Immunotherapeutic potential of nanobody derived from bamboo shark immunoglobulin novel antigen receptor (IgNAR)

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Introduction

The history of the research on immunoglobulin goes way back to the year 1890, when von Behring and Kitasato first identified an agent which had the ability to neutralize the diphtheria toxin. Later on, the so called terms of "Antibody" and "Antigen" were introduced by various scientists (Schroeder & Cavacini, 2010). By the advancement of electrophoresis separation methods, in 1939 two scientists named Tiselius and Kabat reported the globulin fraction; hence the terms "gamma-globulin", "immunoglobulin" was came in to picture. Along with the advancement of technology the investigations of structure and function of immunoglobulins were widely carried out in order to solve the complexity of their nature.

Antibody (Ab) or immunoglobulins (Igs) have the ability to bind to a number of different antigen types through adjustable binding due to alteration of deoxyribonucleic acid (DNA) arrangement in B lymphocytes. There are two types of Igs such as membrane bound form and soluble form. The membrane bound form acts as cell surface receptors, which convey the signal and activate cells after exposure to an antigen. The soluble forms are in plasma and able to bind with antigen individually and neutralize them at a distance (Nikolova *et al.* 2009). The basic structure of Igs includes two heavy chains (H) and two light chains (L). Both have variable (V) region with NH2 - terminal and constant (C) region with COOH – terminal. (Williams & Barclay 1988). Ig H chains have three or more C domains while Ig L consists of only one C domain (Schroeder & Cavacini 2010).

During evolution, adaptive immune system played a key role in jawed vertebrates by high affinity binding to particular antigens and removal of invading pathogen. This can be done due to their characteristic feature of unlimited diversity and development of memory (Flajnik 2002). Based on the environment and antigens exposed, different species evolved various structural features in their immune system. In order to produce Ab against all foreign objects and infectious agents, the required number of genes may exceed the amount of whole genome. Therefore, strategies like V(D)J recombination and somatic hypermutation takes place in all jawed vertebrates (Figure 1).(Tonegawa 1983; Flajnik 2002; Wang *et al.* 2013).

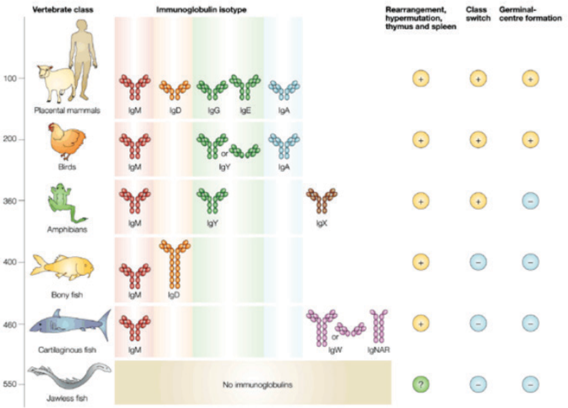

Figure 1. Evolution of immunoglobulins in vertebrates and development of their characteristic features

Shark immunoglobulins

Sharks belong to the class chondrichthyes, subclass elasmobranch. They diverged from the evolutionary common ancestor with jawed vertebrates over 500 million years ago. Cartilaginous fish are the oldest jawed species that possess acquired immune system (Flajnik 2002). The distribution of Ig genes is different in sharks compared to vertebrates. The Ig loci are found in cluster configuration in sharks while higher vertebrates have translocon arrangement (Figure 2). In each cluster V, (D), J and C region exons reside and form three heavy chain Ig isotypes namely, IgM, IgW and IgNAR (novel antigen receptor) (Hinds & Litman 1986; Litman *et al.* 1999). Unlike vertebrate immune system, shark Ig encodes by different clusters and there is no rearrangement among clusters and isotype switching (Dooley & Flajnik 2006).

