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C-C motif chemokine ligand (CCL) production in equine peripheral blood mononuclear cells identified by newly generated monoclonal antibodies

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ABSTRACT

Chemokines are soluble molecules directing immune cell trafficking and homing, mediating inflammation, and initiating immune responses to infection. In horses, the analysis of chemokines has been limited by the lack of specific antibodies. We generated mAbs specific for the equine C-C motif chemokine ligands (CCL) CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES) and CCL11 (eotaxin) using hybridoma technology.

Antibody specificity was confirmed by intracellular staining of Chinese Hamster Ovary cells transfected with expression vectors encoding for CCL2, CCL3, CCL5, or CCL11. Transfectants were stained with the anti-CCL mAbs. Flow cytometric analysis confirmed the specificity of the different mAbs for the respective chemokine. In addition, equine PBMC were stained after isolation, culture in medium, or stimulation with LPS, or PMA and ionomycin.

CCL2 was detected in few cluster of differentiation (CD)14$^+$ monocytes in PBMC stimulated with PMA and ionomycin for 2 h. CCL3 was produced by CD14$^+$ monocytes after 4–6 h culture in medium. After stimulation with PMA and ionomycin for 12–24 h, CCL3 was also expressed in lymphocytes, mainly in CD4$^+$ T cells. Stimulation with LPS reduced the percentage of CCL3$^+$ monocytes in PBMC. CCL5 was detected in PBMC *ex vivo* in CD4$^+$ and CD8$^+$ T cells. Culture of PBMC for longer than 6 h or stimulation with PMA and ionomycin reduced the percentage of CCL5$^+$ cells. CCL11 was produced by CD4$^+$ T cells in PBMC after stimulation with PMA and ionomycin for 4–24 h. After LPS stimulation of PBMC, CCL2, CCL5, and CCL11 production were comparable to culture in medium alone.

ELISAs for each of the four chemokines were developed using pairs of anti-equine CCL mAbs. Supernatants from PMA and ionomycin stimulated PBMC contained detectable amounts of CCL2, CCL3 and CCL5, while CCL11 secretion could be stimulated from equine tracheal epithelial cells in response to IL-4.

The newly generated mAbs for equine CCL chemokines facilitate the quantitative analysis of intracellular chemokine production by flow cytometry and soluble chemokines by ELISA. The CCL mAbs are valuable tools to improve the evaluation of innate immune responses in horses.

1. Introduction

Chemokines are small molecules (7–15 kDD) that mediate tissue homeostasis, leukocyte recruitment and activation. Based on their amino acid structure they are divided into CXC, CX3C, C and CC chemokines. CC chemokines (C-C motif chemokine ligand, CCL) are characterized by two conserved, successive cysteine residues near the N-terminus (IUIS/WHO, 2002). Members of this group stimulate leukocyte migration, particularly in mononuclear cells (MNC). CCLs are important early initiators of various immunological responses.

The chemokines CCL2, CCL3, CCL5 and CCL11 have been designated (pro-) inflammatory chemokines according to their induction and effects in humans and laboratory animals (Mantovani et al., 2004). CCL2 (Macrophage Chemoattractant Protein 1, MCP-1) recruits monocytes into tissues in mice and people (Lu et al., 1998; Mantovani et al., 2004). It is furthermore chemotactic for human T cells (Carr et al.,

Abbreviations: CCL, C-C motif chemokine ligand; CHO, Chinese hamster ovary cells; EEC, equine endothelial cells; ERU, equine recurrent uveitis; ETEC, equine tracheal respiratory epithelial cells; FPLC, Fast Protein Liquid Chromatography; MCP, Macrophage Chemoattractant Protein; MIP, Macrophage Inflammatory protein; MNC, mononuclear cells; mRNA, messenger ribonucleic acid; RANTES, Regulated on Activation, Normal T cell Expressed and Secreted

