTGAGTAAGCCAAAAGAT-3'. Total RNA was isolated from PBMC stimulated with 5 µg/ml concanavalin A (Con A) using a commercially available kit (QIAamp RNA Blood Mini Kit, Venlo, Netherlands). The RNA was then resuspended in 25 µl RNase-free glass distilled water containing 0.1 mM EDTA. Samples were treated with DNase by adding two units of DNase I (Ambion, Austin, TX) to 3 µl of viral RNA, 20 mM MgCl₂, 1 mM of each dNTP, 1X PCR buffer II (Perkin-Elmer, Branchburg, NJ), 20 units of RNase inhibitor, and 2.5 mM of random hexamers in a total volume of 20 µl. The reaction was incubated at 37° C for 30 mins and heated to 75° C for 5 mins to inactivate the DNase. After cooling to 4° C, 50 units of Moloney murine leukemia virus reverse transcriptase were added. Reactions were incubated at 42° C for 45 mins, heated to 99° C for 5 mins, and then cooled to 5° C for 5 mins. Reactions were then brought up to 100 µl with 2 mM MgCl₂, 0.2 mM of each dNTP, 1X PCR buffer II, 2.5 units of Taq polymerase, and 1 µM of each primer. PCR amplification conditions were 37 cycles of denaturation at 94° C for two mins, annealing at 50° C for 1 min, and extension at 72° C for 1 min. The initial and final cycles contained a prolonged extension at 72° C for 5 mins. Two µl of PCR product were ligated into the pCR-8/GW/TOPO-TA vector according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA) and transformed into *E.coli Top 10*® cells (Invitrogen, Carlsbad, CA). Positive clones were identified by PCR by amplifying a subgenomic fragment with internal primers designed for conserved regions of CD154. Plasmids were isolated from positive clones and the CD154 inserts were sequenced bidirectionally with primers to vector sequences flanking the insert. DNA sequencing was performed using an automated DNA sequencer at the Iowa State University DNA Synthesis and Sequencing Facility. Sequences were aligned using Vector NTI, version 10 software (Invitrogen, Carlsbad, CA).

2.5 Transfection of CHO cells and production of recombinant protein

To obtain the recombinant equine CD154 protein for the purpose of in vitro assays, CHO cells (ATCC CCL-61™) were cultured at a concentration of 1×10^6 cells/ml in 60 mm diameter Petri dishes (Fisher Scientific, Waltham, MA) in antibiotic free DMEM with 10% fetal bovine serum. Prior to transfection, the lethal dose of Geneticin® for CHO cells was determined according to the manufacturer's guidelines for the expression plasmid used, pcDNA3.2 V5 DEST (Invitrogen, Carlsbad, CA), which contains a neomycin resistance gene conferring resistance of transfected cells to Geneticin®. The cells were transfected with pcDNA3.2 V5 DEST containing recombinant equine CD154 using chemical transfection (TransIT-LTI, Mirus Bio, L.L.C., Madison, WI) according to the manufacturer's instructions, and cultured for 48 h at 37 °C in a humidified incubator with 5% CO₂ before the first change of media. Selection of stably transfected cells expressing the recombinant equine CD154 protein was performed in media containing Geneticin® (125 µg/ml) after two weeks of incubation. Cell clones were expanded under similar conditions and used as described.

2.6 Immunofluorescence

CHO cells expressing CD154 were fixed with 2% paraformaldehyde for 10 mins. Cells were washed twice with PBS and incubated for 20 mins with PBS containing 10% FBS (blocking buffer). Incubation with the primary antibody, a human CD154-specific monoclonal mouse antibody (24–31) (Millipore, San Diego, CA, USA), diluted 1:500 in blocking buffer, was performed for 1 hr. The cells were washed twice with PBS and a 30 minute incubation with a rabbit F(ab')₂ anti-mouse IgG:FITC secondary antibody diluted 1:100 was performed. CD154 protein was visualized on an Olympus IX-61 inverted microscope equipped with blue, green, and red filter sets with a cooled CCD camera.

