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ISOLATION AND CHARACTERIZATION OF HEAVY CHAIN SINGLE-DOMAIN ANTIBODIES FROM THE HORN SHARK *Heterodontus francisci* AGAINST MURINE CD38

TESIS

que para cubrir parcialmente los requisitos necesarios para obtener el grado de DOCTOR EN CIENCIAS

Presenta:

KARLA OYUKY JUÁREZ MORENO

Ensenada, Baja California, México, 03 de Marzo de 2011

RESUMEN de la tesis de **Karla Oyuky Juárez Moreno,** presentada como requisito parcial para la obtención del grado de DOCTOR EN CIENCIAS DE LA VIDA con Orientación en Biotecnología Marina. Ensenada, Baja California. 03 de Marzo, 2011.

AISLAMIENTO Y CARACTERIZACIÓN DE ANTICUERPOS DE CADENA PESADA DE DOMINIO SENCILLO DEL TIBURÓN *Heterodontus francisci* DIRIGIDOS CONTRA CD38 MURINO

Resumen aprobado por:

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Dr. Alexei Fedórovish Licea Navarro
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Además de los anticuerpos convencionales, los tiburones desarrollaron anticuerpos compuestos por la cadena polipeptídica pesada denominados IgNAR, el fragmento variable de éstos se designa como vNAR y constituye los dominios de anticuerpo más pequeños capaces de reconocer a un antígeno. El largo inusual de su CDR3, les permite penetrar en las cavidades de los sitios activos de las enzimas e inhibirlas. Considerando esta característica, uno de los objetivos de este proyecto consistió en aislar anticuerpos de dominio sencillo del tiburón *Heterodontus francisci,* a partir de una biblioteca inmune específica, capaces de unirse a la enzima CD38 murina. La proteína CD38 actúa como receptor en la sinapsis inmunológica, y como enzima está involucrada en la degradación extracelular del NAD^+. Clínicamente esta proteína se utiliza como marcador de prognosis en la Leucemia Linfocítica Crónica.
En este proyecto se generaron y aislaron vNARs que específicamente se unen a CD38 murino. A partir de un tiburón inmunizado se obtuvo una biblioteca de vNARs altamente diversa, después de varias rondas de selección se aislaron 16 familias. Las proteínas recombinantes así como los fago-anticuerpos fueron utilizados para probar su actividad de reaccionar específicamente contra CD38 en un ensayo de ELISA, los resultados revelaron que todas las familias con excepción de una eran capaces de unirse a CD38. A pesar de esto, ninguno de los vNARs aislados fue capaz de inhibir la actividad hidrolítica de la enzima. Se realizaron ensayos de FACS sobre células que expresaban CD38 en su membrana, como células de bazo de ratón y células de linfoma. De esta forma se identificó que los vNAR tenían una capacidad baja de unirse a células de bazo de ratón, pero se unían de manera favorable a las células de linfoma EL4-R

(CD38^{pos}). Hasta el momento se sabe que la familia SAGTK es la que mejor reacciona contra la enzima CD38 de ratón.
El segundo objetivo del proyecto, fue generar anticuerpos capaces de reconocer moléculas de tipo vNAR, para ello se inmunizaron tres ratones con un vNAR purificado. Los ratones inmunizados desarrollaron una respuesta inmune específica, por lo que se utilizó el bazo y los nodos linfñaticos de uno de ellos para generar anticuerpos monoclonales. De la fusión celular se aislaron clonas capaces de reconocer una variedad de vNARs, después de subclonar las células fusionadas, se aislaron los anticuerpos monoclonales 370 y 533 ambos con un isotipo IgG1k. Usando los anticuerpos monoclonales (MAb) #379 y #533 en ensayos de ELISA y dot blot, se detectó específicamente diferentes vNARs y suero de tiburón. Además se evaluó si ambos MAb eran capaces de detectar alguna molécula presente en el suero del tiburón *H. francisci,* demostrándose que una molécula de aproximadamente 80 kDa fue inmuno-precipitada y detectada por Western blot utilizando el MAb 370. Otras dos proteínas del suero de tiburón fueron precipitadas, pero no detectadas por western blot, la primera con un peso molecular de 160 kDa y la segunda con un peso entre los 160 y 260 kDa. Todas estas moléculas están actualmente siendo analizadas por espectometría de masas para identificar la naturaleza de cada una de ellas, hasta la fecha se tiene la secuencia parcial de la proteína de 80 kDa que se confirmó como un IgNAR de *H. francisci.*
Por otro lado, los MAbs 370 y 533 se utilizaron para determinar si en el curso de una inmunización de un tiburón *H. francisci* existían cambios en la especificidad del suero con el antígeno utilizado. Los resultados obtenidos utilizando el MAb 370 como método de detección en un ensayo de ELISA, muestran que después de 10 días posteriores a la inmunización, existe un reconocimiento específico al antígeno, dicha señal alcanza su punto más alto entre los días 26 y 40. Se demostró también que ante la falta de la administración de antígeno, la respuesta específica disminuye e incrementa de forma rápida ante una nueva administración. Los resultados obtenidos hasta ahora representan el primer acercamiento para investigar si existe una respuesta antígeno específica durante los procesos de inmunización del tiburón *H. francisci* y para determinar si existe maduración de la afinidad de los anticuerpos mediante un proceso dirigido por el antígeno.

Palabras Clave: IgNAR, *Heterodontus francisci*, CD38.

ABSTRACT of the thesis presented by **Karla Oyuky Juárez Moreno** as a partial requirement to obtain the DOCTOR OF LIFE SCIENCES degree in Marine Biotechnology Ensenada, Baja California, Mexico March 3rd, 2011.

ISOLATION AND CHARACTERIZATION OF HEAVY CHAIN SINGLE-DOMAIN ANTIBODIES FROM THE HORN SHARK *Heterodontus francisci* AGAINST MURINE CD38

Besides conventional antibodies, sharks have evolved unusual antibodies compose only of the heavy chain, its variable domains are designated as vNAR, and are the smallest known antigen-binding antibody fragments exhibiting a high degree of chemical and thermal stability. Its unusual long CDR3 allows them to penetrate into the active site of enzymes and inhibit its activity, which makes them excellent enzymatic inhibitors. Therefore, one of the goals of this project was to isolate single domain antibodies from shark immune library specific for murine CD38 with binding and inhibitory activity. The CD38 protein has a dual function, as an adhesion molecule plays important roles in the immunological synapse and as enzyme, acts as an ecto-NADase, involved in extracellular NAD^+ degradation. Clinically CD38 is a prognostic marker in Chronic Lymphocytic Leukemia.

In this work we present the generation and isolation of vNARs that specifically bind to murine CD38. High diversity phage display library was obtained from immunized shark, with it several rounds of selection were performed and 16 vNAR families were isolated. Purified vNAR proteins and vNAR-phages were used to test their reactivity to CD38; revealing that all the families but one, specifically reacts with the enzyme. Although vNAR families bound to soluble CD38 protein, they were unable to inhibit its NADase enzymatic activity. However, to corroborate the binding capacity of isolated vNARs, FACS analysis were further accomplished on cells that express CD38 as cell-surface protein, a low reactivity was shown by vNAR-phages on mouse spleenocytes, whereas on $CD38^{pos}$ mouse lymphoma cell line EL4-R the reactivity was higher and specific. At the very end, the family SAGTK had a better binding reactivity to CD38.

The second major goal of this project was to generate second reagents able to recognize vNAR molecules. Thee mice were immunized with a purified vNAR protein (SAALAK), a specific immune response against it was developed by the mice. To make monoclonal antibodies, the lymph nodes and spleen from one of the mice were taken and fused together with a myeloma cell line. From fused cells, positive clones reacting against a wide variety of VNAR molecules were isolated, after subcloning; two hybridoma cells #370 and #533 retained the ability to specifically react with vNARs. The isotype of both clones were determined as IgG1k.

Monoclonal antibodies #370 and #533 specifically detect vNARs and shark serum by ELISA and dot blot. Furthermore, it was tested whereas MAbs 370 and 533 were able to detect any molecule present in the serum from horn shark *Heterodontus francisci*, and it was shown by an immunoprecipitation assay that a

molecule of ~80kDa was specifically precipitated and detected by Western blot using the hybridoma #370. Two other shark serum´s proteins were precipitated but not detected by western blot (one of 160 kDa and other in between 160 and 260 kDa). Mass spectrometry analysis of all the identified molecules is currently underway, to determine the identity of them and confirm whereas the 80kDa molecule is indeed a IgNAR molecule.
In addition, MAbs 370 and 533 were used to determine if during the course of an immunization protocol of one *H. francisci* shark, there are any changes in the specific reactivity of serum molecules with an specific antigen. The obtained results using MAb 370 to detect the reactivity indicated that there is an specific-antigen response developed by the immunized sharks then after 10 days post immunization and raising the higher point in between days 26 and 40 post immunization, it was also shown that in the absence of antigen, the levels of reactivity decrease, but soon after boost administration, the specific response increase. This represents the first attempts to investigate if there is a specific-antigen response during the immunization of horn sharks and determine whereas an affinity maturation process is taking place by means of an antigen-driven process.

Keywords: IgNAR, *Heterodontus francisci*, CD38.

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tu fuerza y amor siempre motivaron este trabajo,
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CHAPTER I

INTRODUCTION

I.1 The immune system

All vertebrate organisms are protected from infectious agents, hazardous substances and the damage they generate by a variety of effector cells and molecules, all of which as a whole constitute the immune system. This has four main tasks: i) immunological recognition, ii) immune effector functions, iii) regulation, and iv) memory (Abbas and Lichtman, 2004; Murphy et al.2008).

Innate immunity consists of cellular and biochemical defense mechanisms that are in place even before infection, in order to respond rapidly to it. This system provides the first line of defense against microorganisms. The principal components of innate immunity are i) physical and chemical barriers (epithelia and antimicrobial substances produced on epithelia), ii) phagocytic cells (neutrophils, macrophages) and natural killer cells (NK), iii) blood proteins (the complement system and mediators of inflammation), iv) cytokines that regulate and coordinate many of the activities of the cells of innate immunity (Abbas and Lichtman, 2004).

I.2 Adaptive immunity

In contrast to innate immunity there are other immune responses that are triggered by exposure to infectious agents and increase in magnitude and defense capabilities with each successive exposure to a particular antigen. Because this type of immunity is developed during the lifetime of an individual as an adaptation to infection with that pathogen it is called adaptive immunity. In many cases an adaptive immune response also results in the phenomenon known as immunological memory, which confers lifelong protective immunity to reinfection with the same pathogen. This is one of the main features that distinguish an adaptive immune response from naive immune responses (Abbas and Lichtman, 2004; Murphy et al. 2008).

The defining features of adaptive immunity are the exquisite specificity for distinct molecules, and being able to recognize a large number of microbial and non-microbial substances along with the extraordinary ability to distinguish between closely related molecules.

There are two branchs of the adaptive immune response, called cell-mediated immunity and humoral immunity. The former is mediated by T lymphocytes. Intracellular pathogens such as viruses and some bacteria, survive inside the phagocytes and other host cells, where they are inaccessible to circulating antibodies. The defense against such infections is a function of cell-mediated immunity.

Humoral immunity is mediated by molecules in the blood and mucosal secretions called antibodies that are produced by B lymphocytes (B cells). Antibodies recognize antigens, neutralize their infectivity and target them for elimination by various effector mechanisms. Antibodies themselves are specialized molecules and may activate different response mechanisms, such as promoting phagocytosis or triggering the release of inflammatory mediators.

I.3 Antibodies

Antibodies (Abs) are produced in a membrane-bound form by B lymphocytes, and these membrane molecules function as B cell receptors for antigens (Ag). The interaction of an Ag with membrane Abs on naive B cells initiates a response to them, and thus constitutes the recognition phase of humoral immune responses. Abs are also produced in a secreted form by antigen-stimulated differentiated B cells called plasma cells, and are found in the plasma, mucosal secretions and in the interstitial fluid of the tissues. In the effector phase of the adaptive immune response, these secreted Abs bind to antigens and trigger several effector mechanisms that eliminate Ag.

When blood or plasma forms a clot, Abs remain in the residual fluid, called serum, it contains detectable number of these molecules that bind to a particular Ag, and this is called antiserum.

I.4 General features of antibody structure

An antibody (Ab) molecule has a symmetric core structure composed of two identical light chains (L) and two identical heavy (H) chains forming a Y-shaped molecule. Each light chain is about 24 kDa and each heavy chain is around 55 to 70 kDa. In conventional Abs, one or two disulfide bonds link the H chains and one L chain is covalently attached to one H chain by disulfide bonds (Edelman, 1991). The H and L chains contain a series of repetitive immunoglobulin (Ig) domains of approximately 110 amino acids. Each domain has a characteristic β-strand tertiary structure, known as the immunoglobulin domain (Davies and Chacko, 1993).

Both H and L chains consist of an amino-terminal variable (V) region that participates in the Ag recognition, and carboxy-terminal constant (C) regions that mediate effector functions. The C domains of the H chain are designated as C_H and those of the L chain as C_L. The V domains contain regions of variability in amino acid sequence that distinguishes a particular Ab made by one B cell from another Ab made by another clone. The V domains of both pairs of H and L chains of each antibody are associated to form the region that confers the specific antigen binding capacity of the Ab. This site is known as the antigen binding site. In any given conventional Ab the two H and the two L are identical, this guarantees the ability to bind two identical antigens simultaneously. The C domains of the H chains are numbered from the N terminus to the C-terminus as follows: C_H1, C_H2, C_H3 and so on.

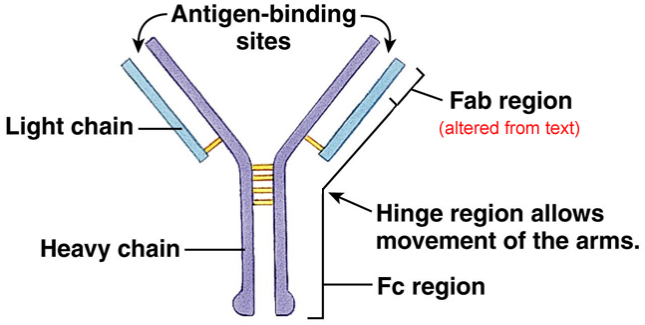

Figure 1. Schematic representation of an antibody general structure. Taken from http://www.colorado.edu/intphys/Class/IPHY3430-200/image/24-12.jpg

There are three segments with high variability, called complementarity-determining regions (CDRs), in all V_L or V_H domains (Abbas and Lichtman, 2004). Proceeding from either VL or VH amino terminus, these regions are called CDR1, CDR2 and CDR3. There is more sequence diversity in the CDR3 than in CDR1 or CDR2, therefore the CDR3 plays an especially important role in Ag binding by an Ab molecule. Crystallographic analysis of Ab molecules revealed that the CDRs form extended loops that are exposed on the surface of the Ab and are thus available to interact with Ags. The differences among CDRs of different Abs make them display unique structures at the surface of the projecting loops, thereby providing the basis for the different specificities for Ags. The CDRs are flanked by conserved sequences, designated as frameworks (FRs) that are mostly responsible for the correct folding of the molecule (Davis & Cohen, 1996).

Abs interact with a small part of a protein or polysaccharide Ag, this structure is referred to as an epitope, while the region of the Ab that complementary binds to the epitope is called paratope. Epitopes can be linear or conformational, depending whether the amino acids that make up the epitope are next to each other in the primary sequence or brought into proximity by the folding (conformation) of the protein. The interactions between Ab and Ag involve hydrogen bonds, van der Waal's, electrostatic and hydrophobic forces. They can be disrupted by means of high salt concentration or pH (Abbas and Lichtman, 2004; Braden and Poljak, 1995).

According to the structure of the C domains, Abs can be divided into different classes and subclasses and also might be referred as isotypes such as: IgA, IgD, IgE, IgG and IgM in humans. IgA and IgG isotypes can be further subdivided into subtypes: IgA1 and IgA2, and IgG1, IgG2, IgG3 and IgG4. Antibody isotypes and subtypes differ in their C regions and therefore in what they bind to and what kind of response they trigger. Ab molecules are flexible, allowing them to bind to different types and arrays of Ags, this feature is mainly mediated by the hinge region located between the CH1 and CH2 regions in some isotypes, the length of the hinge regions varies from 10 to 60 amino acids.

