

CXCL10 production in equine monocytes is stimulated by interferon-gamma

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ABSTRACT

C-X-C motif ligand 10 (CXCL10) is a pro-inflammatory chemokine and has been extensively evaluated in people and mice. In horses, CXCL10 and its involvement in host immunity has rarely been analyzed due to the lack of specific antibodies.

We generated a mAb specific for the equine chemokine CXCL10 using hybridoma technology. Antibody specificity was confirmed by intracellular staining and flow cytometric analysis of Chinese Hamster Ovary (CHO) cells expressing equine rCXCL10, while CHO cells expressing equine rCXCL9 were not detected.

Native CXCL10 expression in PBMC from horses of different age groups was analyzed by flow cytometry after *in vitro* stimulation. CXCL10 expressing PBMC were characterized by triple staining of CXCL10 combined with cell-surface markers.

Stimulation with IFN- γ for 5 h similarly induced CXCL10 production in cluster of differentiation (CD) 14⁺ CD16⁻ MHCII^{high} monocytes of adult horses and weanlings. The newly generated mAb enables the quantitative intracellular analysis of CXCL10 by flow cytometry and provides a new valuable tool to improve the evaluation of inflammatory responses in horses.

1. Introduction

C-X-C motif ligand 10 (CXCL10) belongs to the ELR⁻ CXC subfamily of chemokines. CXCL10 was originally termed IFN- γ inducible protein 10 (IP-10) as its transcription and secretion are induced by IFN- γ (Luster et al., 1985). Various cells can produce CXCL10 in response to cytokine stimulation. Human and murine macrophages, T cells and NK cells are common producers of CXCL10. In several pathological conditions in people and in mouse models, CXCL10 is produced by tissue infiltrating M1 macrophages under the influence of a Th1 response. Soluble or cell associated CXCL10 is therefore commonly interpreted as a pro-inflammatory marker molecule indicative of Th1 biased immune responses in these species, for example during viral infections (Wuest and Carr, 2008; Liu et al., 2011; Antonelli et al., 2014; Vissers et al., 2016). In people and mice, CXCL10 binds to the receptor CXCR3 (IUIS/WHO Subcommittee on Chemokine Nomenclature, 2003). Amongst other functions, CXCL10 mediates chemotactic migration of activated T cells and macrophages into tissues (Mantovani et al., 2004; Shin and Iwasaki, 2013; Antonelli et al., 2014). In addition, CXCL10 has important angiostatic effects during wound healing, as well as cancer

remission (Belperio et al., 2000).

In horses, CXCL10 has been rarely analyzed. A few studies measured mRNA expression of equine CXCL10 and the results point to a pro-inflammatory function comparable to that in people. CXCL10 mRNA expression was induced by IFN- γ in equine dermal fibroblasts (NBL-6) (Bussche et al., 2015), M1 polarized macrophages (Cassano et al., 2018), and mesenchymal stem cells *in vitro* (Hill et al., 2017; Cassano et al., 2018). Likewise, monocytes or bronchoalveolar lavage cells stimulated with high concentrations of poly I:C *in vitro* upregulated CXCL10 expression (Figueiredo et al., 2009; Mignot et al., 2012). Furthermore, increased expression was demonstrated in the endometrium in oestrus compared to dioestrus (Marth et al., 2016b) and after induction of infectious endometritis in mares (Marth et al., 2016a). Infection with equine herpesvirus type 1 *in vitro* induced up-regulation of CXCL10 mRNA in equine endothelial cells (Johnstone et al., 2016) or PBMC (Wimer et al., 2011) and infection with Equine Infectious Anemia Virus had a similar effect in equine monocyte derived macrophages in one study (Covaleda et al., 2010), but not in another (Lin et al., 2011). CXCL10 mRNA was also upregulated in lamina tissue after experimental induction of laminitis compared to controls

Abbreviations: APC, Allophycocyanin; CD, cluster of differentiation; CHO, Chinese Hamster Ovary cells; CXCL10, C-X-C motif ligand 10; FSC, forward scatter; IP-10, IFN- γ inducible protein 10; SSC, side scatter

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(Noschka et al., 2009), and in skin after treatment with complexed DNA vectors (Schnabel et al., 2015). Finally, Mund et al. (2018) analyzed gene expression in skin wound tissues and found indications for CXCL10 and its receptor CXCR3 to be involved in wound healing in horses. These reports point to a similarly pivotal role of CXCL10 during inflammatory conditions in horses as in people.