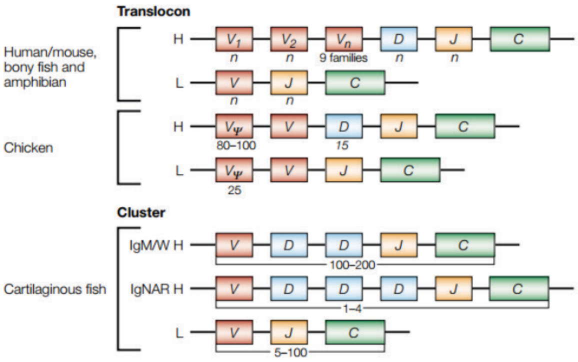

Figure 2. Translocon and cluster organization of immunoglobulin genes among species

Immunotherapy using nanobodies

The antibody based biotherapeutics are on the rise since recent decades. The high specificity and to avoid adverse off-site toxicity on normal tissues such as complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxicity (ADC) are challenges of bioengineering of therapeutic immunoglobulin (Presta 2008; Müller *et al.* 2012). Although there are number of immunotherapeutics available to date, there are disadvantages such as, structural complications and large size of globular proteins which limits the access to cryptic epitopes (Müller *et al.* 2012). Therefore scientists are now concerned on the development of smaller, pH and temperature resistant (stable) antibody with higher affinity and specificity (Kubota *et al.* 2009).

Recent concerns on naturally occurring antibodies with single chain such as nonobodies from camelids (VHH) and IgNAR from cartilaginous fish are promising molecules for immunotherapeutics (Hamers-Casterman *et al.* 1993). Both antibodies are heavy chain homodimers which lacks light chain. Their smaller size (12-13 kDa), higher solubility and stability increased their potential in therapeutics. However, IgNAR variable domain (vNAR) is known as the smallest antigen binding domain available to date in the kingdom of animalia (Figure 3) (Zielonka *et al.* 2015).

Another significant difference between VHH and vNAR is that camelid antibody is an IgG variant while IgNAR is encoded at particular loci (cluster) (Hamers-Casterman *et al.* 1993). Furthermore, charged and polar amino acids in the IgNAR structure enables it to show higher solubility than other antibodies, which is an advantage to reach target epitopes (de los Rios *et al.* 2015). Therefore, the present study, analysed the IgNAR structure of brownbanded bamboo shark (*Chiloscyllium punctatum*), and its potential as a future immunotherapeutic.

Most of the immunogenetic studies were conducted to identify target specific antibodies by using phage display (Flajnik & Dooley 2009), ribosome display (Kopsidas *et al.* 2006) and yeast display (Zielonka *et al.* 2014) technologies. The development of immunoglobulin library was also performed by randomization methods and polymerase chain reaction (PCR) methods followed by sequencing of expression clones (Diaz *et al.* 2002; Dooley *et al.* 2006). Although the use of next generation sequencing (NGS) method can reveal massive amount of information, many studies have not used it probably due to high cost. A recent study published by Feng and colleagues performed NGS of the phage displayed clones of nurse shark vNAR revealing more promising results than conventional methods (Feng *et al.* 2019).

This study conducted next generation sequencing (Illumina, MiSeq) for the first time without conventional cloning methods to identify Ig repertoire and to isolate potentially highest specific vNAR gene sequence of brownbanded bamboo shark.

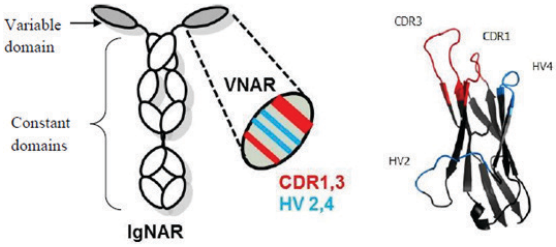

Figure 3. Structure of IgNAR showing variable domains (CDR1, CDR3, HV2 and HV4) and five constant domains

The application of next generation sequencing methods have not been done by many researchers to date on IgNAR except in a study on *Triakis scyllium*, which used Genome Sequencer FLX (GS FLX) in their study (Honda *et al*. 2010) and by Feng *et al*. for naïve nurse shark phage display library preparation (Feng *et al*. 2019). The amplification of IgNAR for the first time of a particular species were performed in almost all the previous studies by primer designing to reference sequence of another species or cloning. These methods may lead to biasness and restrict the volume of data which mask the actual scenario.

