Production of novel camelid anti-CXCL10 specific polyclonal antibodies and evaluation of their bioreactivity

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Abstract

CXCL10 chemokine is a member of CXC chemokine family. It is secreted from a variety of cells in response to IFN-γ and stimulates a range of inflammatory responses via binding to its receptor, CXCR3. CXCL10 has a pivotal role in the pathogenesis of various infectious diseases, cancers, and inflammatory and autoimmune diseases. It has been put forward as a potential biomarker and therapeutic target in diagnosis and treatment of these diseases. In the present study, production of camel heavy chain antibodies (HCAbs) specific for the CXCL10 is reported. In this regard, recombinant CXCL10 was used for immunization of camel and subsequently the CXCL10 HCAbs were obtained. Afterwards, three subclasses of IgG were separated using protein A and protein G affinity columns, characterized with SDS-PAGE and confirmed for specific binding to the CXCL10 using ELISA. These IgG subclasses successfully recognized CXCL10 and a strong and specific reactivity towards CXCL10 were observed. Therefore, the selected HCAbs and their corresponding expression library could be used to develop a recombinant variable domain of these HCAbs (nanobody or VHH) as a new possible strategy for treatment of multiple sclerosis and other autoimmune diseases.

Key words: CXCL10, Polyclonal antibody, Heavy chain antibody, Nanobody, Multiple sclerosis, Chemokine

1. Introduction

CXCL10 or IFN-γ-inducible protein of 10 kDa (IP-10) is a low molecular weight basic chemokine that plays important role in the physiological processes of immune cell maturation and trafficking. It is induced by IFN-γ and is secreted by several types of immune and non-immune cells including monocytes, neutrophils, astrocytes, dendritic cells, endothelial cells, fibroblasts, mesenchymal cells, hepatocytes and keratinocytes. CXCL10 is categorized functionally as a Th1-chemokine and directs migration of many cell subsets such as T cells, eosinophils, monocytes and NK cells, which express CXCR3 as the CXCL10 receptor, into the sites of inflammation in different autoimmune and inflammatory processes (Loetscher et al. 1996; Neville et al. 1997; Vazirinejad et al. 2014; Zlotnik and Yoshie 2000). CXCL10 involves in the pathogenesis of several infectious or autoimmune diseases, chronic inflammation, tumor development, metastasis and dissemination. It has been described as a potential biomarker, prognostic indicator and therapeutic target in various diseases, including multiple sclerosis (MS) (Iwanowski et al. 2017), rheumatoid arthritis (RA), Parkinson's Disease (Lue et al.