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1994) and can activate basophils (Nickel et al., 1999). CCL3 (Macrophage Inflammatory protein 1 α, MIP-1α) is chemotactic for human monocytes, macrophages and to a lesser extent for T cells (Lee et al., 2000; Loetscher et al., 1994). CCL3 furthermore promotes human monocyte adhesion to endothelia (Vaddi and Newton, 1994), eosinophil migration (Rot et al., 1992) and it contributes to Th1 polarization (Nickel et al., 1999). CCL5 (Regulated on Activation, Normal T cell Expressed and Secreted, RANTES) is chemotactic for human monocytes, T memory cells (Schall et al., 1990) and eosinophils (Ebisawa et al., 1994; Kuna et al., 1998). CCL11 (Eotaxin-1) primarily mediates eosinophil chemotaxis in people (Ponath et al., 1996). It is furthermore chemotactic for human Th2 cells and basophils (Nickel et al., 1999) and promotes eosinophil release from the bone marrow in mice (Palframan et al., 1998).

In horses, basal chemokine messenger ribonucleic acid (mRNA) expression has been demonstrated in skin, lung, liver, spleen, jejunum, colon, and kidney (Benarafa et al., 2000). Induction of chemokine mRNA was furthermore analyzed in pathogeneses of different immune-mediated diseases, such as equine recurrent uveitis (ERU) (Gilger et al., 2002), insulin resistance (Burns et al., 2010), laminitis (Steelman et al., 2013), *Culicoides* hypersensitivity (Benarafa et al., 2002), equine herpesvirus type 1 infection (Johnstone et al., 2016; Wimer et al., 2011), and Equine Infectious Anemia Virus infection (Covaleda et al., 2010). However, biological effects are dependent on protein secretion, which can often be regulated beyond mRNA transcription (Wu and Brewer, 2012).

Few reports have evaluated equine CCL chemokines at the protein level. Recently, equine CCL2 was measured by a custom fluorescent bead-based assay and was elevated in serum after LPS infusion (Bonelli et al., 2017). Equine CCL2 was also detected by bead-based assays in serum of horses with recurrent uveitis (Curto et al., 2016) or heaves (Lavoie-Lamoureux et al., 2012). CCL2 correlated positively with lung resistance in challenged heaves-affected horses while it was not indicative of the disease status itself (Lavoie-Lamoureux et al., 2012). In this report CCL2 was explored as a candidate marker of systemic inflammation, but its specific function in heaves pathogenesis was not analyzed. Equine CCL3 analyzed using a commercial bead-based assay could not be detected in serum or aqueous humor of healthy horses or horses with uveitis (Curto et al., 2016). Human *r*RANTES was shown to be bioactive in horses when applied intradermally. Equine CCL5 (RANTES) was then quantified by an ELISA for human RANTES. Its concentration was higher in aqueous humor of ERU affected than in healthy eyes (Gilger et al., 2002) and it was released from equine platelets after stimulation *in vitro* (Dunkel et al., 2012). Equine *r*CCL11 was biologically active and chemotactic for horse eosinophils *in vitro* (Benarafa et al., 2002; Weston et al., 2006).

In summary, the knowledge about the protein expression of equine CCL chemokines is still limited, mainly due to the lack of equine CCL-specific reagents and assays with confirmed specificity for the respective equine chemokine. As a result, CCL chemokines in horses are poorly characterized regarding their cellular sources, induction pathways, and their functional roles during homeostasis and immune responses to infectious and inflammatory diseases. In this report, we describe the development of new mAbs specific for equine CCL2, CCL3, CCL5 and CCL11 and the expression of these chemokines in equine monocytes and T cells after different stimulation conditions.

2. Materials and methods

2.1. Recombinant CCL2, CCL3, CCL5 and CCL11

Recombinant equine CCL2, CCL3, and CCL11 produced in yeast were kindly provided by Joanna LaBresh, Kingfisher, Saint Paul, MN, USA. Additionally, equine CCL2, CCL3, CCL5, and CCL11 chemokine genes were cloned (Table 1) and expressed as fusion proteins with equine IL-4 as previously described (*r*IL-4/CCL expression vectors, Wagner et al., 2012) (Supplementary Fig. 1a).