2.7 Quantitation of production of superoxide

Oxidation of dihydroethidium (hydroethidine) to oxyethidium by superoxide was used as an indicator of intracellular superoxide production. One $\times 10^5$ equine monocyte- derived macrophages were seeded into a 96-well flat bottom plate, incubated overnight in 100 μ l RPMI containing 20% fetal bovine serum without phenol red to avoid interference with fluorimetry readings (37°C., 7% CO₂). Plates were then co-cultured for 24 hrs with 2.0×10^4 CHO cells (both expressing and not expressing CD154) with or without IFN- γ (10 ng/ml) as a positive control. Plates were then incubated for 15 mins with 43.6 μ M dihydroethidium (DHE; Invitrogen, Carlsbad, CA) per well at 37°C in the dark. Supernatants were aspirated and fluorescence of the cellular monolayer was measured with a Fluostar Omega fluorimeter (BMG Biotech) at excitation/emission spectra of 518/606 nm, respectively. Experiments were performed in replicates of four and repeated. Data are presented as mean $\pm$ standard deviation with significance defined as $p < 0.05$.

2.9 Sequence analysis

Multiple clones were sequenced; the CD154 open reading frame was translated, aligned, and analyzed using Vector-NTI (Life Technologies™, Grand Island, NY). Sequences were aligned with and compared to available GenBank data for other species.

3. Results and Discussion

3.1 cDNA cloning and sequence analysis

Sequence alignment of the translated equine sequence [accession number here pending acceptance of manuscript] revealed distinct regions of homology with those of ovine, bovine, porcine, feline, canine, murine and human species (Fig 1). Similar to other species, it appears that equine CD154 also has a hydrophobic signal anchor region typical of Type II membrane proteins. Trimerization of the CD154 subunits is due in large part to these hydrophobic residues (An et al., 2011). In contrast to human CD154, which contains 2 potential N-linked glycosylation sites, equine CD154 contains but one site, with a similar location as described in other domestic species. In the horse it is unknown as to whether or not the putative N-glycan sites are glycosylated.

3.2 Expression of CD154 in Chinese hamster ovary (CHO) cells

For the expression of equine CD154, the DNA product ligated into pCR-8/GW/TOPO-TA was amplified by PCR and subcloned into pcDNA3.2 V5 DEST. Visualization of CHO cells transfected with the equine CD154 construct(s) using the primary human CD154 specific monoclonal murine antibody and secondary rabbit F(ab')₂ anti-mouse IgG:FITC secondary antibody clearly indicated green fluorescence (Fig 2). These results indicate that the human monoclonal antibody recognizes the equine CD154 protein expressed on CHO cells. Absence of fluorescence was evident in mock-transfected cells (data not shown).

3.3 Production of reactive species by CD154-stimulated equine macrophages

Production of reactive oxygen intermediates is an important and well-recognized mechanism whereby activated macrophages limit infection by macrophage-tropic pathogens, including bacteria and parasites (Fang, 2004). We therefore selected this functional response to macrophage activation as a sensitive response to CHO cells expressing equine CD154 protein. We measured relative production of superoxide by fluorometric assay of superoxide-mediated oxidation of dihydroethidium. Zymosan, a strong activator of macrophages via the TLR2 pathway, was used as a positive control (Yang et al., 2012). Similarly, IFN- γ is also an important activator of macrophages, often acting in concert with other stimuli, and known to upregulate CD40 expression on macrophages (Munder et al., 1998); it was therefore included to gain insight into the potential impact of altered CD40 expression on MDM in the presence of ectopic expression of equine CD154 on CHO cells. Relative production of superoxide attributed to ligation of CD40 by CD154 expressed on CHO cells was determined using non-transfected control CHO cells. Mock transfected CHO cells showed no difference compared to non-transfected control CHO cells in previous studies (data not shown). Our data indicate that transfected equine CD154 protein induced increased production of superoxide in primary equine macrophages relative to controls. Moreover, the response was equivalent to superoxide production in macrophages stimulated with opsonized zymosan (positive control) and in macrophages stimulated with both IFN- γ and CHO expressing CD154 ($p > 0.05$) (Fig 3). These results indicate that this approach to expression of equine CD154 in CHO cells was effective and that ligation of CD40 by CD154 can enhance production of superoxide by primary equine macrophages.

CD154 is a co-stimulatory molecule that has been demonstrated in many other species to play a pivotal role in the generation of cellular and humoral immune responses. The equine CD154 coding sequence was isolated by RT-PCR from Con A stimulated non-adherent PBMC. Selection of primers was based upon available GenBank sequence data and the reported high degree of conservation of CD154 among domestic species. Sequence alignments of the translated equine sequence revealed distinct regions of homology with those of the cow, pig, human, mouse, cat, dog, and sheep. As described for other species, equine CD154 also has a 32 amino acid hydrophobic signal anchor region typical of Type II membrane proteins that is immediately downstream of a 13 N-terminal amino acid sequence (Wienhold et al., 2002). In contrast to human CD154, which contains 2 potential N-linked glycosylation sites, equine CD154 contains but one site, with a similar location as described in other domestic species. In the horse it is unknown as to whether or not the putative N-glycan sites are glycosylated. Nonetheless, cross-reactivity of a murine monoclonal antibody

to human CD154 (Calbiochem, San Diego, CA, USA) by immunofluorescence suggests that the extracellular protein domain shares considerable homology with human CD154 as this antibody also recognized recombinant equine CD154.