In addition, few reports on CXCL10 protein detection in horses are available. Curto et al. (2016) used a commercial bead-based assay and detected increased concentrations of CXCL10 in aqueous humor of eyes with Equine Recurrent Uveitis compared to healthy eyes and increased CXCL10 serum concentrations in horses with uveitis compared to healthy controls. Martin et al. (2017) used a similar assay but did not detect a treatment effect of Misoprostol or LPS on soluble CXCL10 concentrations in equine leukocyte-rich plasma. However, the commercial assays applied seem to use mAb pairs for homologous molecules (e.g. murine CXCL10) of the equine chemokine of interest. Such assays relying widely on the homology of chemokines between species and specific cross-reactivity of Abs can produce both, false positive and false negative reactions (Wagner, unpublished observations). Resulting information may not be reliable without thorough assay validation for the specific equine target chemokine. To date, information on the specificity of the commercial assays for native equine CXCL10 is not readily available.

Overall, equine CXCL10 protein quantification is limited because specific antibodies to detect this molecule have not been generated or validated. This has restricted the analysis of its function in horses. To overcome this limitation, we generated a mAb for equine CXCL10, tested its specificity for native equine CXCL10, and developed a method to stimulate CXCL10 expression in equine cluster of differentiation (CD) 14⁺ monocytes including intracellular staining and flow cytometric analysis.

2. Materials and methods

2.1. Recombinant CXCL10

Horse PBMC were used to extract mRNA and generate cDNA as previously described (Wagner et al., 2012; Schnabel et al., 2018). Equine CXCL10 was cloned (forward primer with BamH I restriction site GGCGGATCCAATACCTCTCTCTAGAACTG, reverse primer with Hind III restriction site GGCAAGCTTTTATACTTCTCTCAGTGTTCG) and expressed as an IL-4 fusion protein as previously described for other molecules (Wagner et al., 2012). ExpiCHO-S cells (1.5×10^8 in 25 ml, ThermoFisher Scientific Waltham, MA, USA) were cultured on a 19 mm orbital shaker at 140 rpm, in 8% CO₂ at 36 °C and were transiently transfected with 20 µg DNA of IL-4/CXCL10 gene expression vector with ExpiFectamine reagents (ThermoFisher Scientific) according to the manufacturer's instructions. Cell-free supernatants were harvested 8 days after transfection and were dialyzed 4 times against PBS. The rIL-4/CXCL10 protein was then purified 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) as previously described for other rIL4/chemokines (Schnabel et al., 2018).

2.2. mAb generation

A BALB/c mouse was immunized with purified rIL-4/CXCL10. The immunizations and subsequent cell fusion to generate hybridomas secreting mAbs specific for equine CXCL10 were performed as previously described (Wagner et al., 2003, 2008, 2012). Hybridoma supernatants were initially screened against rIL-4/CXCL10 by ELISA. A polyclonal anti-IL-4 antibody (R&D Systems, Minneapolis, MN, USA) was coated to the ELISA plates. In the next step, rIL-4/CXCL10 supernatant was applied to the plate. Hybridoma supernatants were tested on this matrix and mAb binding was detected with a peroxidase conjugated goat anti-mouse IgG(H + L) antibody (Jackson ImmunoResearch, West Grove,

PA, USA). The resulting CXCL10 ELISA-positive hybridoma clone supernatants were subsequently tested by similar ELISAs against other equine target molecules including rIL-4/CXCL9, rCCL5/IgG1, rIL-2/IgG1, rIL-10/IgG4, rIFN-β/IgG4, rCD14/IgG1 (Wagner et al., 2003; Schnabel et al., 2018). One hybridoma clone that was positive on the CXCL10 ELISA and negative on all other equine target molecules was considered as preliminary CXCL10-specific. This clone was expanded and further characterized.