2016; Rocha et al. 2014), psoriatic arthritis (Abji et al. 2016), Alzheimer's disease, viral encephalitis, colorectal cancer (Liu et al. 2011, Bai et al. 2016), lupus nephritis (Marie et al. 2014), type 1 diabetes (Antonelli et al. 2014), localized scleroderma (Magee et al. 2013), Mycobacterium tuberculosis infection (Strzelak et al. 2012), asthma (Wark et al. 2007), COPD (Quint et al. 2010), Kawasaki Disease (Ko et al. 2015), HTLV-1-Associated Myelopathy/Tropical Spastic paraparesis (Sato et al. 2013), Cystic Fibrosis (Solomon ey al., 2013), Neurosyphilis (Wang et al. 2016a), graft failure and rejection and chronic Graft Versus Host Disease (cGVHD) (Kariminia et al. 2016; Kim et al. 2016; Paczesny and Abu Zaid, 2016; Romagnani and Crescioli, 2012), non-alcoholic steatohepatitis (Zhang et al. 2014), Vitiligo (Richmond et al. 2017; Wang et al. 2016b), Cardiovascular Disease (van den Borne et al. 2014), and Juvenile Dermatomyositis (Bellutti Enders et al. 2014). MDX1100, a human anti-CXCL10 antibody is currently being investigated in phase II clinical trial for RA and ulcerative colitis (Yellin et al. 2012). Furthermore, SCH-546738 has successfully passed preclinical trials for MS, RA, graft rejection and psoriasis (Jenh et al. 2012). The multicenter Clinical Trials in Organ Transplantation 04 (CTOT-04) study demonstrated the prognostic and diagnostic value of the CXCL10 in kidney recipients (Suthanthiran et al. 2013). Several other clinical trials are being in progress for the discovery of CXCL10 inhibitors and its investigation as a biomarker which is beyond the scope of this paper. Antibody-based targeting of molecules is an alluring approach to drug and biomarker discovery, disease prognosis, and therapy. Conventional antibodies consist of two heavy and two light polypeptide chains with a molecular weight of about 150 kDa. Some limitations of the conventional antibodies are their high molecular weight, high immunogenicity, low penetration, long production process and high cost. These are serious bottlenecks in their applications (Holliger and Hudson, 2005; Ma and O'Kennedy, 2015; Nelson, 2010). Camelids (camels, llamas, and alpacas) produce special kinds of antibodies, known as heavy chain antibodies (HCAbs) which overcome these problems. HCAbs devoid of the light chains and consist of only two heavy chains lacking the CH1 domain. Many unique characteristics of HCAbs are attributed to their low molecular weight of 90 KDa and their single variable domain, VHH or nanobody (Hamers et al. 1993) (Fig1). VHH fragments or nanobodies are the smallest known natural intact antigen-binding entities of IgG (15 kDa) harboring specific and superior features including, high affinity, solubility and temperature and pH stability, excellent tissue distribution, non-immunogenicity due to the extensive homology to human VH3 genes, and easy to recombinant production. These features led to intensive research on production of HCAbs and VHH nanobodies for their potential application in diagnosis or immunotherapy of various diseases (Abji et al. 2016; De Meyer et al. 2014; Liu and Huang, 2018). The aim of the present study is production, characterization and assessment of bioreactivity of the novel anti-CXCL10 specific camel polyclonal antibody. This specific antibody and its expression library would be further used in later studies to produce high affinity anti CXCL10 nanobodies with probable healing effects in multiple sclerosis or other autoimmune diseases.

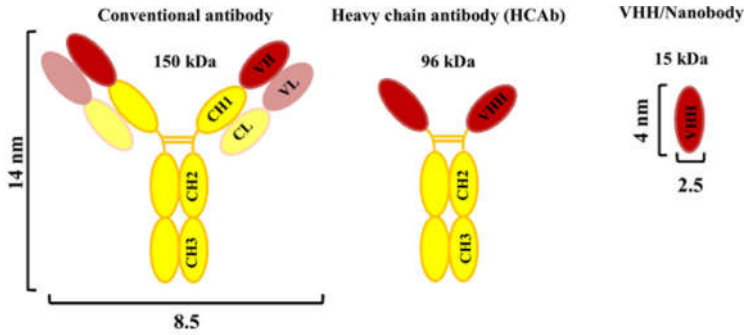

Fig1 Schematic representation of structure of conventional antibody, heavy-chain antibody, and VHH/nanobody.

2. Materials and methods

2.1 Camel immunization with recombinant CXCL10

The recombinant CXCL10 used for camel immunization was produced in the *E. coli* host by Biomatik Company (Cambridge, Canada). Serum samples were collected from a non-immune 7-month-old male camel and stored at

-70 °C. This camel was immunized by six subcutaneous injections of the recombinant CXCL10 at 2-weeks intervals. One hundred µg CXCL10 was mixed with Freund's complete adjuant for the first injection and with incomplete Freund's adjuvant in equal volume for the booster injections. The blood serum samples of the immunized camel were collected 5 days after each injection and stored at -70 °C until further use (Kazemi-Lomedasht et al. 2015).