Therefore in this study, cDNA library was synthesized by the total RNA extracted from peripheral blood leukocytes (PBL) through Illumina (MiSeq) sequencing technique. Transcriptomic analysis using various bioinformatic tools revealed novel information and enhanced the IgNAR gene sequence database.

Characterization of the IgNAR constant domains

As per the transcriptome analysis, a higher homology was revealed and confirmed by the ClustalW sequence identity matrix to be greater than 0.9. Based on the transcriptome sequence of immunized shark, the brownbanded bamboo shark IgNAR was classified mainly into two types, i.e., the *C. punctatum* IgNAR-1 CH type and the IgNAR-2 CH type. A total of seven major polymorphic sites with minor variation frequency above 20 % were distributed in the constant domains of both IgNAR types with six nonsynonymous and one synonymous mutation (Figure 4).

Figure 4. Brownbanded bamboo shark IgNAR heavy chain constant domain sequences indicating the presence of two IgNAR types

Compatibility of the *C. punctatum* IgNAR constant region for immune modifications

Though IgNAR sequences are dissimilar to human immunoglobulin domains, the essential elements in the immunoglobulin fold are conserved. Like the analysis by Feige *et al*. 2014, in this study, the brownbanded bamboo shark IgNAR constant domains (C1 to C5) were aligned with other cartilaginous fish CH domains and human IgG domains.

Similar to the previous study on nurse sharks, brownbanded bamboo shark IgNAR CH also possessed a tryptophan molecule which helps hydrophobic residues to form a tight core around the disulfide bridge (Feige *et al.* 2014). Furthermore, molecules such as cysteine, tyrosine, phenylalanine and tryptophan along the C1-C5 domains in brownbanded bamboo shark IgNAR marked the significance of evolutionary conservation and indicates the stability in nature which in turn can be used to modify human immunoglobulins. However, in IgNAR constant domains, presence of hydrophobic core which surrounds the disulfide bridge and formation of helix interacting with aromatic residues lead to the stability and flexibility which lacks in human IgG.

vNAR nanobody as potential immunotherapeutic

Over the past ten years, use of monoclonal antibody (mAB) became popular by getting approved as therapeutics in clinical applications (Reichert 2012). The conventional antibodies have several drawbacks such as limitation of the systemically accessible targets and less tissue penetration. Therefore, discovery of small binding molecules with high efficiency came to picture in the field of immunotherapy. Among several antibody origin fragments or molecules available to date (Skerra 2007), vNAR from cartilaginous fish draws attention due to its unique characteristics. Leow *et al*, reviewed the currently available vNAR based products and it shows the potential of this smallest naturally available nanobody as an immunotherapeutic.

Synthesis of vNAR based nanobodies for the immunotherapy, diagnostics and biosensors is being investigated by many scientists today. However major concern in such methods was on the specificity, stability and safety of the nanobody. Almost all these drug developmental studies, used cloning or cell display method to obtain diverse sequences (Kovaleva *et al.*, 2014). However, this study proposes the NGS based amplicon sequencing method and molecular screening for selection of the candidate vNAR gene sequences with high affinity to antigen for their future applications as a potential immunotherapeutic.