The murine isotype of this equine CXCL10-specific hybridoma clone (44-2) was determined using mouse monoclonal antibody isotyping reagents (Sigma Aldrich, St. Louis, MO, USA). CXCL10 mAb 44-2 was adapted to serum-free medium (Hybridoma-SFM, Gibco, ThermoFisher Scientific) and purified using a HiTrap Protein G HP column (GE Healthcare) on an FPLC instrument (ÄKTA FPLC, GE Healthcare) as previously described (Wagner et al., 2003; Schnabel et al., 2017). The mAb was conjugated with Alexa Fluor 647 (ThermoFisher Scientific) according to the manufacturer's instructions.

2.3. Transfection of Chinese hamster ovary (CHO) cells

For evaluation of mAb binding and specificity, CHO cells were transiently transfected with IL-4/CXCL10, IL-4/CXCL9, IFN-β/IgG4, or IL-2/IgG1 expression vectors using Geneporter II transfection reagent (Gene Therapy Systems, San Diego, CA, USA) as previously described (Wagner et al., 2005). Transfected cells were harvested after 24 h of incubation and fixed in 2% (v/v) formaldehyde (Sigma Aldrich) prior to intracellular staining.

For generation of rIFN-γ, CHO cells were transfected with an equine rIFN-γ/His expression vector (Wagner et al., 2005) using the same protocol as described above. After 48 h, cell free supernatant was harvested and stored at 4 °C, in the dark, for a maximum of three months. The equine IFN-γ concentration in the supernatant was quantified using an equine cytokine multiplex assay as previously described (Wagner and Freer, 2009; Perkins et al., 2014; Wagner et al., 2017).

2.4. Blood sampling and PBMC isolation

Heparinized peripheral blood was obtained from healthy Icelandic horses by jugular venipuncture. The animal sampling procedure was approved by the Institutional Animal Care and Use Committee at Cornell University (protocol #2011-0011). Thirteen adults (5 mares, 8 geldings; 5–15 years, median 6 years), 11 weanlings (4 fillies, 7 colts, 6–9 months, median 7 months) and two neonates (2 colts, 5 days) were tested. PBMC were isolated from peripheral blood by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare) and used immediately for *in vitro* stimulation.

2.5. PBMC stimulation

PBMC were cultured *in vitro* in cell culture medium (DMEM supplemented with 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 50 µg/ml Gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin, all from ThermoFisher Scientific Waltham, MA, USA; and 10% FCS, Atlanta biological, Flowery Branch, GA, USA) in the presence of the secretion blocker Brefeldin A (10 µg/ml, Sigma Aldrich, St. Louis, MO, USA). In preliminary experiments, CXCL10 expression was stimulated in PBMC by 0.16–16.3 µg/ml rIFN-γ for 4–6 h *in vitro*. A concentration of rIFN-γ (1.63 µg/ml) and 5 h of stimulation were found optimal for CXCL10 expression and intracellular staining (data not shown). These stimulation conditions were used for all data shown in this article. As controls (all with Brefeldin A), incubation in medium, or stimulation with PMA (25 ng/ml) and ionomycin (1 µM; all Sigma Aldrich) were included. After stimulation, PBMC were harvested, fixed in 2% (v/v) formaldehyde (Sigma Aldrich) and stored at 4 °C, in the dark, for a maximum of 24 h until staining.