2.2 Antiserum titer determination by ELISA

The titer of serum samples was assayed using an indirect enzyme-linked immunosorbent assay (ELISA). Maxisorp (Nunc) 96-well microtiter plate was coated with 100 µl per well of recombinant CXCL10 (2 µg/ml in 50 mM bicarbonate buffer, pH 9.6) overnight at 4 °C. The wells were blocked with 200 µl per well of 3% skimmed-milk proteins and plate was incubated overnight at 4 °C. Preimmune and immune serum samples were diluted at 10-fold serial dilutions (1:10 – 1:100000), were added to duplicate wells and incubated at 37 °C for 1h. The plate was incubated with 100 µl per well of HRP-conjugated rabbit anti Llama IgG with the dilution of 1:10000 (Thermo Fisher Scientific) at 37 °C for 1h. The wells were washed three times with PBST (PBS-0.05% Tween 20) for every 5 min after each step. Then, the wells were reacted with 100 µl of 3, 3′, 5, 5′-Tetramemethylbenzidine (TMB) at room temperature in dark room for 20 min and finally the reaction was stopped by 100 µl of 2N H_2SO_4 and absorbance was read at 450 nm (Behdani et al. 2012).

2.3 Fractionation of IgG subclasses

Polyclonal heavy chain antibodies were purified from the high-titer serum collected after 6^{th} injection by differential adsorption on protein A and protein G columns. Five mL of the serum was diluted with equal volume of 20 mM phosphate buffer pH 7.4 (PBS) and loaded on protein G (GenScript) column equilibrated with PBS. The flow-through was collected as it contained the IgG2 subclass of antibodies which do not adsorb to protein-G. Following washing with one column-volume of PBS, the IgG3 fraction was eluted by 150mM NaCl and 0.58% of acetic acid (pH 3.5). Subsequently, the IgG1 fraction was eluted with 100 mM Glycin-HCl buffer (pH 2.7). Next, the column was washed with PBS and the flow through was reloaded on the column to remove the residual IgG1 and IgG3 subclasses. The flow-through of the first step which contained the fraction not absorbed to the protein G was loaded on PBS equilibrated protein A column (GenScript). Afterwards, the IgG2 fraction was eluted using 100 mM glycin-HCl buffer (pH 4.5). All IgG fractions were immediately neutralized with 1 M Tris-base buffer (pH 9), concentrated and subjected to buffer exchange using 10 kDa cut-off filter columns and PBS. The purity and integrity of IgG fractions were verified by reducing and non-reducing SDS-PAGE analysis (Arezumand et al. 2014).

2.4 Functional assay

ELISA was used for assessment of reactivity of the purified camel antibodies with CXCL10. The coating and blocking steps performed as described above. The plate was incubated with 100 µl diluted (1:1000) sera from before immunization and after final immunization as negative and positive controls, respectively, and 3 µg/ml of each IgG subclasses at 37 °C for 1h. The wells were treated with 100 µl per well of HRP-conjugated rabbit anti Llama IgG (1:10000 dilution) at 37 °C for 1h. The wells were washed three times with PBST (PBS-0.05% Tween 20) for every 5 min after each step. Subsequently, the wells were reacted with 100 µl TMB at room temperature in dark room for 20 min. The reaction was stopped by 100 µl 2N H_2SO_4 and absorbance was read at 450 nm.

3. Results

3.1 Immunization and serum response

Specific antibody titers rose gradually after each injections of the recombinant CXCL10 (Fig2). The ELISA results demonstrated successful stimulation of a humoral immune response against CXCL10.