I.5 Antibody production and B cell activation

B and T lymphocytes arise from a common bone marrow-derived precursor that becomes committed to the lymphocyte lineage. B cell maturation proceeds in the bone marrow; early maturation is characterized by cell proliferation induced by cytokines like IL-7. B cell maturation involves somatic recombination of antigen receptor gene segments and the expression of the Ig molecules. Both steps are essential for the survival and maturation of developed lymphocytes and for the selection processes that lead to a diverse repertoire of useful antigen specificities (Abbas and Lichtman, 2004; Murphy et al.2008). Diversity of the antibody repertoire is generated by the combinatorial association of multiple germline V, D and J genes mediated by the RAG-1 and RAG-2 enzymes, and further junction diversity is generated by the addition of random nucleotides to the sites of recombination, mediated by the TdT enzyme. These mechanisms generate most of the diversity at the junctions of the V that form the third hypervariable region of an Ab (Abbas and Lichtman, 2004; Murphy et al.2008).

In the humoral immune response, B lymphocytes secrete Abs in response to the stimulation of either protein or non-protein Ags. This response requires the contribution of $CD4^+$ helper T cells specific for the Ags. The activation of B cells is triggered by the clustering of membrane Ig antigen receptors, whereupon the associated signaling molecules Igα and Igβ initiate signalling cascades that lead to the activation of transcription factors in the cell nucleus and to the expression of various genes.

Helper T cell-dependent B cell responses to protein and Ag require the initial activation of naive T cells in the T cell zones, and of B cells in lymphoid follicles in lymphoid organs. The activated lymphocytes migrate toward one another and interact at the edges of follicles, whereby the B cells present the Ag to the helper T cells. This leads to the activation of the T helper cells and to expression of the molecule CD40L, which binds to CD40 on B cells. This signal allows T cells to secrete cytokines that bind to the cytokine receptors on the B cell surface and

stimulate proliferation and the differentiation into antibody-secreting cells. They also induce isotype switching in B cells, leading to the production of different Ig isotypes that mediate different effector functions.

Germinal centers are formed inside the follicles of spleen and lymph nodes when activated B cells migrate into the follicles and proliferate. The late events in T cell-dependent Ab responses, including affinity maturation and the generation of memory B cells, take place within germinal centers.

The affinity maturation leads to increased affinity of Abs to their Ag during the course of a T cell-dependent humoral response, and is the result of somatic hypermutation of Ig H and L chains genes, followed by the selective survival of the B cells that produce high-affinity Abs and bind to the antigen displayed by the follicular dendritic cells in the germinal centers. Some of the progeny of B cells differentiate into antibody-secreting cells that migrate to extra-follicular regions of secondary lymphoid organs and bone marrow, other B cells become memory cells that live for long periods, recirculate between the lymph nodes and spleen, and respond rapidly to subsequent exposures to Ag by differentiating into high-affinity antibody secretors.

I.6 Antibody diversity

There are two events that determine the diversity of the Ab repertoire. The first occurs during the differentiation of pre-B cells to mature B cells, where gene segments rearrange to create a large diverse pool of V_H and V_L sequences. The second implies somatic hypermutation, which occurs during the differentiation of B cells into plasma cells, the affinity and specificity of the Ab response is accented by the introduction of mutations into the rearranged V_H and V_L sequences.

The diversity of the Ab sequences results from a series of gene rearrengments within the V domains of both the L and H chains (Tonegawa, 1983), during the splicing process, some nucleotides can be lost from the genetic elements or added in a random (N nucleotides) or template-dependent (P nucleotides) fashion. In total, potentially more than 10^9 different V-D-J junctions can be generated, and

further random combinations of H and L chain partners increase the potential pre-immune Ab repertoire to larger than 10^{13} different Ig members. Somatic mutations can add amino acid substitutions that add further diversity (Roitt et al. 1993).

I.7 Antibody engineering and protein scaffolds

Antibodies have multiple applications in research, medicine, and biotechnology. As molecular tools they are used in diverse techniques such as immunoblotting, immunoprecipitation, ELISA, radioimmuno-assays, and as catalysts in various chemical reactions (Winter and Milstein, 1991). In industry, their specific binding properties have been used for immunoaffinity chromatography, as blocking and inhibitor molecules, and for the design of new drugs. In medicine they are used for a variety of *in vivo* therapeutic applications, including use as antivenom molecules, for drug delivery, to deplete certain cell populations, and to interrupt cytokine signalling cascades. In diagnosis they are used as indicator molecules and in imaging assays (Li et al. 1994; Pluckthun, 1990; Weselowski et al. 2008).

For this reason a novel branch of antibody biotechnology has been developed that aims withn Ab modifications, this involves size reduction, humanization, modulation or improvement of binding capacity and the development of heterologous expression systems for high protein production yields.

Since only a small portion of the Ab is involved in Ag recognition and binding, its reduction i.e to the size of the Fv fragment or smaller would be beneficial in immunoaffinity and biosensor applications, because it increases the stability of the ligand (Welling et al. 1990; Welling et al. 1991) and have better tissue penetration and blood clearance (George, 1994; Gruber et al. 1994). **Figure 2**, illustrates among others the classical way to reduce Abs, by digestion with pepsin, yielding bivalent $F(ab')^2$ fragments, or with papaine, yielding monovalent Fab fragments (Roitt et al. 1993). The Fv fragments consist of the V_L and V_H fragments, held together by unstable non-covalent interactions. Preservation of the binding site of the CDR loops is possible by generation of recombinant versions of the Fv fragments that are stabilized by linking the V_H and the V_L domain with a flexible

glycine-rich peptide linker. This allows its expression as a single polypeptide chain, called single chain Fv (scFv) (Bird et al. 1988; Huston et al. 1988).

The ability to mimic the natural process of Ab selection and maturation provides a tremendous potential for the development of protein functions *in vitro* (Simmons et al. 2008). Selection and evolution of protein functions require the construction of large libraries of molecules that share a common structural scaffold but differ in variable regions that may mediate the interaction with other proteins. The rapid isolation of interesting mutants from the library depends on a system that links the phenotype of a mutant with its genotype. In 1985 Smith and coworkers developed the phage display technique, which will be described in more detail below (Smith, 1985).

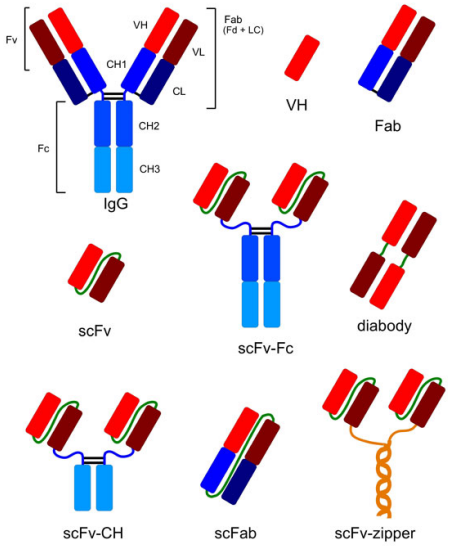

Figure 2. Schematic representation of immunoglobulin molecule and its derived fragments. Taken from http://www.landesbioscience.com/images/journals/mabs/mabs-image02.jpg

I.8 M13 bacteriophage display technique

In the middle of the 80s Smith and coworkers described a novel molecular tool for mapping protein-protein interactions. They determined that the N-terminus of the filamentous bacteriophage M13 pIII minor coat protein could be genetically modified by short peptide sequences without eliminating the bacteriophage's infectivity for its bacterial host (Smith, 1985; Smith and Johnson, 1988). Smith demonstrated that using a fragment of the restriction endonuclease *Eco*RI as fusion partner, it was possible to display it as a fusion-protein on the surface of the bacteriophage. It was shown that bacteriophages could be selected using an antibody against the displayed peptide, and in this way it was possible to enrich the specific particles from an original library of 10^8 non-specific clones (Parmley and Smith, 1988). Other groups have shown that random peptides displayed on the pIII protein could be propagated as library, which could be used to select peptide sequences binding to streptavidine or monoclonal antibodies in an *in vitro* iterative selection process known as biopanning or panning selection (Cwirla et al. 1990; Devlin et al. 1990; Smith, 1991; Smith and Scott, 1993).

Bacteriophage display technique as shown in **figure 3**, coupled the phenotype (the displayed peptide on the bacteriophage surface) with the genotype (the DNA sequence cloned as a fusion of the pIII gene). Since its description many groups have shown that peptides, proteins and peptide libraries can be displayed on the surface of coating proteins of bacteriophage, being pIII, pVI and pVIII the frequently displayed systems (Hoogenboom, 2002; Wilson and Finlay, 1998). The use of the M13 bacteriophage has many advantages: it is produced easily at a high titer (>10^{12} pfu/ml), is physically stable, and represents a characterized phagemid-cloning vector (Wilson and Finlay, 1998).

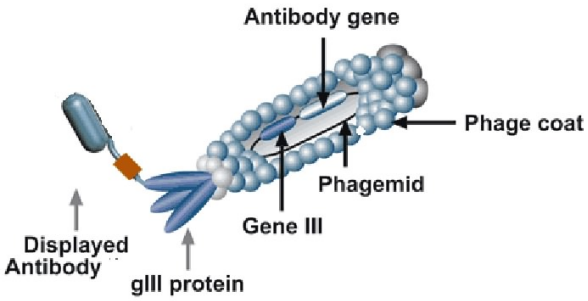

Figure 3. Schematic representation of bacteriophage display of single domain antibodies. As an example of protein displaying scaffold system, bacteriophage display technique couple phenotype with genotype. In this case it is shown the displayed of a heavy chain single domain antibody from shark (VNAR) in the minor bacteriophage coat protein pIII. Modified from http://www.abdserotec.com/uploads/hucal-fig2.gif

Phagemid vectors are plasmids that contain both, bacterial and the M13 replicative origins, an antibiotic resistance gene, and a multiple cloning site followed by the gene of one M13 coat proteins. To generate infectious phage particles, transformed bacteria are superinfected with a helper phage, which is a modified M13 bacteriophage with a low replicative level. Since the machinery for bacteriophage replication and packaging is provided by the helper phage, and the phagemid vector provides the information for the displayed recombinant protein, whole bacteriophages displaying the protein of interest are obtained. The use of a phage library for biopanning selection of specific binding phages is illustrated in **figure 4**.

The first antibody successfully displayed was an anti-lysozyme scFv (McCafferty et al. 1990), which retained the antigen binding site and antigenic specificity. It was also shown that Fab fragments from the same antibody could be assembled on the surface of bacteriophage by linking one chain to the pIII coat protein and by secreting the complementary chains as a soluble chain into the bacterial periplasm (Hoogenboom et al. 1991). Introducing an amber mutation into the phagemid vector between the antibody chain and the coat protein made it possible to use the

system either for display or to produce soluble Fab fragments, using non-suppressor bacterial strains (Hoogenboom et al. 1991).

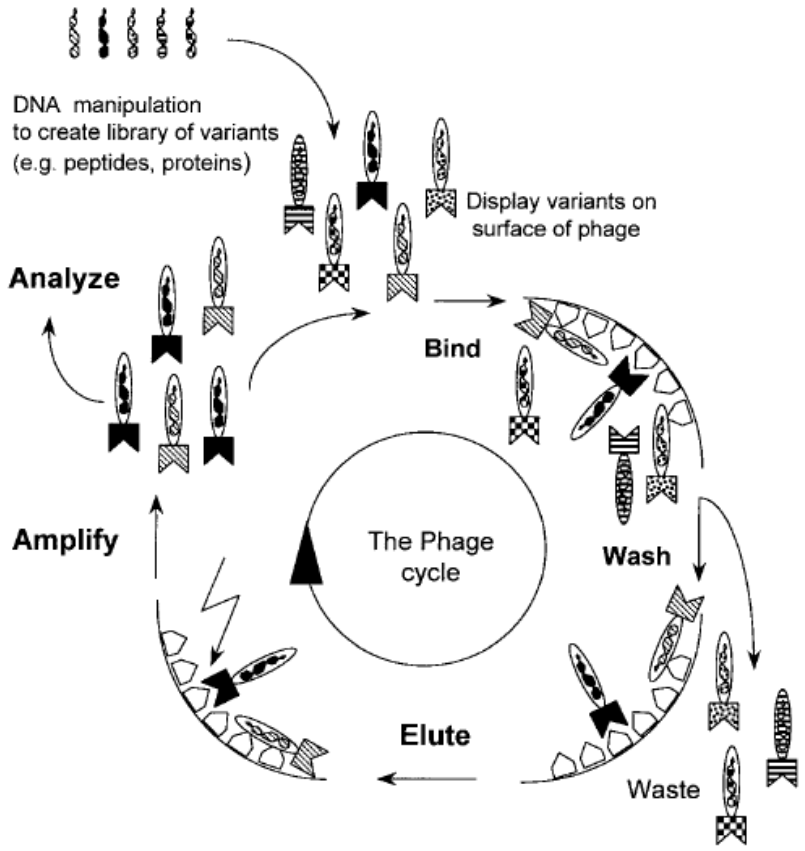

Figure 4. Phage-display cycle. DNA encoding of certain ligands (e.g., peptides or proteins) is cloned into the phage genome as part of one of the phage coat proteins (pIII, pVI, or pVIII). From these repertoire, phage carrying specific-binding ligands can be isolated by a series of recursive cycles of selection on Ag, each of which involves binding, washing, elution, and amplification (Hoogenboom, 2002).

Initial libraries of antibody fragments in a pIII system were successfully constructed, the isolated molecules from them have a dissociation constant of 10 μM (Clarkson et al. 1991). Expression and biopanning selection against immobilized antigen led to the isolation of protein-binding fragments from an anti-tetanus toxoid Fab library (Barbas et al. 1991). A human scFvs-antibody library

was constructed from peripheral blood lymphocytes and displayed on M13-bacteriophage (Marks et al, 1991). This was the first bacteriophage display library used to isolate human antibodies against unknown antigens without the need for immunization (Hoogenboom and Winter, 1992). Synthethic antibody libraries were reported by Barbas and co-workers in 1992, and in the same year Hoogenboom generated a complete synthethic library of scFv domains (Barbas et al, 1992; Hoogenboom and Winter, 1992).

In summary, several bacteriophage-displayed library types can be constructed, based upon the desired requirements and applications. As expected libraries from immunized individuals present a bias towards antibodies specific for the previously encountered antigen (Clackson et al, 1991). However, it has been demonstrated that antibodies can be isolated showing affinities comparable to those from an immune repertoire (Griffiths et al. 1994). Therefore, non-immune or naive libraries can be also be used for the isolation of high-affinity antibodies against many antigens (Marks et al, 1991). Nevertheless, immune libraries represent a secure way to isolate antibodies directed against the antigen of choice in the absence of a large synthetic library.

I.9 General features of horn shark *Heterodontus francisci*

The horn shark, *Heterodontus francisci*, (**figure 5A)** belongs to the family Heterodontidae. It is endemic to the coastal waters from California to the Gulf of California (**figure 5B**). Typically this species measure in between 1 m to 1.5 m, they have two high dorsal fins with large venomous spines, and a brown or gray coloration with many small dark spots.

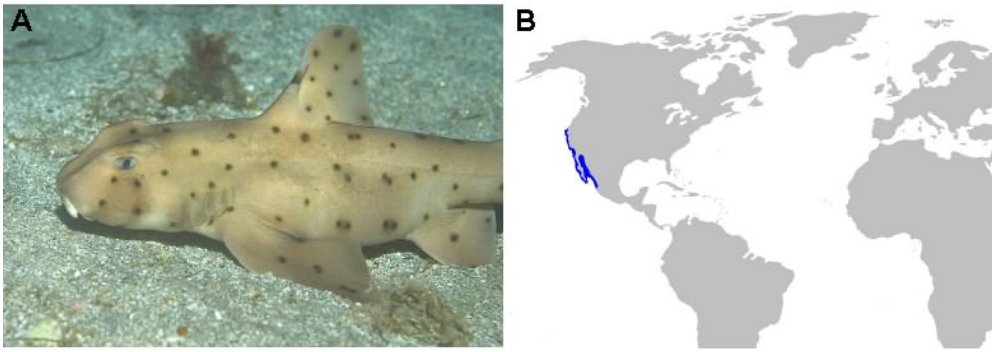

Figure 5. Geographical distribution of *Heterodontus francisci* horn shark. (**A**) A *Heterodontus francisci* horn shark specimen and (**B**) the gepgraphical distribution of horn shark. Taken from http://en.wikipedia.org/wiki/File:Heterodontus_francisci_distmap.png#file

Horn sharks are solitary predators, hunting at night and seems that its and activities are mostly regulated by light levels, they use to live in deeper sandy areas of the costal sea. Their reproduction is oviparous. Horn sharks are harmless unless harassed, and are readily maintained in captivity.