2.6. Intracellular staining and CXCL10 detection by flow cytometry

Transfected CHO cells were permeabilized in saponin buffer (PBS, supplemented with 0.5% (w/v) BSA, 0.5% (w/v) saponin and 0.02% (w/v) NaN_3), incubated with CXCL10 hybridoma supernatants, and mAb binding was detected by a secondary goat-anti-mouse (H + L) antibody conjugated to Alexa Fluor 647 (Jackson ImmunoResearch). Anti-IL-4 (clone 13G7 (Wagner et al., 2006) was used as a positive control to demonstrate successful transfection.

Intracellular staining of PBMC was performed in saponin buffer with CXCL10 mAb 44-2 conjugated with Alexa Fluor 647. An isotype control (murine IgG1) was included for all samples in each experiment. For phenotyping of PBMC, triple staining was performed with intracellular CXCL10 mAb 44-2 combined with mAbs for the cell-surface markers CD14 (clone 105) (Kabir et al., 2010), and CD16 (clone 9G5) (Noronha et al., 2012), or MHCII (clone cz11) (Barbis et al., 1994) conjugated with Alexa Fluor 488 (ThermoFisher Scientific) or biotin. The latter mAbs were detected using Streptavidin-Phycoerythrin (Jackson ImmunoResearch).

For CHO cells 10,000 events per sample and for PBMC 500,000 events per sample were recorded on a FACS Canto II flow cytometer (BD Biosciences, San Diego, CA). Data analysis was performed with FlowJo version 10.2 (FlowJO LLC, Ashland, OR, USA).

CHO were gated by forward scatter (FSC) and side scatter (SSC) characteristics and analyzed for anti-CXCL10 binding (Allophycocyanin, APC channel) (Fig. 1A). For PBMC, duplets were excluded by FSC-area and FSC-height characteristics. Then, lymphocyte scatters and monocyte scatters were gated by FSC and SSC characteristics and CXCL10 expression (APC channel) was analyzed compared to the isotype control (Fig. 2A, C).

2.7. Statistical analysis

CXCL10 expressing cells in the monocyte scatter were analyzed quantitatively. The data was not normally distributed according to D'Agostino and Pearson normality test. Within each age group (adults, weanlings), percentages of CXCL10 expressing cells were compared between medium incubated and IFN- γ stimulated PBMC by Wilcoxon

matched-pairs signed rank tests. Percentages of CXCL10 expressing cells were furthermore compared between adult horses and weanlings for each condition (medium or IFN- γ stimulation) by Mann-Whitney tests. P-values < 0.05 were considered significant. The statistical analysis was performed using GraphPad Prism software version 6 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Specificity of the newly generated anti-CXCL10 mAb

By hybridoma technology one mAb specific for equine CXCL10 was generated. The CXCL10 mAb 44-2 is a murine IgG1 isotype. CHO cells were transiently transfected with the IL-4/CXCL10 expression vector, stained intracellularly with CXCL10 mAb 44-2, and analyzed by flow cytometry. CXCL10 mAb staining was comparable to IL-4 staining of the IL-4/CXCL10 transfected cells, while IL-4/CXCL9 transfected CHO cells were not stained by the CXCL10 mAb (Fig. 1B). CHO cells transfected with IFN- β /IgG4 or IL-2/IgG1 were also not detected (data not shown). In addition, other recombinant equine chemokines or cytokines, including rIL-4/CXCL9, rCCL5/IgG1, rIL-2/IgG1, rIL-10/IgG4, rIFN- β /IgG4, and rCD14/IgG1 were not detected by CXCL10 mAb 44-2 by ELISA, while rIL-4/CXCL10 was recognized (data not shown). Based on these results, the newly generated anti-CXCL10 mAb 44-2 was considered specific for its equine target molecule.