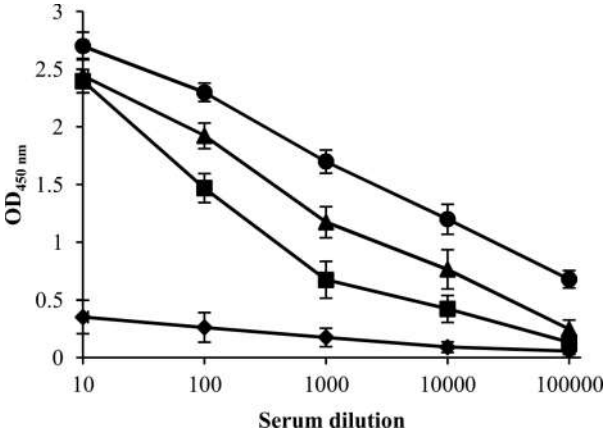

Fig2 Monitoring of camel immune response. Sera from immunized camel were examined by ELISA for pre-immunized sera (♦), after third (■), after fourth (▲) and sixth (●) injections. As it is shown, the titer of the CXCL10 specific antibodies rises after each injection.

3.2 Purification of camel IgG subclasses

Camel has three IgG subclasses including, IgG1, IgG2 and IgG3. IgG1 has a conventional four-chain structure with molecular weight of 170 kDa, which upon reducing condition yields two 50 kDa heavy chains, and 30 kDa light chains (Fig3). In contrast, IgG2 and IgG3, which together account for 75% camel immunoglobulins and 50% llama immunoglobulins, are devoid of light chains and only consist of heavy chains of about 45 and 42 kDa, respectively (Daley et al. 2010). Therefore, IgG2 and IgG3 molecular weight is about 90 kDa under non-reducing condition (Fig3). Results of reducing and non-reducing SDS-PAGE demonstrated that integrity of immunoglobulins conserved during purification.

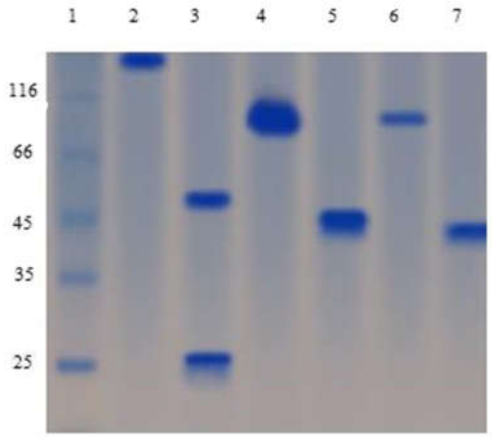

Fig3 SDS–PAGE pattern of purified camel antibodies in reducing and non-reducing conditions. Lane 1: protein molecular weight markers. Lane 2 and 3: camel IgG1 upon non-reducing and reducing conditions, respectively. Lane 4 and 5: IgG2 upon non-reducing and reducing conditions, respectively. Lane 6, 7: IgG 3 upon non-reducing and reducing conditions, respectively.

3.3 Reactivity of camel IgG subclasses with CXCL10

By means of ELISA against CXCL10, the reactivity of the eluted IgG subclasses was assessed. The reactivity of purified IgG1, IgG2 and IgG3 antibodies with the CXCL10 can be observed in Fig4. IgG subclasses successfully recognized CXCL10 similar to the positive crude serum, and a rarely strong and specific reactivity towards CXCL10 was also seen when compared to the negative control.

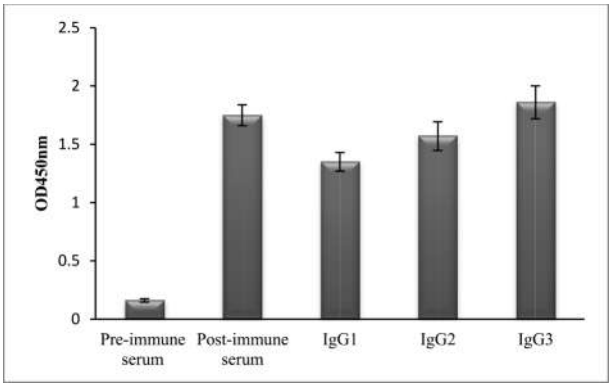

Fig4 ELISA for evaluation of reactivity of the purified camel IgG subclasses to the CXCL10. As it is shown, all the three purified subclasses retained their reactivity to the CXCL10 as was for the crude immune sera.