I.10 Adaptive immunity in jawed vertebrates

Jawed vertebrates (cartilaginous and bony fish, amphibians, reptiles, birds and mammals) share the basic building blocks of an adaptive immune system, including immunoglobulins (Ig), T-cell receptors (TCR) and polymorphic MHC class I and class II molecules, a thymus and compartmentalized secondary lymphoid tissues, as well as recombination-activating gene (RAG)-mediated V(D)J recombination and somatic hypermutation of immunoglobulin genes, as shown on **figure 6** (Flajnik, 2002).

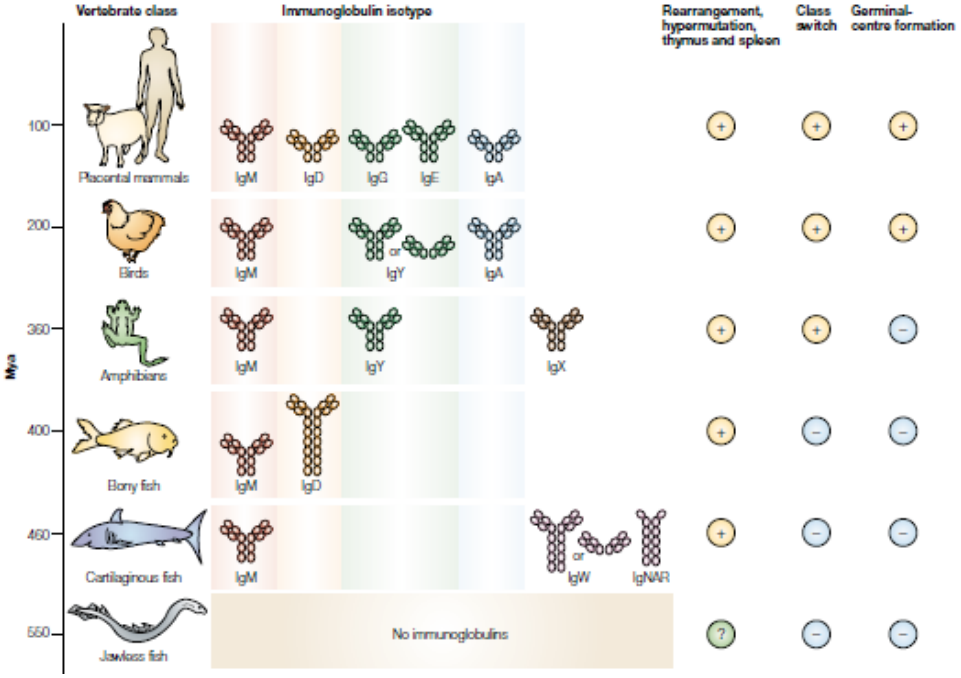

Figure 6. Evolution of features from the adaptive immune system in vertebrates. Taken from (Flajnik, 2002).

Cartilaginous fish lack bone marrow and have unique tissues such as the Leydig and the epigonal organ, both are believed to be the equivalents of mammalian bone marrow **figure 7.** The Leydig organ is attached to the esophagus and contains many immature lymphocytes that seem to be important for myeloid cell development (Fänge, 1984; Flajnik, 2002). The epigonal organ, similar in structure and organization to the Leydig organ, is physically attached to the gonads. Either one or both tissues are present in all cartilaginous fishes, but nurse sharks only have the epigonal organ (Flajnik, 2002).

It was described recently that *RAG1, TdT,* and B-cell specific transcription factors are expressed in the Leydig and the epigonal organs, suggesting that these tissues play a role in lymphopoiesis and are likely to be the bone marrow equivalents of cartilaginous fishes (Flajnik, 2002; Miracle et al. 2001; Rumfelt et al. 2001, 2002; Rowley et al. 1988).

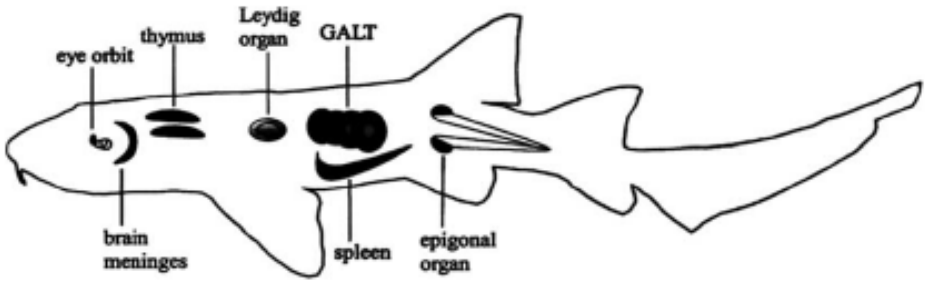

Figure 7. Schematic representation of different immunological organs and their relative positions in the shark (Dooley and Flajnik, 2006).

Three immunoglobulin H chain isotypes have been identified in cartilaginous fish: IgM, IgW and IgNAR, all as transmembrane and secreted forms (**figure 8**).

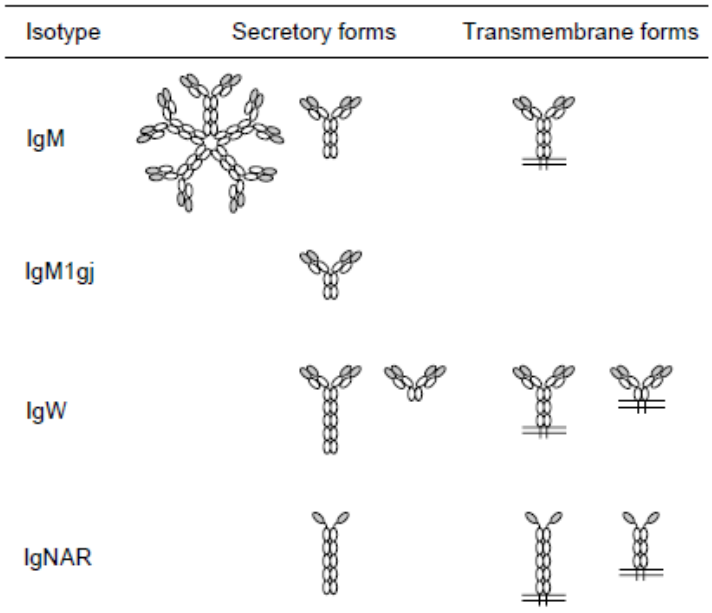

Figure 8. Schematic of the transmembrane and secretory immunoglobulin isoforms found in the cartilaginous fish to date. Constant domains are shown in white and variable domains in grey (Dooley and Flajnik, 2006).

The immunoglobulin genes of elasmobranchs are arranged in a “cluster” conformation, rather than the translocon organization typical in mouse and human (Dooley & Flajnik, 2006; Hinds and Litman, 1986). Each cluster contains one V segment, one to three D segments, one J segment, and the constant regions (**figure 9**). All evidence available to date indicates that VDJ/VJ rearrangements occur exclusively within a cluster and not between clusters (Dooley and Flajnik, 2006; Greenberg, et al. 1995; Lee et al, 2000).

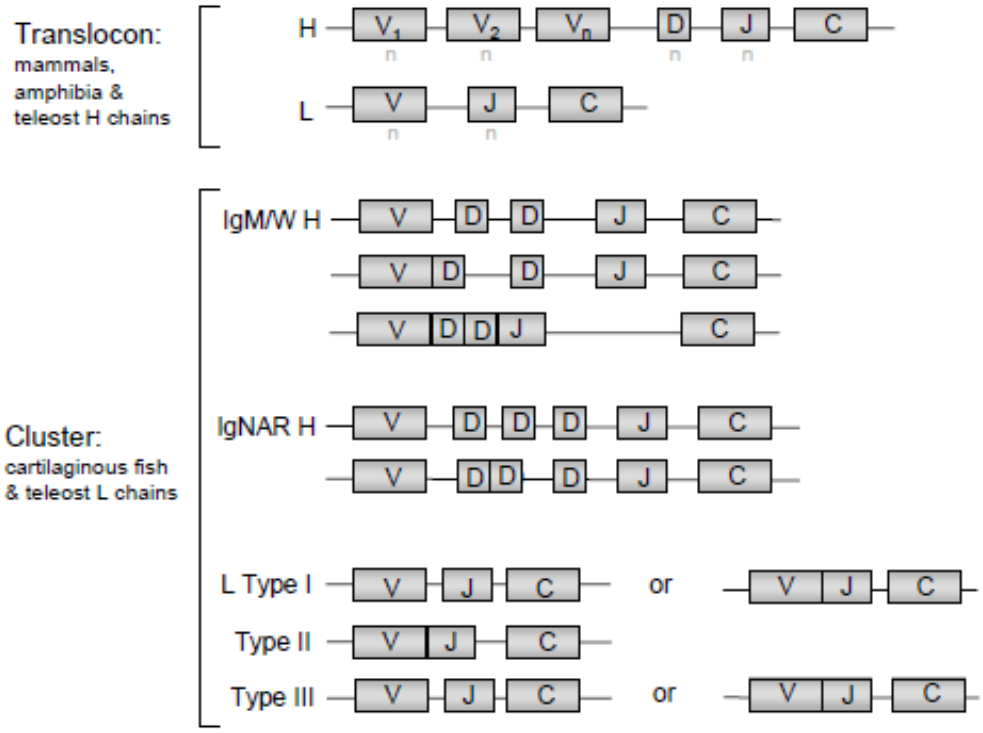

Figure 9. Schematic representation of immunoglobulin gene translocon and cluster configurations. It is also shown the occurrence throughout phylogeny of each immunoglobulins gene arrangements (Dooley and Flajnik, 2006).

I.10.1 IgM

IgM was discovered in the serum of cartilaginous fish 44 years ago (Marchalonis et al, 1966), it is present in all the jawed vertebrates, and originally was thought to be the only isotype present in this group (Dooley and Flajnik, 2006).

Serum IgM is found in two forms: a monomeric (7S) and a pentameric (19S) form, present in roughly equal amounts, and constituting half (>20 mg/ml) of the total serum protein in adult animals (Clem & Small, 1967; Dooley and Flajnik, 2005). It has been proposed that pentameric IgM provides the primary humoral response, whilst monomeric IgM together with IgNAR mediate the secondary immune responses in cartilaginous fish (Flajnik and Rumfelt, 2000; Dooley and Flajnik, 2006).

I.10.2 IgW

All elasmobranchs studied to date have this isotype. It was first reported in skates as a non-IgM-secreted isotype called IgR (Hsu et al, 2006; Kobayashi, et al, 1984). IgW exists in two forms, a long seven-domain and a short three-domain form (**figure 8**). The short form was originally discovered in two different species of skate and named IgR in the ocellate spot skate (*Raja kenojei*) (Dooley and Flajnik, 2006). Later, cDNA transcripts for the long form were detected in the sandbar (IgW) and nurse sharks (IgNARC) (Bernstein, et al. 1996; Dooley and Flajnik, 2006).

There are few IgW genes in the nurse shark, but many more in the skate. IgW is alternatively spliced to encode either a five or a three-domain molecule (Dooley and Flajnik, 2006; Harding et al. 1990; Rumfelt et al. 2004). Very little is known about the function of this isotype, since IgW specific monoclonal antibodies have not been generated (Hsu et al. 2006). IgW is presumably the orthologue of mammalian IgD in reptiles and fish (Ohta and Flajnik, 2006).

I.10.3 IgNAR

It was thought that cartilaginous fish posses only two immunoglobulin isotypes, IgM and IgW, until the novel immunoglobulin isotype, IgNAR (immunoglobulin new antigen receptor) was discovered in the serum of the nurse shark (*Ginglymostoma cirratum*) as a homodimeric heavy-chain complex similar to the camelid VHH species, which also naturally lack light chains (Greenberg et al. 1995). A similar isotype has also been found in the serum of the wobbegong shark (*Orectobulus maculatus*) (Nuttall et al. 2000; 2001), smooth dogfish (*Mustelus canis)* and spiny dogfish (*Squalus acanthias*) (Liu et al. 2007).

IgNAR molecules are composed of two protein chains, each with one variable and five constant domains (**figure 10**), and occur in both cell-bound and secretory forms (**figure 8**). The lack of associated light chains has been confirmed by immune-electron microscopy, and the variable domains of IgNAR (VNAR) do not contain a typical V_H/V_L-style interface, implying that, binding to the antigen is solely mediated by the VNAR domains (Roux et al. 1998).

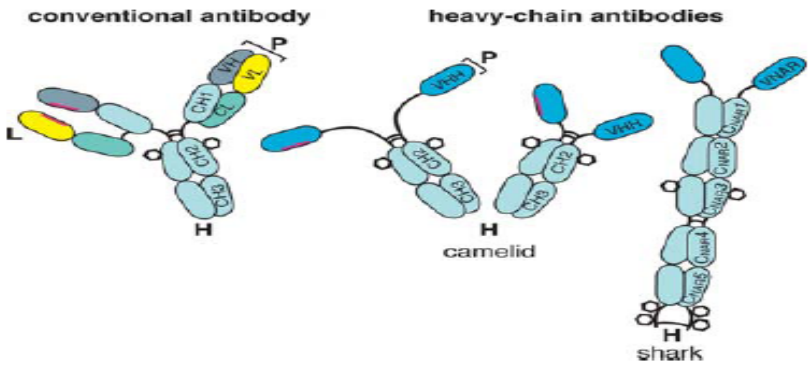

Figure 10. Distinguishing structural features of conventional antibodes, camelid and shark heavy chain antibodies (Wesolowski et al. 2009).

Similarities at the level of molecular structure, such as the presence of charged instead of hydrophobic residues at the conventional V_L interface of V_H framework regions, larger CDR3 loop regions than those found in murine or human antibodies, and the presence of disulphide bonds within the CDR3 loop or between

CDR1 and CDR3 loops, have provided evidence for evolutionary convergence between the camelid VHH and the VNARs. The immunoglobulin fold is well preserved in the VNAR structure, and shows a finger-like loop extension formed by the CDR3 (Dooley et al. 2003).

Despite these similarities, camel and shark variable domains are clearly different in terms of the contribution of the different CDR regions to antigen binding, and in the distribution of non-canonical cysteine residues (Diaz et al. 2002).

As described above, IgNAR genes are found in the cluster conformation typical of cartilaginous fish immunoglobulins (**figure 9**). To date, two clusters of IgNAR genes (type I and type II) have been described (Greenberg et al. 1995). As rearrangements only occur within a cluster (Greenberg et al. 1995; Hinds & Litman, 1986; Kokubu et al. 1988; Lee et al, 2000), and only a single functional cluster is present for each IgNAR type (Greenberg et al. 1995), the diversity encoded by the V germline is limited. However, the repertoire is expanded greatly through the generation of enormous diversity in the CDR3 through four rearrangement events that include N region additions at each joining region, and by a high rate of antigen-driven somatic hypermutation (Diaz and Flajnik, 1998; Diaz et al. 1999; Dooley et al, 2006; Nutall et al. 2004).

It has yet to be formally proven that the shark VNAR binding domain plays a role in immunological surveillance *in vivo*. However, there is evidence supporting a role in antigen binding by analysis and comparison of mutational patterns between membrane-bound and secretory forms of nurse shark VNAR regions that suggests that affinity maturation occurs through somatic hypermutation (Diaz et al. 1998; 1999). Furthermore, a VNAR isoform (type III), recently identified in neonatal shark primary lymphoid tissues, is hypothesized to function as a protective low-specificity antibody in early development, prior to the maturation of the type I/II VNAR antigen-driven response (Diaz et al. 2002). Characteristic of the Type III VNAR topology is a large CDR3 loop of limited diversity that is stabilized by a conserved tryptophane residue within the CDR1 loop (Diaz et al. 2002; Liu et al. 2007).

More recently, the most compelling evidence showing a role of IgNAR in immunological surveillance has been provided by the observation that nurse sharks (*G. cirratum* produce not only IgM, but also highly antigen-specific IgNAR responses (Dooley and Flajnik, 2005). There is also a differential expression of pentameric and monomeric IgM, whereby the pentameric IgM provides the initial defense with a low-affinity driven reaction, followed by a highly specific antigen-driven response provided by monomeric IgM and IgNAR (Dooley and Flajnik, 2005).

Nuttall and coworkers have shown the presence in wobbegong sharks of naturally occurring IgNAR antibodies that bind the Kgp protease of the pathogenic bacterium *Porphyromonas gingivalis* with affinities within nanomolar range (Nutall et al. 2002). The same group has isolated VNAR domains specific for other targets from synthetic CDR3-based libraries through *in vitro* selection (Nutall et al. 2001; 2003; 2004). This demonstrates that synthetic (or partially synthetic) CDR3 libraries can generate VNAR domains with antigen-binding affinities comparable to those of natural systems (i.e. immunization of animals followed by isolation of the variable genes repertoire). This approach can be important in situations where conventional (murine) antibodies are difficult to generate.