In the studies presented here, we demonstrate that CHO cells expressing equine CD154 are able to efficiently upregulate production of superoxide in equine monocyte- derived macrophages. These results indicate that production of reactive oxygen species is a relevant biological assessment of the CD40-CD154 interaction in this system, and support others' work suggesting that activation of macrophages through CD40-CD154 is sufficient for the upregulation of superoxide. Indeed, two of the most important effector systems of macrophages that are tantamount to innate immune responses include the NADPH phagocyte oxidase (NOX2) and inducible nitric oxide synthase (iNOS) pathways which account for the generation of superoxide ($O_2^- \bullet$) and nitric oxide ($NO \bullet$) radicals, respectively. Together, superoxide and nitric oxide can form the highly reactive peroxynitrite, the molecule implicated in the intracellular killing of *R. equi* by IFN- γ activated macrophages (Bogdan et al., 2000; Darrah et al., 2000). These three reactive intermediates have broad antimicrobial effects and their use is conserved across the plant and animal kingdoms (Fang, 2004). Reactive oxygen species also participate in host defense via induction of NF- κ B and release of proinflammatory cytokines, including IL- 1 α , IL-6, MCP-1 and TNF- α , indicating that the superoxide production elicited by CHO cells transfected with eqCD154 could impact broader *in vivo* immune responses (Yang et al., 2012).

In this study, we did not evaluate the kinetics of the CD154/CD40 interaction as the expression of equine CD40 on adult macrophages was not investigated. However, we were able to isolate the role of CD154 in co-culture experiments. Constant ectopic expression of equine CD154 in this controlled system permitted detection of differences in MDM reactive intermediate production due solely to presence or absence of CD154. Under these conditions, we demonstrate that equine MDM respond to ectopic equine CD154 with an increased production of superoxide.

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Highlights

- PMA/ionomycin stimulated foal PBMC produce less superoxide than adult PBMC
- We cloned equine CD154 and stably expressed it in Chinese Hamster Ovary cells
- Ectopically expressed reqCD154 elicits robust production of superoxide in MDM
- Levels of CD154 expression may impact susceptibility to intracellular infections

Fig 1:

Alignment of deduced amino acid sequence of CD154 from horse, cow (GenBank accession no. [Z48469](#)), pig (GenBank accession no. [AB040443](#)), human (GenBank accession no. [X67878](#)), mouse (GenBank accession no. [AY416063](#)), cat (GenBank accession no. [AP079105](#)), dog (GenBank accession no. [AP086711](#)), & sheep (GenBank accession no. [DQ054533](#)). The conserved TNF domain includes amino acids 123–262 (bold type). The hydrophobic signal anchor region is underlined. Putative N-glycan sites are in gray shading. Dots denote exact sequence identity with the reference equine sequence (top) and dashes denote amino acid gaps in the alignment.

Fig 2:

CHO cells transfected with equine CD154 visualized with CD154-specific monoclonal mouse antibody and rabbit F(ab')₂ anti-mouse IgG:FITC fluorescence prior to neomycin selection of positively transfected cells. CHO cells were transfected with recombinant construct and expressed CD154 was detected using indirect immunofluorescence microscopy. Using a 20x objective, both transfected and non-transfected cells in the confluent monolayer can be seen in the same field of view. Transfected cells are easily distinguishable from non-transfected cells using α -CD154 primary antibody followed with FITC labelled secondary antibodies. Image is representative of observations from 3 separate transfection experiments.

Fig 3:

Equine monocyte-derived macrophages (MDM) have increased production of superoxide when co-cultured with Chinese Hamster Ovary (CHO) cells transfected with equine CD154. Zymosan (opsonized, 0.5 mg/ml); IFN- γ (10 ng/ml); CHO POS, CHO cells transfected with cloned equine CD154; CHO NEG, CHO cells not transfected; No stim: MDM alone. (a vs. b = $p < 0.001$; other comparisons $p > 0.05$).