3.2. Detection of native equine CXCL10 in PBMC

In non-stimulated PBMC, cultured in medium *in vitro*, typically few cells produced CXCL10 (median 0.2%; 0.1–1.8%) after 5 h of incubation compared to the isotype control (Fig. 2A,B). After stimulation of PBMC with rIFN- γ , increased CXCL10 expression (median 0.9% positive cells; 0.3–3.8%) was observed (Fig. 2B). CXCL10 was detected in cells of high SSC and FSC, presumably monocytes, while lymphocytes did not express CXCL10 (Fig. 2B). Notably, PMA/ionomycin stimulation did not stimulate CXCL10 production in cells in the monocyte scatter. In PMA/ionomycin stimulated PBMC of some individual horses minor binding of CXCL10 mAb 44-2 to lymphocytes was observed. In these individuals,

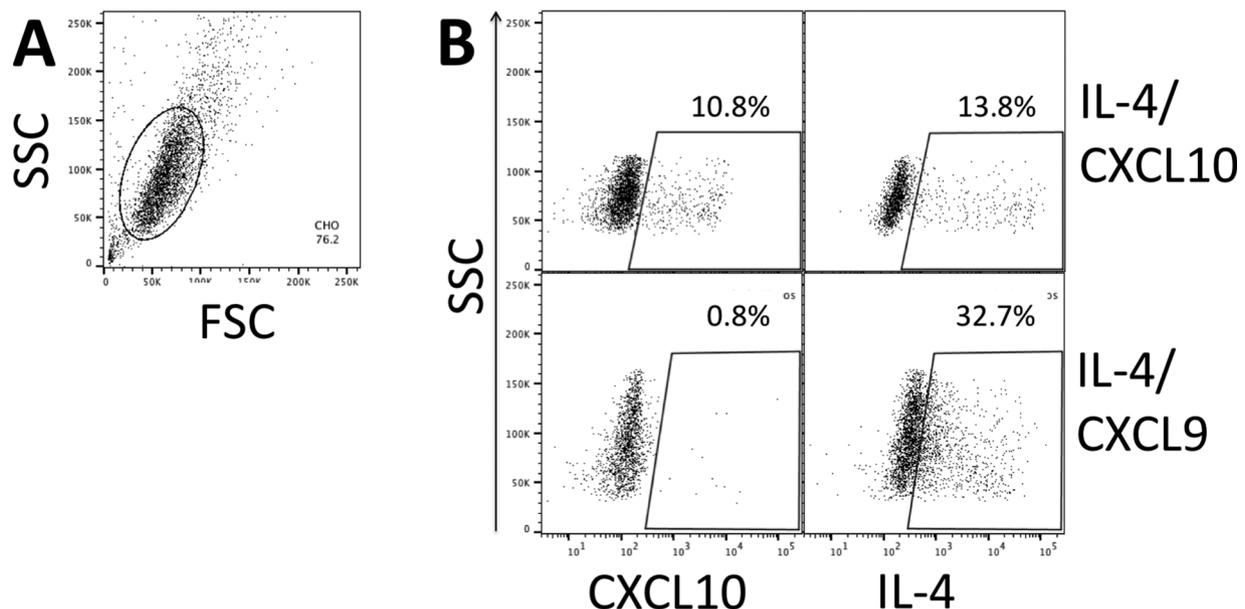
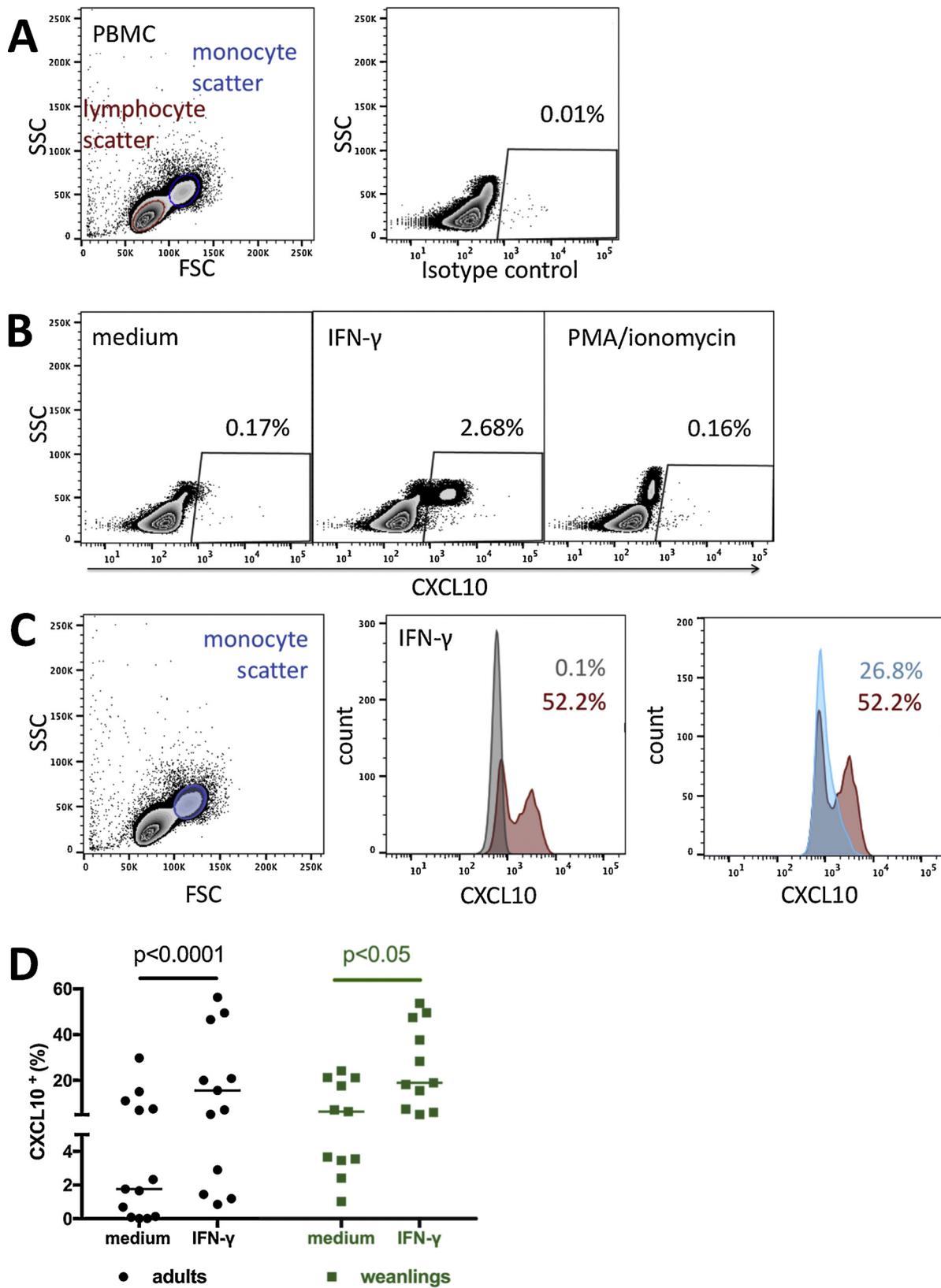