The evolutionary origin of IgNAR as single domain antibodies is open to speculation; however there are at least two valid hypotheses (Richards and Nelson, 2000; Roux et al, 1998; Nuttall et al. 2003). The first is that VNARs represent ancestral immunoglobulins, possibly derived from primitive cell-surface molecules co-opted as soluble antibodies in the serum. In this hypothesis, the IgNARs represent a unique antibody lineage, or alternatively an ancient version of a single-domain antibody existant before the adoption of heterodimeric pairing in the V_H/V_L chain configuration. Alternatively, IgNARs may have evolved to a single-domain format from a primitive heterodimeric ancestor based on antibody (V_H/V_L) or T cell receptor (Vα/Vβ) forms. Such re-evolution to a single variable domain has been observed for camelid VHH antibodies over far shorter period of evolutionary time (Su et al. 2002). Recent evidence has suggested that the IgNAR antibody has

evolved from a primitive cell-surface adhesion molecule due to its similarity to the I-set family of the immunoglobulin super-family (IgSF) proteins, such as the cell adhesion molecules (CAMs) (Streltsov et al. 2004). However, whether IgNAR has evolved from a primordial antigen-binding receptor, or was derived later in phylogeny will remain unresolved until IgNAR-like molecules are found in more phylogenetically distant species, such as the jawless vertebrates (Stanfield et al. 2004).

I.11 CD38 protein

CD38 belongs to a family of structurally conserved enzymes found in a wide variety of organisms including worms (schistosomes), sea urchins, birds, and mammals (Schuber and Lund, 2004; Lund, 2006). A prominent feature of this enzyme family is the ability to produce cyclic adenosine diphosphoribose (cADPR) from nicotinamide adenine dinucleotide (NAD^+); cADPR is known to induce calcium mobilization from intracellular calcium reservoirs such as the endoplasmic reticulum (Lee, 2001; Lund, 2006). This metabolite regulates many cellular signal transduction pathways in plants, invertebrates and vertebrates; therefore, a great interest in CD38 as a cADPR-producing enzyme has been raised (Lund, 2006). Additionally, CD38 can also directly hydrolyze NAD+ to ADPR and nicotinamide, as well as catalyze a base-exchange reaction that leads to the production of NAADP from NADP and nicotinic acid.

I.11.1 CD38 structure

CD38 is a highly conserved type II transmembrane glycoprotein that is expressed on most bone marrow-derived cells (Lund et al, 1998; 2006). The treatment of CD38 with the V8 *Staphylococcus aureus* protease yields a dominant band of 38 kDa, which is recognized by an anti-CD38-monoclonal antibody (A10/IB4) (Malavasi et al. 1985; 2008). The structure of CD38 is an “L” shape, and it can be divided into two separate domains: the amino terminal (residues 45-118 and 144-

200) and the carboxyl terminus (residues 119-143 and 201-300) **figure 11** (Malavasi et al. 2008).

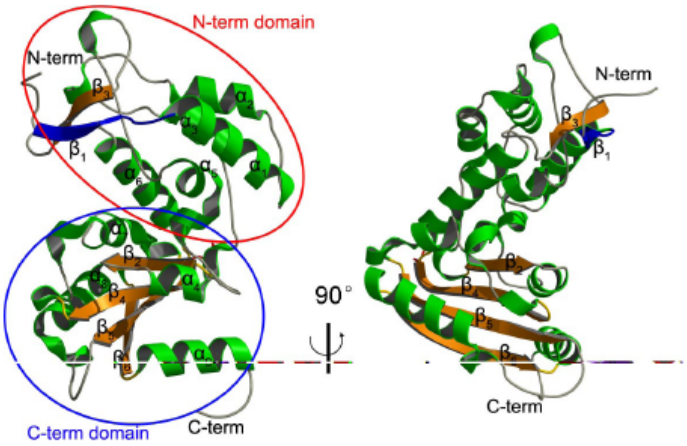

Figure 11. Ribbon representation of soluble human CD38 structure (Malavasi et al, 2008).

Using site directed mutagenesis it was reported that the cyclase activity of CD38 is completely lost when the residue Glu-226 is changed to Asp, Asn, Gln, Leu or Gly, implying that Glu-226 is crucial for the catalytic activity. The crystal structure of human CD38 reveals that this residue plays an important role in positioning cADPR within the catalytic site through strong hydrogen bonding interactions, and that the binding of cADPR to the active site induces a significant structural rearrangement in the peptides Glu146-Asp147 (Liu et al. 2007; Malavasi et al. 2008).

Structural analysis revealed a high homology between the active sites of CD38 and the ADPR-cyclase of *Aplysia californica*, with critical residues clustered in a pocket near the center of the CD38 molecule (Malavasi et al. 2008; Munshi et al. 2000;).

Besides the membrane-bound form, CD38 is also found as a soluble protein, and has been identified in cell culture supernatants of activated T lymphocytes and several tumor cell lines, as well as in serum and ascites samples from patients with

multiple myeloma or the acquired immune system deficiency syndrome (AIDS) (Funaro et al, 1996; Malavasi et al. 2008).

I.11.2 CD38 activity

CD38 protein has a dual activity as an ecto-ezyme involved in the catabolism and scavenging of extracellular nucleotides and as a receptor molecule (Malavasi et al. 2008).

As an enzyme, CD38 is involved in the degradation of extra cellular NAD^+., By producing second messengers,, such as cADPR, NAADP, and ADPR-, it regulates the effector functions of dendritic cells. These molecules are mobilize calcium from intracellular reservoirs, modify protein functions, and play critical roles in cell homeostasis and metabolism (Lund, 2006; Malavasi et al. 2008; Partida-Sanchez, 2004; Seman et al. 2004;).

CD38 is also a cell surface molecule with receptor functions. Since the intracellular domain of CD38 is small and does not contain any known signal-transducing motifs, signalling through CD38 is considered to work in synergy with signal-transducing modules of other molecules (Deaglio, et al. 2006; Lund, 2006; Malavasi et al. 2008). In the immunological context, CD38 acts as an adhesion molecule capable of binding to endothelial cells and playing important roles in the immunological synapse (Deaglio, et al. 2008; Muñoz et al. 2008). CD38 is recruited into lipid rafts on activated B lymphocytes, and interacts with neighboring proteins such as CD19 or CD81 to mediate intracellular signals (Deaglio et al, 2007). Silencing of CD19 directly impacts on the ability of CD38 to mediate intracellular Ca^{2+} fluxes, while leaving CD38-surface expression unmodified (Deaglio, 2007).

As shown in **figure 12**, CD38 has multifunctional roles in the modulation of immunological and inflammatory responses not only on lymphocytes but also on dendritic cells (DCs), granulocytes and multiple non-bone marrow derived cells, including air-way smooth muscle cells (ASM) and pancreatic β-cells (β-cells) (Lund, 2006). CD38 mediates calcium signaling on DCs and ASM cells by producing calcium-mobilizing second messengers like cADPR, $NAADP^+$ and ADPR

(Lund, 2006). In other cell types, like β-cells and T cells, CD38 possibly additionally indirectly regulates signaling by catabolizing NAD^+ and thus inhibiting the activity of other NAD^+-dependent enzymes such as ART2 (Krebs et al. 2005; Lund, 2006; Seman et al. 2004). In B lymphocytes, CD38 regulates cell signaling by an enzyme-independent mechanism. It was shown that crosslinking of CD38 induces proliferation of mature follicular B cells and enhances the proliferation and differentiation of immature translational 2 (T2) B cells. Furthermore, it was observed that receptor functions of CD38 were independent of the formation of calcium-mobilizing metabolites, but depended on tyrosine kinase activation and the localization of CD38 to lipid raft microdomains on the plasma membrane (Deaglio et al. 2007; Lund, 2006; Lund et al. 2006).

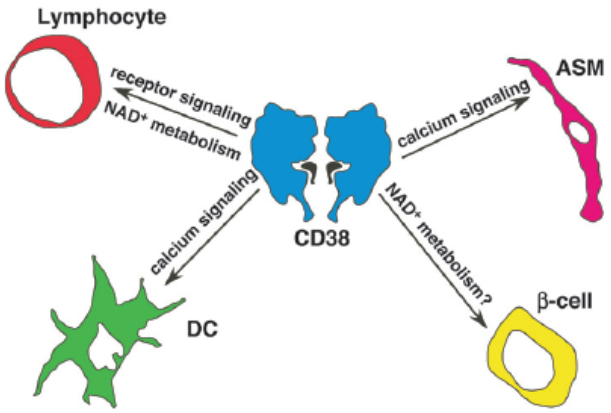

Figure 12. Multi faceted roles of CD38 in immune responses and inflammation (Lund, 2006).

I.11.3 CD38 in human diseases

Analysis of CD38 is currently used in the diagnosis of leukemia and myeloma. In this setting it is used as a prognostic marker for chronic lymphocytic leukemia (CLL), a lymphoproliferative disease with a highly variable outcome. Some patients experience a slowly progressive clinical course, but most will eventually enter an

advanced phase and require recurrent treatment. Although disease progression of patients with CLL may be followed using clinical parameters that reflect disease burden, these do not predict the outcome for those with early stage disease. Together with other biomarkers, high expression of CD38 by lymphoma cells is considered to be a marker for poor prognosis (Patten et al. 2008). Unlike most other B-lymphoproliferative disorders, little is known about the pathogenesis of CLL (Deaglio et al. 2006). It is therefore of enormous interest to elucidate the cellular origin of this disease, but until today no common key cytogenetic abnormalities have been identified that might offer pathogenetic clues (Deaglio et al. 2006).

In addition, CD38 expressed by CD4+ and CD8+ cells constitutes a reliable, economic, and easy to use prognostic tool for the progression to AIDS in HIV-infected patients, and abnormalities of CD38 expression have been linked to the development of type II non-insulin-dependent diabetes mellitus (NIDDM) (Malavasi et al. 2008).

Since CD38 is expressed in high amounts on a variety of lymphoid tumors, including most cases of myeloma and some cases of AIDS-associated lymphoma (Malavasi et al, 2008), these differences in cell surface expression between normal cells and their leukemic counterparts make CD38 an attractive target for immune-therapeutic treatment.

Until today none of the monoclonal antibodies available in mouse and rat that have been raised against the protein shown inhibitory effect, and there is a lack of specific and effective CD38 inhibitors. Therefore, the development of new specific reagents against the enzyme that have a functional blocking activity or that can be used for *in vivo* bio-imaging and CLL detection is of interest.

CHAPTER II

OBJECTIVES

II.1. Main goal of the project

The main goal of this project is to obtain and characterize single domain antibodies from sharks able to bind and inhibit the activity of the murine CD38 ecto-enzyme (mCD38).

We use shark single domain antibodies (vNAR) as a model, because up today they are together with the llama antibodies (VHH) the smallest molecules able to recognize an antigen. The vNAR and VHH in contrast from the conventional antibodies, only have the heavy chain polypeptide, this feature makes them small molecules (~12kDa), thermal and chemically stable (7), they also have a long CDR3 region which allows them to act as an excellent enzymatic inhibitors (8). Using shark antibodies has also a good advantage; due to the evolutionary distance, we are able to immunize sharks with antigens that probably would be toxic or lethal for mammals, so we can use a wide range of antigens to generate shark single domain antibodies.

II.2. Specific goals

The specific goals for this work are:

1. Construction of vNAR antibody library from previously immunized *Heterodontus francisci* shark against mCD38
2. Bio-panning selection by phage display of specific vNARs against mCD38
3. Isolation and purification of the specific vNARs from biopanning
4. Characterization of binding and inhibitory activity of vNARs against mCD38
5. Generation of secondary antibodies directed against vNARs from *Heterodontus francisci* horn shark
6. Characterization of monoclonal antibodies against vNAR.

CHAPTER III

MATERIAL AND METHODS

III.1. Animals

Sharks from the species *Heterodonthus francisci* (“horn shark”) were maintained in captivity in indoors sea water tanks at 22°C with constant oxygenation.

III.2 Shark immunization

Two sharks were immunized with different antigens, one with murine protein CD38 and the other with the protein from *Schistosoma mansoni* SmNACE.

Protocol for shark immunization was approached initially via sub-cutaneously with an emulsion cocktail of 1µg/ml of recombinant protein in 1.5 ml of 1x PBS with complete Freund´s adyuvant (Sigma Cat. F-5881). After 15 days a second immunization was performed using the same amount of antigen and administration via, but in a different emulsion including incomplete Freund’s adyuvant. Boost at 2 weeks intervals intravenously into the caudal vein as soluble antigen (250 µg) in PBS were performed during 3 months.

III.3 Isolation of total RNA

After finishing the immunization protocol, both sharks were euthanized, the spleen was taken and immediately washed with DEPC-water and transferred into a new falcon tube containing 15 ml of TRI-reagent for further homogenization.

The sample was incubated at RT for 5 min., afterwards 3 ml of chloroform was added and mixed vigorously for 15 sec and incubated at RT for 15 min. Thenafter the sample was centrifuged at 12,000 rpm for 30 min at 4°C. Three layers of different density were formed due to the centrifugation: the aqueous (which contains the RNA), interphase and organic phase.

The aqueous phase was transferred into a new falcon tube with 7.5 ml of pure isopropanol. The components were mixed for 15 sec and led them stand at RT for

10 min. The tube was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was removed and the RNA pellet was washed with 15 ml of 75% ethanol (H_2O-DEPC), the sample was mixed by vortex and then centrifuged at 12,000 rpm for 20 min at 4°C, the supernatant was discard and the pellet was air dried at RT for no more than 5 min. Finally the RNA pellet was resuspended by pipetting with 500 µl of H_2O-DEPC, aliquots of 50 µl were made and stored at -80°C.

The amount and purity of isolated RNA was determined by its absorbance at the wave lengths of 260/280 nm, if the final preparation of RNA is free of DNA, the ratio of 260/280 nm should be ≥1.7

III.4 cDNA Synthesis

cDNA was obtained by reverse transcription, using 1.5 µg of isolated RNA and mixed together with 3 µl of random primers (Invitrogen Cat. 48190-011) in a final reaction volume of 12 µl. The reaction was incubated at 70°C during 10 min afterwards 5 µl were taken for further agarose gel analysis. The 7 µl reaming from the reaction were mixed up with the following components: 1µl of dNTP's (1mM), 4µl of first strand 5x buffer, 2 µl DTT (0.1M) and 1µl of M-MLV Reverse transcriptase (200U/µl) in a final volume of 20µl.

Reverse transcription was performed in a T3 Thermal cycler (Biometra), the standard reaction comprised the following incubation steps at 24°C for 10 min, 42°C for 20 min and finally 2 min at 95°C. At the end, 5 µl of the cDNA was used for further analysis on agarose gel and the rest of the reaction was used for future PCR reactions.

III.5 Construction of immune vNAR library

III.5.1 Construction of a variable single domain fragment library

Construction of the bacteriophage M13-VNAR library (vNAR) has been done by isolation of total RNA from spleen of immunized *H. francisci* shark (described previously in section III.3). PCR products obtained from cDNA using species-specific oligonucleotides were then cut with restriction endonucleases Not*I* and Nco*I* and cloned into similarity cut ends of phagemid vector pHEN-2. Library ligations were then purified, pooled and transformed into electrocompetent *E. coli* XL1-Blue cells.

III.5.2 Polymerase Chain Reaction (PCR)

PCR was used to amplify specific DNA sequences using FastStart Taq DNA Polymerase (Roche Cat.12 032 929 001). Reactions were carried out in 0.2 µl siliconised PCR tubes containing: cDNA template (0.1 – 0.5 µg), 0.4 mM each dNTP, 2.5 µl of 10x PCR reaction containing $MgCl_2$, 1 µM each of the oligonucleotide primers to the 5' and 3' ends of the DNA to be amplified, 1.5 U of Fast start Taq enzyme and sterile distilled H_2O to a total volume of 25 µl.