4. Discussion and Conclusion

This is the first report on the production, purification, and evaluation of functional activity of camel polyclonal conventional IgG1 and HCAbs against CXCL10. HCAbs was discovered in camelid serum in 1993. These newly discovered antibodies consisted of VHH and CH2 and CH3 constant domains. Lower molecular weight and the VHH domain confer many unique features to the HCAbs which led to intensive research toward generation of HCAbs and nanobodies against toxins, various bacterial, viral, helminthic and protozoal pathogens as well as immunogenic peptides and proteins for diagnosis and treatment of diseases (Desmyter et al. 2015). Ablynx, a pioneering pharmaceutical company in the development of nanobody-based drug candidates, has eight nanobodies in clinical development and the first potential product launch, caplacizumab a bivalent nanobody for treatment of acquired thrombotic thrombocytopenic purpura, is expected in 2018 (Elgundi et al. 2017). Because of increasing evidence and knowledge about the pivotal role of chemokines and chemokine receptors in establishment and pathogenesis of several diseases, they are one of the main focus of pharmaceutical companies researches as promising therapeutic targets, and some of the major companies have a row of chemokine/chemokine receptors potential antagonists in clinical development for different indications (Tschammer 2015). Up to now, two monoclonal antibodies (mAbs) targeting the chemokine network members came to the market, including Mogamulizumab, a humanized anti-CC chemokine receptor 4 (CCR4) approved for treatment of leukemia/lymphoma, and AbCream, a mAb directed against CXCL8 chemokine (IL8) for topical use in psoriasis (Qidwai 2016; Yoshie and Matsushima 2015). Many researchers have introduced the chemokine CXCL10 as a potential therapeutic target for treatment of the several infectious diseases, autoimmune diseases, chronic inflammation and tumor development, metastasis and dissemination (Liu et al. 2011). Several CXCL10-CXCR3 antagonists such as MDX1100, NI-0801, TAK-779 and SCH-546738 have been discovered yet and some of them are currently undergoing preclinical and clinical trials for treatment of MS, RA, ulcerative colitis, HIV, Psoriasis, graft rejection and etc. (Roberto Solari and Malcolm 2015). CXCL10 expression level increases in several diseases and can also be regarded as a promising and ideal biomarker to detect psoriatic arthritis (Abji et al. 2016), colorectal cancer (Liu et al. 2011, Bai et al. 2016), graft failure and rejection and chronic Graft Versus Host Disease (cGVHD) (Kariminia et al. 2016; Kim et al. 2016; Paczesny and Abu Zaid 2016; Romagnani and Crescioli 2012), localized scleroderma (Magee et al. 2013) and etc. In the present study, a camel was immunized with the recombinant CXCL10. It was mainly aimed to develop high-titer HCAbs which is specific for CXCL10, and the final objective was to develop the recombinant camelid nanobody. In fact, the camel HCAbs (IgG2 and IgG3) specific for the human CXCL10 have been produced in this study (Fig3). The whole antiserum and affinity purified conventional and heavy chain antibodies showed significant positive reactions with CXCL10. To conclude, the anti-CXCL10 polyclonal HcAbs can be used for detection of the CXCL10 chemokine which is suitable for development of the diagnostic assays. Furthermore, the findings reported here may pave the way for the development of a specific nanobody against CXCL10 for in vivo therapeutic applications and currently, we are attempting to develop recombinant nanobodies as new therapeutic agents for treatment of autoimmune diseases such as MS.

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Conflict of interest

Tahereh Sadeghian-Rizi, Mahdi Behdani, Hossein Khanahmad, Pooria Ghasemi-Dehkordi, Hamid Mirmohammad Sadeghi and Ali Jahanian-Najafabadi declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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