Fig. 1. Flow cytometric analysis of transfected cells expressing rCXCL10 by CXCL10 mAb 44-2.

Chinese hamster ovary (CHO) cells were transiently transfected with expression vectors encoding equine IL-4/CXCL10 or IL-4/CXCL9. Cells were harvested 24 h post transfection, fixed and stained intracellularly with CXCL10 mAb 44-2 or IL-4 mAb 13G7. (A) CHO cells were gated by forward scatter (FSC) and side scatter (SSC) characteristics. (B) IL-4/CXCL10 or IL-4/CXCL9 transfected cells were stained with CXCL10 mAb 44-2 or IL-4 mAb 13G7. The percentages of positive cells with the respective mAbs are given on top of the analysis gate.



(caption on next page)

the same minor binding was usually observed for the isotype control and was thus considered non-specific.

The induction of equine CXCL10 by IFN- γ agrees with previous reports of CXCL10 mRNA expression in equine cells in response to this stimulus (Bussche et al., 2015; Hill et al., 2017; Cassano et al., 2018).

Likewise, IFN- γ directly induces CXCL10 secretion in people and mice (Antonelli et al., 2014). Accordingly, CXCL10 is used as a quantitative marker for a Th1 dominated immune response in people in certain pathological conditions (Mantovani et al., 2004; Antonelli et al., 2014). In horses, a similar use of the CXCL10 mAb may be possible, for

Fig. 2. Stimulation of CXCL10 expression in equine PBMC.

PBMC of Icelandic horses were cultured in medium, stimulated with IFN- γ , or with PMA and ionomycin *in vitro* for 5 h. Then, the cells were fixed and stained intracellularly using CXCL10 mAb 44-2 conjugated with Alexa Fluor 467. (A) After duplet exclusion, gates were set on cells with high forward (FSC) and side scatter (SSC) characteristics called ‘monocyte scatter (blue)’ and lower FSC and SSC characteristics called ‘lymphocyte scatter (red)’. The gate for CXCL10 positive cells was set in comparison to the isotype control. (B) Representative example of CXCL10 detection within PBMC with high SSC (monocyte scatter) after incubation in medium, or stimulation with IFN- γ , or PMA/ionomycin. (C) Example of a histogram analysis of cells in the monocyte scatter (left panel) for intracellular CXCL10 production. CXCL10 staining of cells in IFN- γ stimulated PBMC (red) are shown in comparison to the isotype control (grey, center panel) or to cells in medium incubated PBMC (turquoise, right panel). (D) Cells in the monocyte scatter expressing CXCL10 were quantified in PBMC from adult horses (n = 13; black circles) and those from weanlings (n = 11; green squares). The gating and analysis was performed as shown in C. Distributions and medians are graphed per age group and stimulation. Significant differences are indicated by p-values.

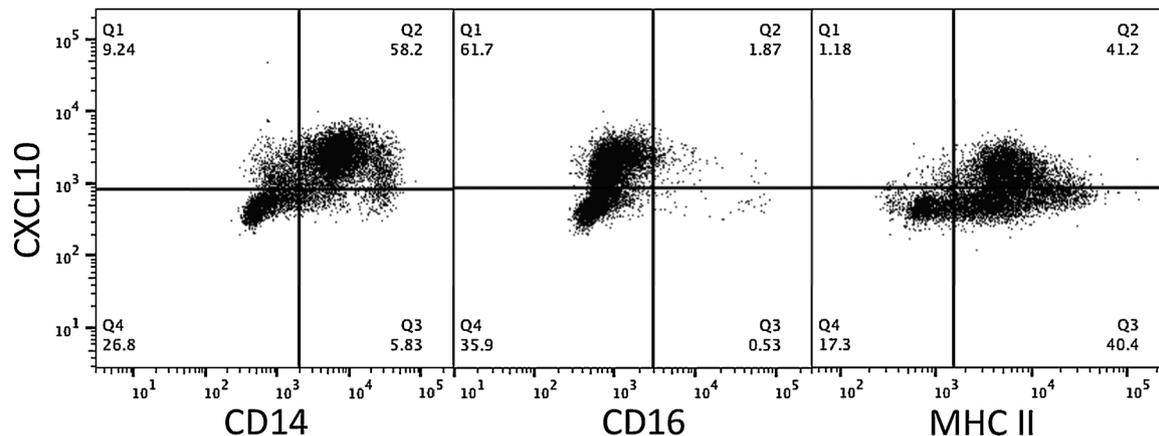


Fig. 3. IFN- γ induced CXCL10 expressing cells are monocytes.