PCR reactions were performed using the following steps:

Table I. PCR conditions for VNAR fragments amplification

Step	Temperature (°C)	Time	Cycles
Denaturation	95	5 min	1x
Denaturarion	95	45 sec	30x
Primer annealing	57	45 sec	
Elongation	72	45sec	
Final extension	72	7 min	1x
Incubation	4	hold	

Heterodontus francisci species-specific oligonucleotides used for PCR reactions are listed on the table below:

Table II. Specific oligonucleotide sequences to amplifly VNAR fragments from horn shark *Heterodontus francisci*

Name	Restriciton Site	Sequence	Amino acid sequence
557(→)	Nco*I*	GCA CAT GCC ATG GCC GCA CGG CTT GAA CAA ACA CC	MAARLEQT
558(→)	Nco*I*	GCA CAT GCC ATG GCC CAA CGG GTT GAA CAA ACA CC	MAQRVEQT
559(→)	Nco*I*	GCA CAT GCC ATG GCC ACA AGG GTA GAC CAA ACA CC	MATRVDQT
560(→)	Nco*I*	GCA CAT GCC ATG GCC GCA AGG GTG GAC CAA ACA CC	MAARVDQT
561(→)	Nco*I*	GCA CAT GCC ATG GCC GCA TGG GTA GAC CAA ACA CC	MAAWVDQT
562(→)	Nco*I*	GCA CAT GCC ATG GCC GCA AGC CTG GAC CAA ACA CC	MAASLDQT
563(→)	Nco*I*	GCA CAT GCC ATG GCC GCA TTG ACG GAC CAA ACA CC	MAALTDQT
564(←)	Not*I*	ATA GGG GCC GCG TTC ACA GTC AGC ACG GTG C	

III.5.3 Plasmid DNA isolation

All plasmids isolation were prepared using cells obtained from single colonies that were inoculated in 5 ml LB broth with the corresponding antibiotic (100 µg/ml of ampicilin or 50 µg/ml of kanamycin). Bacterial cultures were incubated with shaking at 230-250 rpm overnight at 37°C.

The overnight cultures were centrifuged at 4,500 rpm during 15 min at 4°C and pellet cells were treated for “mini-preps” using the QIA-prep plasmid isolation kit (QIAGEN, Cat. 27106 Hilden, Germany), according to the manufacturer’s instructions for purification up to 15 µg of plasmid DNA. For larger plasmid purification “maxi-prep” was used (QIAGEN Cat. 12362). The columns were eluted for the mini-preps with 50 µl or for the “maxi-prep” with 500 µl of ddH_2O and stored at -20°C.

III.5.4 Restriction digestion of DNA fragments

To generate cohesive DNA ends for ligation purposes, DNA fragments (PCR products or linearized vector) were digested with restriction enzymes purchased from New England BioLabs and were used according with provider’s specifications.

Either 1 µg of vNAR-PCR fragments or pHEN-2 plasmid was double digested with 15U of Not *I* (NEB Cat. R0189L), 15 U of Nco *I* (NEB Cat. R0193L), 2 µl NEB 10x Buffer 3, 0.2µl 100x BSA (NEB Cat. B9001S) in a final reaction volume of 20 µl. Reactions were incubated for 4 hrs at 37°C and further purified by preparative agarose gel electrophoresis (section **III.5.6**).

III.5.5 Phosphatase treatment of DNA

To reduce the frequency of self-ligation, dephosphorilation of digested pHEN-2 vector was made with Antartic Phosphatase (NEB Cat. MD2895 5U/µl) the reaction was performed with 1µg of purified-digested pHEN-2 vector with 20 U of Antartic Phosphatase and 2 µl of 10x Antartic Phosphatase Buffer in a final volume reaction of 20 µl. The reaction was incubated at 37°C for 4 hrs and inactivated at 65°C for 20 min. The treated plasmid was then extracted by preparative agarose purification from agarose gel (section **III.5.7**).

III.5.6 Agarose gel electrophoresis of RNA and DNA

Agarose gels at 1% or 2% were typically used to separate DNA or RNA. Agarose gels were prepared in Tris-Acetate-EDTA buffer 1x and pre-stained using 0.01 µl of 1mg/ml ethidium bromide solution in the molten agar. Unless stated otherwise, all gels to separate plasmid DNA were performed contained 1% agarose, gels used for RNA analysis and for PCR purification or digestions were done using 2% agarose. Once polymerized the gel was immersed in 1x TAE buffer in a horizontal electrophoresis apparatus.

RNA or DNA samples were mixed in the appropriate volume of 6x DNA gel-loading dye (Fermentas Cat. R0611) for loading. Electrophoresis was routinely performed at 100V for 30 – 40 min depending on the level of desired resolution. The size of the samples was determined by comparison with DNA nucleotide base pair size markers such as SmartLadder (Eurogentec Cat. MW-1700-10).

Samples were visualized using a UV trans-illuminator and photographed in a gel-doc system (GVision Capt. Version 14.3).

III.5.7 Elution of DNA fragments from agarose gel

DNA fragments of interest were separated using preparative agarose gels ranging from 1% to 2% (for PCR products or plasmid respectively) in TAE buffer. The DNA fragments were excised by a sterile scalpel blade and further purified by NucloeSpin Extract II kit (Macherey-Nagel Cat. 740609-250) following the manufacturer's protocol. The DNA fragments were eluted with at least 35 µl of buffer NE or ddH_2O and used for quantification.

III.5.8 Quantification of DNA

The concentration of DNA was performed either by (i) comparison of fluorescence intensity of ethidium bromide stained DNA with that of DNA standards of known concentration such as SmartLadder MW (Eurogentec Cat. MW-1700-10) or (ii) by UV spectroscopy by absorbance at 260 nm using the following conversion factor:

$$1U\ Abs_{260nm} = 50\ \mu g/ml\ dsDNA$$

As well the ratio between A260/A280 was used for determined the purity of the DNA, sample with a ratio ranging from 1.8 to 2.0 were considered pure.

III.6 Ligation of DNA fragments

Ligation reactions of linearized vector and vNAR-PCR products were performed according to standard procedures; the quality of linearized vector and PCR products was estimated in a 2% agarose gel and the amount of DNA by its absorbance at 280nm.

For every ligation reaction a ratio of vector-insert of 1:3 was used and were accomplished using 1.3 µg of linearized and dephosphorilated pHEN-2 vector, 400 ng of digested-vNAR-PCR, 4 µl of 5x T4-DNA ligase buffer, 20U of T4-DNA ligase (Invitrogen Cat. 15224-041) in a final volume reaction of 10 µl, was incubated for 16 hrs at 16°C and store afterwards at -20°C until use for bacterial transformation.

III.7 Transformation of bacteria by electroporation

Electrocompetent XL1-Blue *E.* coli cells were used to transform the ligation reaction of the vNAR library, to approach this procedure, 100 µl of electrocompetent cells were thawed on ice and 2 µl of ligation was added by mixing slightly with the pippet tip, the mixture were incubated for 1 min and then placed on a cold 0.2 cm gap Gene Pulser electroporation cuvette (Biorad Cat. 1652086) then the cells were chilled out for 1 min and then a electric pulse at 2.5kV, 25 µF, 200 Ω for 2 sec was given. 900 µl of pre-warmed SOC media was added to the cells and allowed them to recover at 37°C for 1 hr at 240 rpm. In between 50 – 100 µl of transformed cells were plated out in selective-LB-agar plates and incubated overnight at 37°C. To estimate the size of the library, after incubation the total number of colonies was counted.

III.8 Screening of positive clones by colony PCR

Transformed bacterial cells resulting from the vNAR library were screened by PCR to estimate the diversity of the library using the pHEN-2 vector specific primers LMB3 and fdseq1. Briefly, individual colonies were picked up from the plate with a pippet tip and resuspended in 10 µl of $_{dd}$H2O. For setting the colony PCR reaction 5 µl of this bacterial solution was used as a template for a PCR reaction following the same procedure as in section **III.5.2**. PCR products were analyzed by agarose gel electrophoresis, in the case of positive VNAR carrying clones a PCR fragment of around 600 pb was obtained.

After PCR analysis, positive clones were selected for further plasmid isolation and sequentiation as well as for preserving in glycerol stock.

III.8.1 DNA sequencing

DNA sequencing analysis was performed using the Sanger chain termination method with the ABI Prism BigDye terminator cycle sequencing kit (Applied

Biosystems Cat. 4337455). BigDye is mixture of polymerase, buffer, dNTPs and rhodamine labeled fluorescent dideoxymucleotides (dye terminators).

For the sequencing of fragments cloned into the pHEN-2 vector, the primers LMB3:5'-CAG GAA ACA GCT ATG AC-3´ as forward and fedseq1: 5'-TGA ATT TTC TGT ATG AGG-3' as reverse oligonucleotides were used.

In between 300 to 500 ng of DNA template was mixing with 2 µl of BigDye, 2 µl of sequencing primer (50 ng/ µl) and ddH_2O for a final volume reaction of 20 µl. The PCR reaction was performed at 96°C for 40 sec, 50°C for 15 sec and 60°C for 4 min during 28 cycles.

The PCR products were precipitated before sequencing by adding 16 µl of sodium acetate 3M pH5.2, 160 µl $_{dd}$H2O and 500 µl ethanol 100%. Samples were centrifuged at 13, 000 rpm for 15 min at 4°C. Supernatant was aspirated by vacuum and sample was air-dried at RT and sent to the sequencing service.

III.8.2 Cryopreservation of bacteria cells

For long-term storage of bacterial cultures, glycerol stocks were made using molecular biology grade glycerol at 50% (v/v). Bacterial cells were grown in selective liquid media overnight and centrifuged for 5 min at 4,500 rpm at 4°C, supernatant was discarded and pellet cells were resuspended in 500 µl of LB media and 500 µl of pure glycerol. Final cell suspension was placed on cryotubes and shock frozen in liquid nitrogen and stored at -80°C.

III. 9 Bacteriophage Display of vNAR library

There are several ways to obtain a library as plamidic DNA, as bacterial glycerol stock or as bacteriophages particles that display in the surface of its capsid the protein of interest. For approaching the bacteriophage display technique it is important to have phage library. In the following section the methods depicted this strategy are listed.

III.9.1 vNAR bacteriophage library rescue and amplification

Electrocompetent cells transformed with vNAR library were plated out in LB-Carbenicillin (100 µg/ml) agar plates and incubated overnight at 37°C. Grew colonies were scrapped from the plate using a sterile spreader and transferred into a new falcon tube contained 20 ml of 2xYT-Carb media, cells were agitated at 4°C during 30 min. Afterwards an aliquot of 1 ml of cells was taken for cryopreservation in glycerol.

The remaining cell culture was replaced in a 250 ml-corning flask with 100 ml 2xYT-Carb media and incubated in agitation at 240 rpm at 37°C for 1hr and 30 min.

Cells were then centrifuged at 4500 rpm at 4°C and supernatant was discarded, pellet cells were resuspended in 100 ml 2xYT-Carb media and super-infected with 100 µl (1* 10^{10} pfu) of M13KO7 Helper Phage (NEB Cat. N0315S) and incubated at 240 rpm at 30°C for 1 hr. After adding 50 µl of Kanamycin (50 µg/ml) to the super-infected culture it was let stand on the same conditions overnight to amplify the phages in culture.

III.9.2 Polyethylene glycol precipitation of bacteriophage particles

To amplifly bacteriophage populations from glycerol stocks, transformed *E.coli* cells or phages rending for every panning round it is necessary to superinfect them with helper bacteriophage and harvest them after growth overnight at 30°C.

To obtain the amplified phages from vNAR library, the overnight bacterial culture from the previous section was pelleted by centrifugation at 4,500 rpm for 20 min at 4°C, the supernatant (containing bacteriophage particles) transferred to fresh centrifugation tube.

Precipitation of bacteriophage particles was accomplished by adding 8% (w/v) PEG 8000 and 6% (w/v) NaCl to the isolated supernatant fraction and incubated on ice for 2 hrs, The precipitated mixture was centrifuged at 15,000 rpm for 30 min at 4°C and the supernatant was discarded, precipitated bacteriophages were

resuspended in 2 ml PBS 1x, centrifuged at 10,000 rpm for 10 min at 4°C and filtered through a nitrocellulose low-protein adherence syringe filter (0.22 µm). The resuspended PEG-bacteriophage solution was suitable for panning and kept at 4°C.

III.9.3 Rescue of bacteriophage and amplification of eluted bacteriophage

Eluted bacteriophages from each panning were amplified for successive rounds by infecting exponentially growing *E. coli* TG1 cells.

A 100 ml of *E.* coli TG1 cells at log phase ($OD_{600\ nm}$0.6) was infected with 100 µl of eluted bacteriophages at RT for 15 min following by an incubation step at 30°C for 1 hr at 240 rpm. Afterwards the culture was centrifuged, pelleted cells were resuspended in at least 2 ml of 2xYT media and plated on big LB agar plates and incubated overnight at 37°C.

For the next day bacterial colonies were taken for bacteriophage rescue following the procedure previously described in section **III.9.1**.

III.10 Biopanning of bacteriophage display libraries

Selection of specific-binding phages to murine CD38 protein was approach by biopanning selection rounds by two different slection ways: solid phase panning and antigen-biotinylation selection. In the following parts the procedure to achieve this methods are described.

III.10.1 Biopanning on solid phase using microtitre plates

Standard Nunc-Immuno plate MaxiSorp (Nunc Cat. 456529) were coated overnight with 100 µl/ well of coating buffer (2 µg/ml of antigen in 1x PBS) and incubated at 4°C. Antigen coating buffer was then removed and plate rinsed twice with 1x PBS and blocked with 200 µl per well of 4% (w/v) skim-milk powder blotting grade (Roth Cat. T145.2) in 1x PBS (MPBS) added to each well. The plate was incubated for 2 hrs at RT, and MPBS was washed away with washing buffer [1x PBS/0.05% Tween 20(v/v)] three times prior to the addition of bacteriophage for biopanning.

Following bacteriophage precipitation by PEG (section **III.9.2**) 100 µl of bacteriophage solution was added to each well (50 µl of obtained bacteriophage from section **III.9.2** plus 50 µl of 2%MPBS). Plated was sealed and incubated for 2 hr in shaking at RT.

Bacteriophage solution was then removed and the plate was washed with washing buffer by pippeting into the wells and washes by pippeting up and down five times, the final wash was carried out using 1xPBS following removal of buffer the plate was dried. Remaining bacteriophages bound to the plate were eluted by competition using 100 µl of 1xPBS with 1µg/ml of soluble antigen, the plate was then incubated for 30 min at RT and eluted phages were recovered from the supernatant and used for bacteriophage amplification step (section **III.9.3**).

Biopanning was carried out in three rounds with some modifications between each round in the number of washes and the amount of coating antigen as follows: 20 washes in the first round, 30 in the second and 40 in the third round, in the case of antigen 2, 1.5 and 1 µg/ml of coating antigen respectively was used.

III.10.2 Antigen biotinylation

Using the kit EZ-Link Sulfo-NHS-Biotin (Thermo Scientific Cat. 21326) 166 µg of murine CD38 protein were biotinylated using 10 mM of biotin reagent and followed the manufacturer's instructions. Efficient antigen biotinylation was checked out by western blot, running 1 µg of biotinylated mCD38 in a 12% SDS-PAGE and detected it by streptavidine conjugated with peroxidase diluted 1:200 in 1xPBS (R&D Systems Cat. 890803). As positive control unrelated biotinlylated antigen and as negative control no-biotinylated mCD38 were used.

III.10.3 Biotinylated-antigen panning

20 µl of Streptavidine-Agarose (Thermo Scientific Cat. 20347) were washed twice with 1xPBS before incubation with 2 µg (30 µl) of the biotinylated mCD38 protein in 200 µl of 1x PBS. The mixture was incubated at RT in constant agitation during 30 min, after that agarose was washed three times with 1x PBS and resuspended in

200 µl of 1x PBS and added to it 10 µl of the bacteriophage recovered library and incubated at RT for 30 min in constant agitation.

After the incubation time, agarose was centrifuged and supernatant was discarded, following by 10 washes with 1 ml of 1x PBS/0.05% Tween 20, in the last rinse step the resin was transferred into a new eppendorf tube and further 10 washes with 1 ml of 1x PBS were performed. The agarose was then resuspended in 100 µl of 1x PBS and put into a new tube, bound specific-antigen-bacteriophages were competitive eluted by adding 2 µg of non-biotinylated mCD38 to the tube and incubated overnight at 4°C in constant agitation.

At the next day, agarose resin was centrifuged and the supernatant (that contains eluted bacteriophages) was kept in a new tube and used for TG1 cells infection and for further panning rounds.

III.10.4 Bacterial colony isolation

Individual bacterial colonies were picked up from the inoculated LB-agar plates derived from each biopanning round (section **III.9.3**) and were used for plasmid isolation and sequentiation to estimate the enrichment and main features of the CDRs of every sequence. Clones carrying a plasmid with recognized vNARs in a correct frame-shift were used for transformation in HB2151 *E. coli* cells for protein expression experiments and isolated plasmid was used to transform XL10 Gold competent cells (Stratagene Cat. 200314) for individual bacteriophage production.

