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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.

1. 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 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, 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

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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 × *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 ml of whole blood were obtained by jugular venipuncture from each foal. 450 ml 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 × 108 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 h 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 conconavalin 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, 1× 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 min and heated to 75 °C for 5 min 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 min, heated to 99 °C for 5 min, and then cooled to 5 °C for 5 min. Reactions were then brought up to 100 μl with 2 mM MgCl2, 0.2 mM of each dNTP, 1× 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 minutes, 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 min. Two microlitres 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 × 106 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 d etermined according to the manu-