References

- Diaz, M., Stanfield, R.L., Greenberg, A.S. & Flajnik, M.F., 2002. Structural analysis, selection, and ontogeny of the shark new antigen receptor (IgNAR): Identification of a new locus preferentially expressed in early development. *Immunogenetics*, 54(7), 501–512.
- De los Rios, M., Criscitiello, M. & Smider, V. 2015. Structural and genetic diversity in antibody repertoires from diverse species. *Current Opinion in Structural Biology*, 33, 27-41.
- Dooley, H. & Flajnik, M.F., 2006. Antibody repertoire development in cartilaginous fish. *Developmental and Comparative Immunology*, 30(1–2), 43–56.
- Feige, M.J., Grawert, M.A., Marcinowski, M., Henning, J., Behnke, J., Auslander, D.Herold, E.M., Peschek, J., Castro, C.D., Flajnik, M., Hendershot, L.M., Sattler, M., Groll, M. & Buchner, J., 2014. The structural analysis of shark IgNAR antibodies reveals evolutionary principles of immunoglobulins. *Proceedings of the National Academy of Sciences of the United States of America*, 111(22), 8155–60.
- Feng, M., Bian, H., Wu, X., Fu, T., Fu, Y., Hong, J., Flemming, B.D., Flajnik, M.F. & Ho, M., 2019. Construction and next-generation sequencing analysis of a large phage-displayed VNAR single-domain antibody library from six naïve nurse sharks. *Antibody Therapeutics*. 2 (1), 1-11.
- Flajnik, M.F., 2002. Comparative analyses of immunoglobulin genes: surprises and portents. Nature reviews. *Immunology*, 2(9), 688–98.
- Flajnik, M.F. & Dooley, H., 2009. The generation and selection of single-domain, v region libraries from nurse sharks. *Methods in molecular biology (Clifton, N.J.)*, 562, 71–82.
- Hamers-Casterman, C., Atarhouch, T., Muyldemans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman N. and Hamers, R., 1993. Naturally occurring antibodies devoid of light chains. *Nature*, 363(6428), 446–448.
- Hinds, K.R. & Litman, G.W., 1986. Major reorganization of immunoglobulin VH segmental elements during vertebrate evolution. *Nature*, 320(6062), 546–9.
- Honda, Y., Kondo, H., Caipang, C.M.A., Hirono, I. & Aoki, T., 2010. cDNA cloning of the immunoglobulin heavy chain genes in banded houndshark Triakis scyllium. *Fish and Shellfish Immunology*, 29(5), 854–861.
- Kopsidas, G., Roberts, A.S., Coia, G., Streltsov, V.A. & Nuttall, S.D., 2006. *In vitro* improvement of a shark IgNAR antibody by Qß replicase mutation and ribosome display mimics in vivo affinity maturation. *Immunology Letters*, 107(2), 163–168.
- Kovaleva, M., Ferguson, L., Steven, J., Porter, A. & Barelle, C., 2014. Shark variable new antigen receptor biologics - a novel technology platform for therapeutic drug development. *Expert opinion on biological therapy*, 14(10), 1527–1539.
- Kubota, T., Niwa, R., Satoh M., Akinaga S., Shitara, K. & Hanai, N., 2009. *Engineered therapeutic antibodies with improved effector functions. Cancer Science*, 100(9), 1566–1572.
- Litman, G.W., Anderson, M.K. & Rast, J.P., 1999. Evolution of antigen binding receptors. *Annual Review of Immunology*, 17(1), 109–147.
- Müller, M.R., Saunders, K., Grace, C. et. Al., 2012. Improving the pharmacokinetic properties of biologics by fusion to an anti-HSA shark VNAR domain. *mAbs*, 4(6), 673–685.
- Nikolova, V., Koo, C., Abrahim, S.A. *et al.*, 2009. Differential roles for membrane-bound and soluble syndecan-1 (CD138) in breast cancer progression. *Carcinogenesis*, 30(3), 397–407.
- Presta, L.G., 2008. Molecular engineering and design of therapeutic antibodies. *Curr Opin Immunol*, 20(4), 460–470.
- Reichert J.M., 2012. Marketed therapeutic antibodies compendium. *MAbs*. 4, 413-415.
- Schroeder, H.W. & Cavacini, L., 2010. Structure and Function of Immunoglobulins. *Journal of Allergy and Clinical Immunology*, 125(202) S41-S52.