III.10.5 Individual bacteriophage production

Individual bacteriophage particles were produced from identified plasmids carriying vNARs sequences. Briefly, XL10 Gold Competent cells were transformed by heat-schock protocol incubating the cells with the DNA mixture on ice for 30 min and afterwards a heat schock step at 42°C for 30 seconds following by an incubation of 2 min at 4°C. Transformed cells were then resuspended in 1ml of SOC medium and incubated at 37°C for 1 h at 240 rpm, cells were plated out in LB-Carb agar plates and incubated overnight at 37°C. Individual clone were picked up and used

to inoculate 100 ml of 2xYT-Carb media incubated at 37°C in agitation (240 rpm) until a OD $_{600nm}$ 0.5- 0.7 was reached. Cells were centrifuged at 4400 rpm for 20 min at 4°C and supernantant discarded, and resuspended in 2xYT-Carb media and infected with 100 µl (1* 10^{10} pfu) of M13KO7 Helper Phage (NEB Cat. N0315S) and incubated at 240 rpm at 30°C for 1 hr. After adding 50 µl of Kanamycin (50 µg/ml) to the super-infected culture it was let stand on the same conditions overnight to amplify the phages in culture. Afterwards phage recovering and precipitation were followed according with the protocol on sections **III.9.1** and **III.9.2**.

III.10.6 Bacteriophage tittering

Bacteriophage particles precipitated by PEG were then tittered to determine the amount of total obtained phages. Firstly, 1 µl of produced phages were diluted in 1ml of media and was used to make serial dilutions of 10^{-6}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} and 10^{-12} all in 2xYT media. In the meanwhile XL10 *E. coli* cells were grown in 10 ml of 2xYT media until an OD_{600nm} of 0.5-0.7 was reached. To titer the phages 50 µl of bacteria cells were infected separately using 1 µl of every single dilution of the phage preparation. Incubated those 15 min at RT and plated them out on LB-Carb-agar plates.

Agar plates were incubated overnight at 37°C and on the next day, the number of grew colonies in every plate were counted to calculate the output tittering by multiplying the number of colonies by the culture volume and dividing by the plating volume.

III.11 vNAR protein expression in bacteria

Plasmids of confirmed vNARs in the appropriate framework were purified and used to transform HB2151 *E. coli* strain; recombinant proteins were expressed under the control of lactose IPTG inducible promoter. vNARs were produced as fusion with the c-Myc tag and 6x histidine tail.

III. 11.1 vNAR protein expression cultures in HB2151 *E. coli* strain

Individual colonies were picked up from LB-agar plates supplemented with antibiotic and used to inoculate 5 ml of 2xYT-Carb media and incubated in agitation at 240 rpm at 37°C overnight. The inocule was centrifuged at 4500 rpm for 10 min at 4°C and pellet was resuspended in 1ml of 2xYT media and used for inoculating 100 ml of 2xYT-Carb in at 1:100 dilution. Cells were grown at 37°C at 240 rpm until an $OD_{600\ nm}$ of 0.5- 0.7 was reached, at this point the cultures were induced for protein expression with IPTG at a 100 µM final concentration and induced for 4 hrs at 240 rpm at 37°C, before analysis and purification of the expressed products.

III.11.2 Periplasmic lysate extraction (PPL)

Expressed cultures were then centrifuged at 4500 rpm for 25 min at 4°C and cell pellets were harvested for extracting periplasmic fractions using a modification of the Minsky method (Minsky *et al*., 1986), essentially using the sucrose osmotic shock. Briefly, harvested cell pellets from a 100 ml HB2151 *E. coli* expression culture were resuspended in 20 ml of cold TS buffer (30mM Tris-HCl, pH 8.0, 20% Sucrose, 0.5 mM AEBSF, 10 mg/ml Lysozyme) and let the stand for 30 min on ice. Insoluble material was removed by a centrifugation step at 15,000 rpm at 4°C during 30 min. Supernatant containing PPL extract was filtered through a 0.22 µm filter and kept at 4°C for further protein purification.

III.11.3 vNAR protein purification by metal affinity chromatography

Recombinant vNAR proteins produced in bacteria were purified from PPL fractions by metal affinity chromatography using Hi-Select Nickel Affinity Gel (Sigma Cat. P6611). Briefly the matrix was pre-cleaning with 1x PBS and 1 ml of it was used to fill a plastic column. The packed column was washed with 5 ml of Elution Buffer (50 mM $NaPO_4$, pH 8.0, 250 mM imidazole, 0.3 M NaCl) and equilibrated with 5 ml of Washing Buffer (50 mM $NaPO_4$, pH 8.0, 10 mM imidazole, 0.3 M NaCl). PPL sample was loaded and let it pass through the matrix by gravity flow, after that a washing step with 10 ml of Washing Buffer was made and elution was

accomplished using 5 ml of Elution Buffer and collected 1 ml individual fractions. Column was rinsed with 5 ml Elution Buffer and followed by 5 ml of $_{dd}H2O$ and stored with 20% ethanol at 4°C, purified protein fractions were kept at 4°C as well.

III.12 vNAR protein expression in *Pichia pastoris* yeast

Selected vNARs were expressed as recombinant proteins in *Pichia pastoris* yeast strain X-33 by using the EasySelect Pichia Expression Kit (Invitrogen Cat. K1740-01).

Induction of protein expression was achieved by methanol and recombinant proteins were produced as fusion with the c-Myc flag and 6x histidine tail.

III.12.1 Protein expression vector pPICZαA for *P.pastoris*

To obtain soluble secreted forms of vNARs in to the media, the plasmid pPICZαA (**figure 13**) was selected. Using the restriction sites of the enzymes Xba *I* and Xho *I* as the cloning sites, specie-specific oligonucleotides for amplifying vNAR regions of *H. francisci* were designed.

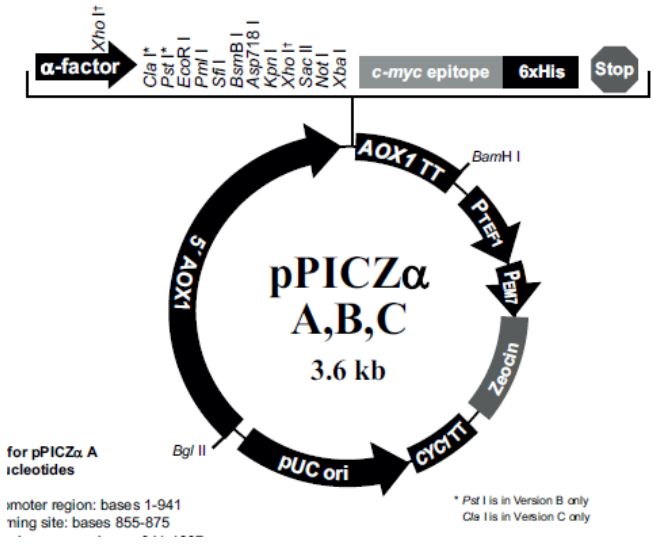

Figure 13. Schematic representation of *Pichia pastori* pPICZαA

III.12.2 Enzymatic restriction of vector pPICZαA

The vector was purified from XL-1 Blue-pPICZαA transformed cells using the MaxiPrep DNA Plasmid Purification kit from QIAgen (Section **III.5.3**), around 1.2µg/ml of purified plasmid was obtained.

Restriction digestion was performed with 2µg of pPICZαA vector, 20 U of Xho *I* (NEB Cat. R0146L), 20 U of Xba *I* (NEB Cat. R0145L) in the presence of 1x NEB Buffer 4, and 2 µl of 10x BSA in a final volume of 20 µl. The reaction was incubated for 4 h 30 min at 37°C and 20 min at 65°C to inactivate the enzymes, after that the reaction was cleaned using the QIAquick PCR purification kit (QIAgen Cat.28104). Plasmid was stored at -20°C until usage.

III.12.3 PCR amplification of vNARs

To amplify the vNAR fragments specific oligonucleotides bearing both restriction sites Xho *I* in the forward sense and Xba *I* for the reverse primer were designed (**Table III**).

Table III. Specific oligonucleotide sequences to amplifly vNAR fragments from horn shark *Heterodontus francisci* and to clone into the yeast vector pPICZαA

Name	Restriciton site	Sequence
557(→)	Xho *I*	CCG CTC GAG AAA AGA GCA CGG CTT GAA CAA ACA CC
558(→)	Xho *I*	CCG CTC GAG AAA AGA CAA CGG GTT GAA CAA ACA CC
559(→)	Xho *I*	CCG CTC GAG AAA AGA ACA AGG GTA GAC CAA ACA CC
560(→)	Xho *I*	CCG CTC GAG AAA AGA GCA AGG GTG GAC CAA ACA CC
561(→)	Xho *I*	CCG CTC GAG AAA AGA GCA TGG GTA GAC CAA ACA CC
562(→)	Xho *I*	CCG CTC GAG AAA AGA GCA AGC CTG GAC CAA ACA CC
563(→)	Xho *I*	CCG CTC GAG AAA AGA GCA TTG ACG GAC CAA ACA CC
564(←)	Xba *I*	GCT CTA GAG AGT TCA CAG TCA GCA CGG T

A PCR reaction was set using 250 ng of vNAR purified plasmid, 25 mM $MgCl_2$, 1x PCR Buffer without magnesium, 25 mM dNTP's, 1U FastStart Taq DNA

Polymerase (Roche Cat.12 032 929 001), 10 mM forward primer, 10 mM reverse primer in a final volume of 50 µl. The program used was: 95°C for 4 min, and 30 cycles of 95°C 30 seconds, 54°C 30 secs and 72°C 1 min and a final elongation step at 72°C for 7 min.

PCR products were cleaned up and quantified. Approximately 1 µg of vNAR-PCR product was digested with 20 U of Xho *I* (NEB Cat. R0146L), 20 U of Xba *I* (NEB Cat. R0145L) in the presence of 1x NEB Buffer 4, and 2 µl of 10x BSA in a final volume of 20 µl. The reaction was incubated for 4 h 30 min at 37°C and 20 min at 65°C to inactivate the enzymes, after that the reaction was cleaned using the QIA quick PCR purification kit according with the provider's intructions and stored at -20°C.

III.12.4 Ligation of vNAR fragments into the pPICZαA vector and transformation

Using the digested DNA fragments, a ligation reaction was performed using 150 ng of digested vector pPICZαA, 30 ng of vNAR PCR fragments, 4 µl of 5x T4-DNA ligase buffer, 20U of T4-DNA ligase (Invitrogen Cat. 15224-041) in a final volume reaction of 10 µl, was incubated for 16 hrs at 16°C.

2 µl of the ligation reaction was then transformed by electroporation to XL1-Blue electrocompetent cells (Stratagene Cat. 200228) and 200, 100 and 50 µl of recovered media was plated out in LB-low salt agar plates with Zeocyn at 250µg/ml according with manufacture´s instructions.

III.12.5 Screening of positive clones and plasmid purification of selected clones

Individual clones grown from the transformed cells were picked up and used to screen for positive clones bearing vNAR fragments, a colony PCR was performed according with the method in section **III.8** using the sequencing oligonucleotides for *P. pastoris* vector pPICZαA: 5'*AOX1*-GACTGGTTCCAATTGACAAGC-3' and 3'*AOX1*-GCAAATGGCATTCTGACATCC-3'. If the tested clones were positive of

carried a vNAR fragment then a PCR product of ~900 bp was amplified. Reactions were analyzed by agarose gel and sent to the sequence service to confirm the presence of a vNAR fragment.

III.12.6 vNAR-pPICZαA linearization

According with the provider's protocol from the EasySelect Pichia Expression Kit, to start the protein expression in *P. pastoris* it is important to transform the strain X-33 with at least 10 µg of linearized vector. Digested reaction was performed as follows:

10 µg of pPICZαA-vNAR vector was incubated with 15U of Not *I* (NEB Cat. R0189L), 40 U of Sac *I* (NEB Cat. R0156L), 5 µl NEB 10x Buffer 1, 5µl 10x BSA (NEB Cat. B9001S) in a final reaction volume of 50 µl. Reactions were incubated for 12 hrs at 37°C and at 65°C for 20 min to inactivate the enzyme. The linearized vector was then further purified by ethanol and sodium acetate precipitation

III.12.7 *Pichia pastoris* X-33 competent cells and transformation

To make competent cells of X-33 yeast strain 5 ml of YPD media (**Appendix I)** was inoculated with a single colony of the yeast and led it grew overnight at 30°C in agitation (230-240 rpm). At the next day 500 µl of overnight culture was used to inoculate 500 ml of YPD media, the culture was incubated until an OD 600 nm of 1.3 – 1.5 was reached.

Grew cells were pelleted 2 times by centrifugation at 3000 rpm for 5 min at RT and resuspended the first time in 500 ml and the second time in 250ml of ice-cold sterile water. Cells were then centrifuged twice and resuspended in 20 ml and 1ml of ice-cold 1M Sorbitol, cells were ready at this point to be electroporated.

To transform X-33 competent cells with the digested vNAR-pPICZαA constructs, 100 µl of cells were taken and incubated with 10 µg of linearized plasmid and transferred to a ice-cold electroporation cuvette and incubated them on ice for further 5 min, cell were pulsed according with the Yeast program stablished in the Micropulser Biorad electroporation machine (Biorad Cat. 411BR). Transformed

cells were recovered with 1ml 1M Sorbitol ice-cold and placed into a 15ml falcon tube, which was let stand at 30°C for 2 hrs without agitation. After incubation 50 and 100 µlof the cells were plated out in low-LB-agar plates containing 100 µg/ml of Zeocin (**Appendix I**). Plates were incubated at 30°C for 3 days until white spot-colonies appeared.

III.12.8 Analysis of *Pichia* transformants

Individual transformed clones were taken to analyze the presence of vNAR fragments by PCR and by plasmid sequencing.

To set PCR reaction it is necessary firstly to lysate the cells taking one loop of the colony and dissolved in 10 µl of destiled H_2O plus 5 µl of a 5U/ µl lyticase solution (Sigma Cat. L4025) and incubated for 10 min at 30°C and then 10 min at -80°C. The PCR reaction was set with 1x PCR Buffer, 1U of FastStart Taq Polymerase, 25mM MgCl2, 25mM dNTP's, 10mM 5'AOX1 primer, 10mM 3' AOX1 primer and 5 µl of lysate cells in a total 50 µl volume reaction.

The PCR program used comprised a denaturation step at 95°C for 5 min, 30 cycles of 95°C for 1 min, 54°C for 1 min and 72°C for 1 min, a final extension step at 72°C for 7 minutes was also given. 10 µl of PCR products were analyzed by gel electrophoresis.

Once the *Pichia* transformants were analyzed clones were used to induce the expression of the desired VNARs.

III.12.9 vNAR protein expression in *Pichia pastoris*

To express recombinant vNARs in yeast one isolated colony was used to inoculate 100 ml of MGYB media (**Appendix I**) which was incubated at 30°C in agitation of 240 rpm until an OD_{600nm} of 4 was reached. The cells were harvested by centrifugation at 3000 rpm for 5 min at 4°C and resuspended in 100 ml of BMMY (**Appendix I**) media and placed into a 2 l flask.

A pre-induction sample of 1 ml was taken from each culture, after that 100% methanol was added to each culture to a final concentration of 2%. Methanol was

added every 24 h to maintain the induction. Cell culture was incubated at 30°C at 240 rpm for 2.5 days.

At the last day, cells were pelleted at 3000 rpm for 5 min at RT, supernatant was taken and centrifuged at 15,000 rpm for 15 min at 4°C a small white pellet appeared and the supernatant was placed into a new flask and adjusted the pH to 7.8 with 2N NaOH. The supernatant containing the soluble vNARs was used for metal affinity chromatography.

III.13 Protein purification and detection

Periplasmic lysate extracts (PPL) derived from protein expression in bacteria or supernatant containing soluble vNAR obtained from yeast protein expression were used for metal affinity chromatography to purify them by its 6x histidine tail.

Detection of PPL and purified proteins was approached by SDS-PAGE used for staining with coomassie blue or for immunodection using the c-Myc tag.

III.13.1 Protein purification by metal affinity chromatography

Recombinant vNAR protein was purified from PPL fractions or from media-supernatant of yeast transformed by metal affinity chromatography using Hi-Select Nickel Affinity Gel (Sigma Cat. P6611).