PBMC were stimulated with IFN- γ *in vitro* for 5 h. Then, cells were fixed and stained intracellularly with CXCL10 mAb 44-2 combined with cell-surface marker staining for CD14, CD16, or MHCII and analyzed by flow cytometry. A monocyte scatter gate was set by FSC and SSC characteristics. Cells in this gate were then analyzed by quadrant gates for CXCL10 and cell surface marker expressions. A representative example (adult horse) is shown.

example in monitoring immune responses to viral infection, in wound healing or in autoimmune pathologies.

Next, PBMC of 13 adult horses and 11 weanlings were stimulated with IFN- γ . A median of 20% (1–66%) of the cells in the monocyte scatter expressed CXCL10 compared to 5% (0–30%) of these cells after culturing in medium. The percentages of CXCL10 expressing cells in the monocyte scatter were significantly higher after IFN- γ stimulation compared to non-stimulated cells cultured in medium in each age group (Fig. 2D). Although large differences in individual responses were observed in intracellular CXCL10 expression, IFN- γ stimulation increased the percentage of CXCL10 producing cells in each individual horse.

Beyond IFN- γ , other inflammatory stimuli, such as different Toll-like receptor agonists also induce CXCL10 expression in murine cells (Bandow et al., 2012). In experiments with PBMC of four adult horses, however, stimulation with LPS resulted in CXCL10 expression similar to medium incubation (data not shown). This result matched findings on CXCL10 mRNA which was not induced by LPS stimulation *in vitro* in equine peripheral blood leukocytes (Martin et al., 2017), or in peripheral blood leukocytes enriched for equine monocytes by density gradient isolation (Figueiredo et al., 2009). The absence of equine CXCL10 up-regulation after polyclonal stimulation of PBMC with LPS or PMA and ionomycin, a potent stimulus for equine lymphocytes mimicking T cell receptor stimulation (Wagner et al., 2010), suggests that equine CXCL10 expression is initiated through the IFN- γ receptor signaling pathway.

By comparing adult horses and weanlings, quantitative differences in CXCL10 stimulation between these age groups were not observed (Fig. 2D). PBMC from two neonatal foals (5 days of age) were subsequently stimulated with IFN- γ and also showed quantitatively similar results for CXCL10 expression (data not shown). This underlines the role of CXCL10 as an innate immune mediator that is not dependent on maturation of adaptive immunity. Previous experiments using PBMC from neonatal foals showed that they have lower percentages of IFN- γ producing cells after stimulation with PMA/ionomycin compared to

adult horses (Breathnach et al., 2006; Wagner et al., 2010; Perkins and Wagner, 2015). Despite the reduced percentages of neonatal IFN- γ producing cells, IFN- γ receptor expression and signaling in neonatal cells seems to be sufficient to trigger comparable CXCL10 induction in neonatal cells and those of weanling or adult horse monocytes. Due to the low number of neonates available for the present experiments the latter statement still requires confirmation using more individuals.

3.3. Phenotyping of CXCL10 producing cells

Cells in the monocyte scatter producing CXCL10 in response to IFN- γ stimulation were subsequently characterized with different cell surface markers as CD14⁺CD16[−]MHCII^{high} cells (Fig. 3). This matches the phenotype of the majority of monocytes in equine PBMC (Mauel et al., 2006; Kabithe et al., 2010) and earlier descriptions of equine CXCL10 expression (Figueiredo et al., 2009). Similarly, monocytes and monocyte derived macrophages stimulated with IFN- γ are typical producers of CXCL10 in people and mice (Mantovani et al., 2004; Antonelli et al., 2014).

4. Conclusion

CXCL10 is a potential marker of Th1-polarized immune responses in the horse. We generated a new mAb specific for equine CXCL10 and characterized the mAb by intracellular staining and flow cytometric analysis of recombinant and native equine CXCL10. Equine peripheral blood monocytes express CXCL10 in response to IFN- γ stimulation independent of the age of the horse. The CXCL10 mAb 44-2 can be used as a new immune reagent tool to characterize inflammatory responses in equine cells.

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