After generation of stable transfectants of Chinese hamster ovary (CHO) cells (Wagner et al., 2005) *r*IL-4/CCL fusion proteins were purified from serum-free cell culture supernatants using an anti-IL-4 affinity column (Wagner et al., 2012) and an ÄKTA Fast Protein Liquid Chromatography (FPLC) instrument (GE Healthcare, Piscataway, NJ, USA).

2.2. Generation of mAbs specific for equine CCL chemokines

One BALB/c mouse per recombinant chemokine was immunized with equine *r*CCL2, *r*CCL3 or *r*CCL11 produced in yeast (Table 2). Additionally, one mouse each was immunized with purified *r*IL-4/CCL3, or *r*IL-4/CCL5 (Table 2). The immunizations and subsequent cell fusions were performed as previously described (Wagner et al., 2012, 2008b, 2003) (Supplementary Fig. 1b, c).

The supernatants of hybridoma clones were tested against respective *r*CCL proteins by ELISA as previously described for mAb development against other equine immune targets (Wagner et al., 2003). Briefly, ELISA plates (Nunc, Maxisorb, Sigma Aldrich, St. Louis, MO, USA) were either coated with *r*CCL produced in yeast or coated with polyclonal anti-equine-IL-4 antibody (R&D Systems, Minneapolis, MN, USA). The latter plates were subsequently incubated with CHO cell supernatants containing *r*IL-4/CCL fusion proteins. In the following steps, incubation with hybridoma supernatants and detection by peroxidase-conjugated goat anti-mouse (H + L) Ab (Jackson ImmunoResearch, West Grove, PA, USA) and tetramethylbenzidine substrate (Sigma Aldrich) were performed (Supplementary Fig. 1d).

Equine CCL chemokine specific hybridomas were selected for mAb production by limiting dilution and the resulting anti-CCL mAb clones were adapted to serum free medium (Hybridoma-SFM, Gibco, ThermoFisher Scientific, Waltham, MA, USA). From serum-free hybridoma supernatants mAbs were purified using a HiTrap Protein G HP column (GE Healthcare) on an FPLC instrument (ÄKTA FPLC, GE Healthcare) as previously described (Schnabel et al., 2017; Wagner et al., 2003). The isotype of each anti-CCL mAb was determined using mouse monoclonal antibody isotyping reagents (Sigma Aldrich).

2.3. Chemokine expression by CHO cell transfectants

For screening of mAb binding and specificity, CHO cells were transiently transfected with each of the four *r*IL-4/CCL expression vectors using Geneporter II transfection reagent (Gene Therapy Systems, San Diego, CA, USA) as previously described (Wagner et al., 2005). The transfected cells were harvested after 24 h of incubation and fixed in 2% (v/v) formaldehyde (Sigma Aldrich).

2.4. Horses and blood sampling

Heparinized peripheral blood was repeatedly obtained from six healthy adult Icelandic horses (4–6 years old) by jugular venipuncture. Samples from four of the horses (two non-pregnant mares and two geldings) were used for flow cytometric detections of native equine chemokines. Samples from two pregnant mares (first trimester) were used for detection of secreted native CCLs by ELISA. The animal sampling procedure was approved by the Institutional Animal Care and Use Committee at Cornell University (protocol #2011-0011).

2.5. PBMC isolation and stimulation

For the confirmation of anti-CCL mAb specificity for native equine chemokines, PBMC were isolated from peripheral blood by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare). PBMC were aliquoted and used under the following conditions: (i) immediately after isolation (PBMC *ex vivo*), or (ii) after incubation in tissue culture plates (Costar, Corning, Corning, NY, USA) with cell culture medium