Briefly the matrix was pre-cleaning with 1x PBS and 1 ml of it was used to fill a plastic column. The packed column was washed with 5 ml of Elution Buffer (50 mM $NaPO_4$, pH 8.0, 250 mM imidazole, 0.3 M NaCl) and equilibrated with 5 ml of Washing Buffer (50 mM $NaPO_4$, pH 8.0, 10 mM imidazole, 0.3 M NaCl). PPL or supernatant sample was loaded and let it pass through the matrix by gravity flow, after that a washing step with 10 ml of Washing Buffer was made and elution was accomplished using 5 ml of Elution Buffer and collected 1 ml individual fractions. Column was rinsed with 5 ml Elution Buffer and followed by 5 ml of $_{dd}$H2O and stored with 20% ethanol at 4°C, purified protein fractions were kept at 4°C as well.

III.13.2 Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE)

Analysis of protein samples was made under reduction conditions by SDS-PAGE using NuPAGE Bis-Tris Gels at 10 or 12% (Invitrogen Cat. NP0342BOX and NP0341BOX) which were run routinely with 1x MES buffer (Invitrogen Cat. NP0002) at 200 V, 110 mA for 33 min using a XCell II Blot Module (Invitrogen Cat. EI9051).

Samples to be analyzed by SDS-PAGE were prepared with 1x LDS Sample Buffer (Invitrogen Cat. NP0007) and 10% Sample reducing agent (Invitrogen Cat. NP0009) and incubated at 75°C during 15 min and briefly centrifuged to pull down the mixture before loaded into the precast gel. The size of the bands was estimated with protein standards such as Novex Sharp Pre-Stained Protein Standards Invitrogen Cat. P/N 57318) ran in parallel for molecular weight comparison.

III.13.3 Coomasie blue staining

Protein visualization was accomplished by staining gels in Novex Colloidal Blue Stain Kit (Invitrogen Cat. LC6025) overnight, and then rinsed with $_{dd}$H2O and distained with distaining bags with active coal (Ameresco Cat. E732-25). Gels were dried by using the Gel Drying Solution (Invitrogen Cat. LC4025-4) using the manufacturer's instructions.

III.13.4 Protein quantification

After analyzed by SDS-PAGE and selected the eluted fractions containing recombinant vNAR proteins, those were pool together into a PD-10 Desalting Columns (GE Healthcare Cat. 52-1308-00) for changing the buffer for 1x PBS following the provider´s instructions. Samples were then quantified using the Pierce BCA Protein Assay kit (Thermo Scientific Cat. 23225). According with the supplier´s protocol samples were compared together with a 2mg/ml BSA standard and measured at 562 nm wavelength.

III.13.5 Western Blot transfer and detection

Electrophoretic transfer of proteins from an acrylamide gel was accomplished by sandwiching it in between a Nitrocellulose Transfer Membrane (Whatman Protran Cat. 10401396) (0.22 µm) and a PVDF Transfer Membrane (0.45 µm) (Amersham Pharmacia Biotech Cat. RPN303F) and surrounded by two 3MM Whatman filter papers (the nitrocellulose membrane was closed to the catode and PVDF to the anode). Western blot transference was ran with 1x NuPAGE Transfer Buffer (Invitrogen Cat. NP0006-1 plus 10% (v/v) Methanol, 0.1% (v/v) Antioxidants) and routinely achieved in a Biorad Power PAC 200 apparatus (Biorad Cat. 165-5052) applying 300mA, 30V during 90 min.

PVDF membrane was blocked with 4% skim milk powder in 1x PBS during 1 hr at RT then washed with 1x PBS. For detecting recombinant vNAR proteins an anti-cMyc-tag antibody HRP-conjugate (Santa Cruz Biotechnology Cat. Sc-789 Clone A-14) was used at 1:10,000 dilution in 2% MPBS/ 0.025% Tween 20 and incubated for 1hr at RT, following by 4 washes of 5 min each one with 1x PBS/ 0.05% Tween 20.

For western blot developing the kit ECL Western Blotting Detection Reagents (Amersham GE Healthcare Cat. RPN2106) was used following the instructions of the manual, exposition was accomplished with the Amersham Hyperfilm ECL High Performance Chemiluminescence film (GE Healthcare Cat. 28906836) for 2 min and developed with the help of Developer processor apparatus (Fuji Cat. FPM-100A).

Nitrocellulose membrane was placed in a plastic reservoir containing 9 ml $_{dd}H_2O$, 0.5 ml of 40% Sodium Citrate, 0.4 ml 20 % Fe_2SO_4 and 0.1 ml 20% $AgNO_3$ and incubated in constant agitation until dark brown bands appeared in the gel. Unspecific silver stained was washed away with 2 washes with $_{dd}H_2O$.

III.14 Enzyme linked immune absorbent assay (ELISA)

ELISA assays were done in three different formats to identify either protein expression or recognition to its antigen.

III.14.1 vNAR protein expression detection

An ELISA plated was coated with 150 ng of purified recombinant vNARs in 100 µl 1x PBS per well and incubated overnight at 4°C. After washing the plate with 1x PBS it was blocked with 4% MPBS for 1 hr at RT. Blocking solution was pour away and ELISA plated was washed with washing buffer. The expression of vNARs was performed by detecting the cMyc-tag in the recombinant proteins, using 100 µl per well of an anti-cMyc-peroxidase antibody (SantaCruz Biotechnology Cat. A-14:sc-789) diluted at 1:10,000 in MPBS and incubated for 1h at 37°C, followed by 4 washes with Washing Buffer. Detection was achieved by using 100 µl per well of TMB substrate (Sigma T0440) and incubated it in darkness for 30 min after that time 50 µl of 0.5N sulfuric acid was added to each well to stop the reaction. ELISA plates were read at 450 nm in an ELISA reader (Perkin Elmer, Victor^3 1420 Multilabel counter)

III.14.2 Phage ELISA

A 96-star-wells nunc-immuno plate MaxiSorp with plate was coated with 100 µl of antigen (1 mg/ml diluted in 1x PBS) and incubated overnight at 4°C, after washing out the antigen solution and rinsed the plate with 1x PBS, 200 µl of MPBS as blocking solution were added for 1 h at 37°C. After blocking step the plate was washed with washing buffer and then 50 µl of either bacteriophage pool solution (~1 x 10^{12} cfu/ml) or individual-produced bacteriophages (~1 x 10^{12} cfu) diluted in 50 µl of MPBS were added to each well and incubated during 1 h at 37°C. Unbound phages were washed away 4 times with washing buffer and then 100 µl of anti-M13-HRP antibody at a 1:5000 dilution in MPBS was added to each well (Ge Healthcare Cat. 27-9421-01). As a control of unspecific binding unrelated antigens were used (3% milk, 3% BSA, 1mg/ml ART2.2 mouse protein). To detect

the binding of phages to the antigens 100 µl of TMB substrate was added to each well and let it stand for 30 min in darkness after that 50 µl of 0.5N sulfuric acid was added to each well to stop the reaction and the plate was read at 450 nm.

III.14.3 Recognition ELISA to murine CD38 protein by recombinant vNARs

This ELISA assays was quite similar to the Phage ELISA with barely changes, in fact the antigen coating and blocking procedures were the same. The only significant difference was that instead of phages in this case 100 µl containing 150 ng of specific vNARs as recombinant protein were added to each well to test its recognition to mCD38 protein. Detection was accomplished by using the c-Myc-tag of each recombinant vNAR with an anti-cMyc-peroxidase antibody diluted in MPBS at 1:10,000. Further steps were similar. ELISA plated reading was done in the same way as in section **III.14.1**.

III.15 Fluorescent Activated Cell Sorting (FACS)

FACS analysis were performed to characterize the binding capacity of the obtained purified vNARs or vNAR-displayed phages obtained from each panning round against mCD38 protein. FACS assays were performed using the FACSCalibur apparatus (Becton Dickinson).

III.15.1 Cell lines for FACS analysis

Two different types of cell line from mouse were used: CTLL-2 cells were kept in culture in complete RPMI media supplemented with IL-2 (**Appendix I)** and EL4-R cells used as well Complete RPMI media suplmented with Gentamicin (GIBCO, Cat. 15750). Cell cultures were maintained in 10cm Petri disches at 37°C, 5% CO_2. Freshly isolated spleenocytes from mouse CD38 wild type (wt) and knock-out (ko) were also used.

III.15.2 Isolation of spleenocytes from wild type and knock-out CD38 mice

Spleen from C57Bl6 mice in between 6-8 weeks with a genotype of CD38-wt and CD38-ko was taken to isolate spleenocytes for further FACS analysis.

Spleen was taken and put it in a Petri dish containing 5ml 1x PBS, spleen was mashed and 10 ml of cold 1x PBS was added to the dish and transferred to a new 50 ml falcon tube. Cells were centrifuged at 1100 rpm for 5 min at 4°C and supernatant was discarded, 5 ml of lysis buffer (**Appendix I**) was added to the cells and incubated them together for 5 min at RT. Lysis reaction was stopped by adding 12 ml of cold 1xPBS/0.2% BSA (w/v). Cells were then strained using a cell strainer with 70 µm Nylon mesh (BD Falcon Cat.352350) and pelleted at 1100 rpm for 5 min at 4°C. The supernatant was discarded and the pellet resupended in 5ml cold 1x PBS. Cells were counted with a Neubauer chamber and kept on ice until usage.

III.15.3 Blockage of the Fc-receptor

Isolated spleenocytes from CD38 wt and ko mice were treated for blocking the Fc-receptor with 0.5µl /sample of an anti-Fc receptor antibody and 1 µl/ sample of normal rat serum incubated the mixture for 5 min at RT and then kept the cells on ce until usage.

III.15.4 FACS analysis with purified vNAR protein

To identify if the purified VNARs from different families were able to recognize CD38 expressed on the cell surface, CTLL-2 as negative CD38-expression cells and EL4-R as positive cell line were used, as well as spleenocytes isolated from CD38-wt and ko mice.

Briefly, 100 ng of purified vNARs were pre-incubated with 700 ng of anti-cMyc-FITC conjugated antibody (Invitrogen Cat. 460307) in 100 µl of RPMI media at RT for 30 min. Afterwards $1x10^6$ cells per sample were used and either incubated in darkness during 45 min at 4°C with the 100 µl vNAR-anti-cMyc mixture or with an rat-anti-mouse CD38-FITC (BD Pharmingen Cat. 558813). Cells were washed twice with 1 ml of 1x PBS and centrifuged at 1200 rpm for 5 min at 4°C and finally

resuspended in 300 µl of 1x PBS and let stand on ice and darkness for further FACS analysis. As negative controls unrelated purified VHH was used.

III.15.5 FACS analysis with phage-displayed vNARs

In order to characterize the binding capacity of vNARs displayed on the surface of phages particles a FACS analysis using CTLL-2, EL4-R and spleenocytes cells were done. To achieve this $1x10^6$ cells tube were incubated during 45 min at 4°C with ~$1x\ 10^{12}$ phages particles per sample. Cells were then washed fourfold with 1 ml of 1x PBS by centrifugation at 1200 rpm for 5 min at 4°C. Cells were resuspended with 300 µl of 1x PBS and incubated in darkness during 45 min at 4°C with 1 µl of an anti-M13g8p-FITC (Acris Cat. BM5516F). Following four wash steps as described previously cells were resuspended in 300 µl of 1xPBS for FACS analysis. In regards of having a negative control, unrelated phage from VHH was produced as well as a commercial M13 Helper phage was used as irrelevant binding particle.

III.16 Inhibitory assays of murine CD38 activity

Having in consideration that murine CD38 protein has an intrinsic NADase activity, one suitable approach to test the activity of the isolated vNARs from the biopanning rounds is by inhibiting the hydrolysis of P^{32}labeled-NAD by CD38. In the following section a precise description of CD38 titration and inhibition assay are given.

III.16.1 CD38 titration

The enzymatic activity of murine CD38 was tritrated by using two fold serial dilutions of the protein in presence of radioactive labeled NAD and using Thin Layer Chromatography (TLC). Previously, 400 µl of Strataclean resin (Stratagene Cat. 400714) was rinsed with 1x PBS and resuspended in PBS in the same initial volume. For the other side, NAD at 100 µM stock concentration was diluted 1:100

in 1x PBS/0.1% BSA (w/v) and 72.6 µl were taken in a new falcon tube and put it on ice.

Serial twofold dilutions of mCD38 from 1.56 ng to 50 ng, were performed in 50 µl of 1x PBS/ 0.1% BSA and incubated on ice. A TLC plastic sheets PEI cellulose membrane (Merck, Cat. 0B559056) was prepared and labeled with pencil spots to assign the place in which the drops were put.

Once in the radioactivity laboratory, 2.5 µl of P_{32}-NAD was added to the NAD 1:100 dilution, from this mixture, 6 µl were added to every single tube containing dilutions of mCD38 protein and incubated at RT for 5 min. Hydrolysis reaction was stopped by adding 20 µl of Stratagene cleaning resin. Samples were centrifuged and the supernatants were put in a new falcon tube.

For making a TLC, a special pre-labeled membrane was taken and 1 µl of the P^{32}-NAD-CD38 supernatant mixture was put on it, and let it dried for some minutes. Afterwards TLC membrane was soak in Buffer A (1M acetic acid) for 3 min and then transferred to soak in Buffer B (0.9 M acetic acid/ 0.3 M lithium chloride) form 9 min, after soaking TLC membrane was dried at RT and covered by a plastic bag and put it into a developing cassette with a layer of film (GE Healthcare Cat. 28906836) and incubated at -80°C for 2 hrs and then developed.

III.16.2 CD38 enzymatic inhibition assay

To characterize if the purified vNARs inhibit the NADase activity of mCD38, 100 ng of purified vNAR were pre-incubated with 9 ng of mCD38 in a 50 µl of 1x PBS/ 0.1% BSA (v/w) volume reaction at RT during 30 min.

After that, 6 µl od P^{32}-NAD were added to each reaction and incubated them 5 min at RT followed by the addition of 20 µl of Strataclean resin to stop the reaction. Following steps for TLC assay were the same as described before in section III.16.1.

III.17 Generation of second reagents to detect VNAR molecules

III.17.1 Animal immunization

Threee BALB/c mice were immunized for 3 months every third week with 20 mg of endotoxin-free purified vNAR-SAALAK protein in an emulsion with the adjuvant Specol.

III.17.2 Preparation of mouse sera

Blood samples from each mouse were obtained to isolate the pre-immune sera (p.i.s) and immune sera (i.s), the blood sample was centrifuged fro 10 min at 2000 rpm, and the serum (on the upper part of the sample) was taken and placed in a new tube. Inactivation of sera was done by incubating the samples for 15 min at 65°C. Aliquots of the sera were taken and diluted 1:10 in 1x PBS and stored at 4°C and at -80°C for long-term mainteinance.

III.17.3 Cell culture

Myeloma cell line SP20 was kept in culture in RPMI 1640 media at 37°C with 5% CO_2. In preparation for cell fusion eight full Petri dishes were cultivated and cells were taken and washed twice with RPMI media by centrifugation at 1600 rpm for 5 min at RT, the cells were resuspended in 50 ml of RPMI media, before starting with the fusion procedure, obtained SP20 cells were counted to have at least $2x10^7$ cells in total, celles were kept on ice until usage.

III. 17.4 Lymph nodes preparation

To proceed with hybridoma cell fusion, axial and inguinal lymph nodes as well as spleen from immunized mouse 3537 were taken and placed in a Petri dish with RPMI 1640 media. After extraction, lymph nodes and spleen were cut in small pieces and mashed by passing through a cell strainer with 70 µm Nylon mesh (BD Falcon Cat.352350), cells were centrifuged at 1600 rpm for 6 min at RT. The supernatant was discarded and the pellet resupended in 20 ml RPMI media, an

aliquot of 10 µl was taken to calculate the amount of isolated cells using a Neubauer chamber.

It was added 30 ml of RPMI media to the cells and was filtered through the Nylon mesh. Cell preparation was washed twice and centrifuged at 1600 rpm for 5 min at RT, in the last wash, the supernatant was discarded and cells were resuspended gently in 5 ml of RPMI media.

III.17.5 Fusion of myeloma SP20 cell line with mouse lymph nodes and spleen cells

To achieve the fusion of myeloma SP20 cells with lymph nodes and spleen cells from immunized mouse, the SP20 cell line in section **III.17.3** were placed together with the spleen and lymph nodes obtained in section **III.17.4**. Both cell lines were placed in a new falcon tube containing 50 ml of RPMI media.