- Skerra A. 2007. Alternative non-antibody scaffolds for molecular recognition. *Curr Opin Biotechnol*, 18, 295-304.
- Tonegawa, S., 1983. Somatic generation of antibody diversity. *Nature*, 302(5909), 575–581.
- Wang, F., Ekiert, D.C., Ahmad, I., Yu, W., Zhang, Y., Bazirgan, O., Torkamani, A., Raudsepp, T., Mwangi, W., Criscitiello, M.F., Wilson, I.A., Schultz, P.G. & Smider, V.V. 2013. Reshaping antibody diversity. *Cell*, 153(6), 1379-1393.
- Williams, F., A. & Barclay, A.N., 1988. The immunoglobulin superfamily domains for cell surface recognition. *Annual Review of Immunology*, 6, 381–405.
- Flajnik, M.F., 2002. Comparative analyses of immunoglobulin genes: surprises and portents. Nature reviews. *Immunology,* 2(9), 688–98.
- Zielonka, S., Weber, N., Becker, S. *et al*., 2014. Shark Attack: High affinity binding proteins derived from shark vNAR domains by stepwise *in vitro* affinity maturation. *Journal of Biotechnology*, 191, 236–245.
- Zielonka, S., Empting, M., Grzeschik, J., *et al*., 2015. Structural insights and biomedical potential of IgNAR scaffolds from sharks. *mAbs*, 7(1), 15–25.

Guest Articles

Role of medium chain triglycerides in paracellular permeation

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Introduction

Medium chain triglycerides (MTCs) consists of three fatty acids chains of carbons ranging from 6 to 12 and a glycerol molecule. MCTs are easily absorbed into the bloodstream unlike other lipid molecules that retains long period of time in the stomach for a complex digestion process. Since these class of lipid are fully digested and absorbed into the body, there are no any safety issues identified. (Pouton CW, *et al,* 2008) These features confer unique benefits of MCTs. At the same time MCTs have a potential to reduce obesity, neurological diseases and cardiovascular diseases. (Roger E, *et al* 2011) This article provides a comprehensive summary on role of MCTs in paracellular permeation, its proposed mechanisms and other factors which effect paracellular permeation.

Some physicochemical properties of compounds restrict or reduce the absorption through the intestine. Properties such as presence of strong charged functional group, low octanol/aqueous partition, a substantial number of hydrogen-bonding functional groups, high molecular weight and high polar surfaces affect the poor membrane permeability. (Sunil P, *et al*, 2005) Compounds which have any of these properties may benefit most from the property of MCTs in the paracellular permeation. Nowadays pharmaceutical industry focuses on excipients which can carry the drug molecules to target sites that are poorly absorbed through active transport. Generally, absorption enhancers or absorption excipient are related to its concentration at the site of drug absorption. For this purpose, both the excipient and the drug molecule should be intestinal site dependent. The basic property which benefits as a solvent for drugs is decides by its effective ester group concentration. (Cao Y, *et al*, 2004). MCTs have higher solvent capacity when compared with long chain triglycerides at the same time they are less prone to oxidization. (Kaukonen AM, *et al*, 2004) Different vegetable sources have different compositions of fatty acids. Some of them are listed in the Table 1. Pure triglycerides are available in refined vegetable oils. Coconut oil and palm seed oil are rich sources of MCTs. (Collnot EM, *et al* 2004 and Collnot EM, *et al*, 2007).

Hydrophilic compounds which contain strongly charged functional groups and that are of high molecular weight confer the importance of paracellular pathway as these molecules are refrain from passing through the intestinal barrier by intracellular permeation.(Hayashi,