Cells were centrifuged at 1600 rpm for 5 min at RT and the supernatant was discarded, the cell pellet was resuspended gently by small agitation and placed into a water bath at 37°C. Afterwards 1.5 ml of 50% PEG/1xPBS solution was added in drops directly above the cell suspension during 1 min after that, the cell solution was mixed gently during 1 min and 14 ml of RPMI media were added in slowly drops directly to the cells in a lapse of 6 min with constant gentle agitation. Cell solution was incubated at 37°C for 10 min to allow the myeloma cell to fuse with spleen and lymph nodes by the effects of PEG on the cell membrane. After incubation time, cells were centrifuged at 1600 rpm for 5 min at RT, supernatant was throw away and cells resuspended in 5 ml of frozen media for hybridoma cells (**Appendix I**), cell aliquots of 1 ml each were made and stored at -80°C.

III.17.6. Plating hybridoma cells

To isolate hybridoma cells, an aliquot of fused cells were plated out in twelve 96-well cell culture plates in different dilutions, four of these plates had a “HIGH” dilution whilst the remaining eight plates had a “LOW” dilution of the cell preparation.

Cell dilutions were prepared as follows: 40 ml of HAT media were added to 1 ml of fused cell from section **III.17.5**, from this solution, 20 ml were taken and placed in a new tube which we named as "**A"** and the other tube was named "**B".**

Cells containing in the **"A"** tube were used to achieve the "HIGH" cell dilutions by mixing them with 60 ml of HAT media and plating out 200 µl of this mixture to four 96-wells cell culture plates, labeled as I, II, III and IV high dilutions.

Furthermore cells on the **"B"** tube were used to perform the "LOW" cell dilution by mixing them with 140 ml of HAT media and plating out 200 µl of this mixture to eight 96-wells cell culture plates, labeled as V to XII low cell dilutions. All the plates were incubated at 37°C, 5% CO_2 for 14 days or until grow cells were macroscopically evident in the wells.

III.17.7. Selection of fused hybridoma cells

Hybridoma cells from fusion of myeloma cells with spleen and lymph nodes were picked from the cell culture plates.

Macroscopically grew cells were individually selected and 80 µl of the cell suspension were placed in a new 96-wells cell culture plate containing 160 µl of HAT media. From this cell suspension, 40 µl were taken and placed in another 96-wells cell culture plated containing 200 µl of HAT media, the first plate is named "test" plate and the former the "culture" plate. As their names indicated, test plate was used for testing the reactivity of the fused cells, while the culture plate is an identical copy of the selected clones and is used to select the positive reactive cells for further culture. Both plates were incubated at 37°C, 5% CO_2 for three days to analyze their reactivity.

III.17.8. Reactivity of hybridoma cells

Selected hybridoma cells were tested whether they have the capacity to react with specific vNAR proteins using an ELISA assay. To do this an ELISA plate was coated with 120 ng of purified proteins and incubated overnight at 4°C, at the next day 200 µl of blocking solution (3% milk powder in 1x PBS) were added to each

well and incubated for 2 hrs at 37°C. After three washing steps with 200 µl of washing solution (0.05%Tween 20 /1x PBS) 100 µl of the supernatants from hybridoma cells were added to each well and leave them at 37°C for 2 hrs, four washing steps were accomplished to rinse the unbound molecules and to detect the positive reactive cells 100 µl of an anti-mouse IgG peroxidase antibody diluted in 1% milk/1x PBS at 1:5000 dilution was added to each well and incubated for 1 hr at 37°C. Four washing steps were made and 100 µl of TMB develop solution was added to each well and incubated in darkness for 30 min, afterwards 50 µl of stop solution (0.5M sulfuric acid) were added to the wells to stop the reaction and the plate was read at 450 nm. Threshold of positive clones was 0.2 Abs 450 nm, since it was the absorbance amount of the negative control (no vNAR protein).

Reactivity assays were performed several times either to test selected clones or to verify the ability of isolated clones to bind to different antigens such as diverse vNAR families, VHH antibody, protamine, milk or ART2.2 proteins.

III.17.9 Subcloning of hybridoma cells

Hybridoma cells which reacted specifically with vNAR molecules were selected for subcloning, and in this way isolate individual immunoglobulin secreting clones. To approach this, selected hybridoma cells were grew until confluence in HT media. Cells were harvested and counted to have $1x10^5$ cells/ ml. From this cell preparation 1 ml was taken and added to a falcon tube containing 9 ml of HT media, two same further dilutions were made and from the last one (containing 10 cells in 100 µl) 3ml were taken and added to a tube with 27 ml of media (this dilution contained 1 cell in 100 µl), to make the last dilution 10 ml from the last tube were taken and added to 20 ml of HT media, this tube contained 0.33 cell per 100µl.

Three 96-wells cell culture plates were made using the last three dilutions the first row of the plate was filled with the dilution with 10 cells/100 µl and it was used as positive control of growing. Three rows of the plate were placed with 1 cell in each well and the last 4 rows were filled with the dilution of 0.33 cell in 100 µl. This same

procedure was followed for each clone that wanted to be subcloned. Plates were incubated at 37°C, 5% CO_2 for 14 days or until macroscopically grow cells were visible. Afterwards grown cells were selected and used for further reactivity test as indicated in section **III.17.8**.

III. 17.10 Isotype of hybridoma cells

The isotype of secreted immunoglobulins from subcloned hybridoma cells, were investigated by a sandwich ELISA using the Mouse Immunoglobulin Isotyping ELISA kit (BD Pharmingen Cat. 550487). Following the provider's instructions, the ELISA plate was coated with the specified dilutions of each isotype-specific rat anti-mouse purified monoclonal antibodies directed with the mouse isotypes IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, Igκ and Igλ and incubated overnight at 4°C, after washing, 200 µl of blocking solution was added to each well and incubated for 1 hr at 37°C, rinse steps were performed and afterwards 100 µl of hybridoma cell supernatant was added to each well, as the specified scheme on the kit recommend. After incubation for 2 hrs at 37°C and washed steps, the secondary antibody was added to detect the mouse Ig and plate was developed as indicated in the section **III.17.8**.

III.17.11 Hybridoma cell culture

After determined the isotype of subcloned fused cells, this were selected and cultivated in complete RPMI 1640 media, some aliquots of this cells were used for long-term storage (at -120°C) and for regular culture maintenance.

III.17.12 Hybridoma cell supernatant production

Selected hybridoma cells were cultivated on RPMI 1640 media until confluence, afterwards cell culture media was replaced slowly but constantly to 100% RPMI 1640 low IgG media. Hybridoma cells in 100% RPMI low IgG media were cultivated in 10 cm Petri dishes until confluence and transferred to 20 cm Petri dishes containing 60 ml of low IgG RPMI media, cells were further cultivated for 7

days and supernatant containing secreted immunoglobulins were harvested by centrifugation at 1600 rpm for 5 min at 4°C. Supernatants were then filtered through a 0.22 µm sterilecup filter and kept at 4°C until usage.

III.17.13 Immunopreciptation of vNAR molecules

Immune sera from mice 3535, 3536 and 3537 were used to immunoprecipitate vNAR proteins as follows: 15 µl of protein G-sepharose were incubated with 10 µl of a 1:10 diluted mouse sera in a 200 µl 1% TritonX-100/1xPBS solution, for 30 min in constant agitation. After incubation samples were centrifuged at 10,000 rpm for 1 min and supernatant was discarded, resin was washed four times with 1 ml of washing solution (1%TritonX-100/1xPBS), finally sepharose was resuspended in 200 µl of washing solution and incubated with 3 µg of purified vNAR-SAALAK (Y2) for 30 min at RT in constant agitation. Washing steps were repeated four times and pellet was resuspended in 20 µl of SDS-PAGE loading buffer for a further SDS-PAGE gel electrophoresis analysis using an 12% acrylamide gel.

SDS-PAGE gel were either stained with coomassie blue or transferred blotted to make silver staining procedure or western blot detection.

III.17.14 Western blot detection of immunoprecipitated vNARs

SDS-PAGE gel from immunoprecipitation procedures from section **III.17.13**, were used to transfer them to a PVDF membrane to perform a Western blot detection. After blocking overnight the membrane with a solution containing 3% milk in 1xPBS at 4°C, the precipitated vNAR molecules were detected by an anti c-Myc peroxidase antibody used at 1:10000 dilution. After this a standard western blot developing procedure was performed as described previously in section **III.13.5**.

III.17.15 Dot blot detection of shark serum and vNAR proteins

A nitrocellulose Hybond ECL membrane (Amersham, Cat. RPN63D) was used to perform the dot blot detection by placing on it 2 µl of each antigens either purified proteins (vNAR, VHH, protamine, ART2.2), 3% Milk solution in 1x PBS or animal

sera (shark, llama or goat), the membrane was dried at RT and was blocked overnight at 4°C with 3% Milk/1x PBS. Afterwards, three rinses of 3 min each were performed with washing buffer (0.05% Tween 20/1x PBS) and slides of membrane were incubated with either immune mouser sera or hybridoma cell supernatant.
In the case of assays with pre and immune mouse sera, membrane slides were incubated with 1 ml of mouse sera diluted in 1x PBS at 1:1000 dilution. In the other case 1 ml of hybridoma cell supernatant was used to incubate the membrane both were let stand for 2 hr at RT in constant agitation. Four “long washes” of 6 min each and four “short washes” of 3 min each one were carried out in agitation using washing solution. Secondary anti-mouse IgG peroxidase antibody was added to the samples at 1:5000 dilution in 1% Milk/1x PBS solution and incubated for 1 hr at RT in agitation. Washing steps were repeated and afterwards membranes were in developed using the Amersham western blotting detection reagents and Hyperfilm ECL films.

III.17.16 Immunoprecipitation of shark serum

This assay is a variant of the method previously described in section **III.17.13,** in this case the experiment was performed to identify serum shark proteins that react with monoclonal antibodies #370 and #533 (isolated in section **III.17.**12).
Immunoprecipitation was achieved by incubating 20 µl of protein G-sepharose with: (A) 1 ml of naive shark serum, (B), (C) and (D) 1 ml of hybridoma cell supernatant (either clone #370 or #533). Samples were incubated overnight at 4°C and then centrifuged; supernatant from each sample was kept at 4°C for further analysis. Sepharose matrix was washed with washing solution (1%Tx-100/1x PBS) four times in a long and four times in a short manner as described previously (section **III.17.15**). After washing steps, sample “C” was further incubated with 1 ml of naive shark serum and sample “C” with 1 ml containing 1 µg of purified vNAR-SAALAK (Y2) protein, both samples were placed in constant agitation overnight at 4°C. Afterwards, samples were centrifuged and supernatant was kept in new tubes for further analysis.

Samples were washed as described before, and matrix resin as well as 1 µl of each kept supernatant were resuspended in 20 µl of SDS-PAGE loading buffer and heated at 75°C for 15 min, then after samples were loaded into a 10% acrylamide gel to perform an SDS-PAGE electrophoresis. Gel was staining with coomassie blue or transferred into an immunoblot membrane for further western blot detection.

III.17.17 Western blot detection of immunoprecipitated shark serum molecules

Immunoblot membrane from previous section was blocked with 3% Milk/1x PBS for 2 hrs at RT in constant agitation and immediately rinsed with washing solution. To detect shark serum bound molecules 10 ml of #370 hybridoma cell supernantat was used to incubate the membrane overnight at 4°C, after this 4 washing steps were made and an anti-mouse IgG-peroxidase antibody was added in a 1:5000 dilution in 1% milk/1x PBS solution. Four washes were accomplished and membrane was developed as previously described.

III.17.18 Specific immune response in sharks

To evaluate the specific immune response of three immunized sharks during an immunization protocol of 149 days, their pre-immune and immune sera were taken. ELISA plates were coated with 1 µg per well of tested antigens (glycophorin-A, hen-egg-lysozyme or a synthethic peptide) and incubated overnight at 4°C, the plate was rinse with washing solution and then 200 µl of blocking solution 3% milk powder in 1x PBS or 3% BSA in 1x PBS were added to the plates and incubated for 2 hrs at 37°C. After rinse the plate, 100 µl of two serial fold dilution (from 1:50 to 1:1600) of each shark serum was added to the wells and incubated for 2 hrs at 37°C, four washing steps were performed and after that 100 µl per well of hybridoma cell supernatant from clone #370 were added and incubated for further 2 hrs at 37°C. After four washing steps, 100 µl of secondary antibody anti-mouse IgG-peroxidase was added to the wells in a 1:5000 dilution in blocking solution and

let it stand for 2 hr at 37°C, rinses were performed as described and plate was developed by adding 100 µl of TMB substrate and incubating the plate for 30 min at RT in darkness and reaction was stopped by adding 50 µl of 0.5 M of sulfuric acid. Plate was read at 450 nm.

CHAPTER IV

RESULTS SECTION I

Isolation of fragments from single domain antibodies (vNARs) from horn shark *Heterodontus francisci* against murine CD38 protein

This section deals with the generation and characterization of an immune library of vNAR fragments against murine CD38. After several months of immunizing one *H. francisci* shark against murine CD38, its spleen was taken and RNA was isolated from it to generate cDNA for making PCRs using species-specific oligonucleotides to amplify vNAR fragments. An immune library of vNARs against CD38 was obtained and after several rounds of panning selection using the phage display technique, several vNAR were isolated and sorted out in families on the basis of its similarities in the CDR3 region. Individual phages from families and recombinant vNAR protein were obtained to use them for further recognition experiments. Specific binding to mCD38 by phage-vNAR or recombinant vNAR protein was performed by ELISA assays. Selected vNARs as binders were used to test if they also have the capacity to block the NADase activity of mCD38 by a thin layer chromatography using ^{32}P-NAD as a substrate. Although the vNARs families were able to recognize CD38 bound to an ELISA plate, they did not inhibit the activity of the enzyme. vNAR binders as phage or purified protein were used to test if they bind to CD38 on the surface of intact cells by FACS analysis, using either wild type and knock-out CD38 spleen cells or lymphoma CTLL-2 and EL4-R cells. Most vNAR-carrying phages showed weak binding to CD38^{pos}, but not to CD38 ^{neg} mouse lymphoma cell lines. Surprisingly, most vNARs (either as phages or purified proteins) did not bind to mouse spleen cells, although the expression level of CD38 is higher on spleen cells than on the CD38^{pos} EL4 lymphoma cells. So far, only clone SAGTK (D6) was found to weakly discriminate wt and CD38 ko spleen cells.

IV.1 Generation of a phage-display library of single-domain antibody fragments (vNARs) from a *Heterodontus francisci* shark

A library of vNARs from was generated from total RNA isolated from the spleen of a horn shark (*Heterodontus francisci*) that had been immunized against murine CD38. The details of the library construction and characterizaton are presented below.

IV.1.1. Total RNA isolation

Total RNA was obtained from the spleen of one shark immunized with murine CD38 protein as described in Materials and Methods. Approximately 1.3 mg of total RNA was obtained, to evaluate the integrity of the sample it was run into a 2% agarose gel as shown in **figure 14** Samples were stored in aliquots of 50 µl at -80°C until further use.

IV.1.2 cDNA Synthesis

For generating complementary DNA specific for *H. francisci* VNARs fragments, a reverse transcription reaction was performed using the isolated RNA from spleen and 1.5 µg of RNA were reverse transcribed in a 20 µl reaction. To assess the quality a sample of the reaction was analyzed by 2% agarose gel electrophoresis as shown in **figure 1**4

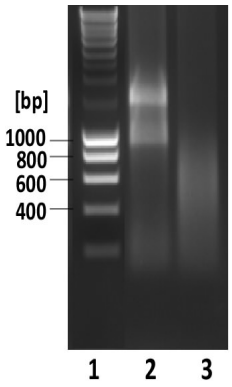

Figure 14. Total RNA and cDNA from the spleen of an immunized horn shark *Heterodontus francisci*. After completing the immunization protocol with murine CD38 protein, the spleen from a horn shark was obtained and total RNA was isolated from it. Using this as template a reverse transcription reaction was assessed and the quality of each reaction was checked in a 2% agarose gel and compared with standard molecular weight markers (lane 1). In lane 2 it is shown 400 ng of total spleen RNA and in lane 3, the quality of 5 µl of cDNA containing 200 ng was loaded.

IV.1.3 Polymerase chain reaction for amplifying vNAR fragments

To amplify specific vNAR fragments, a PCR reaction was performed using the species-specific oligonucleotides from **table II** and the cDNA described above.

The reaction product was analyzed by electrophoresis on a 2% agarose gel. The reaction contained a single band of the expected size (around 400 bp), which was subsequently purified and digested with the enzymes Not*I* and Nco*I* to generate appropriate ends for cloning. After enzymatic digestion, the PCR products were purified with the Nucleospin extract kit to remove any traces of enzymes or buffer. Digested fragments were quantified by their absorbance at 260 nm, yielding around 150 ng/µl. Also, the quality of the products was reviewed by gel electrophoresis as depicted in **figure 15**.

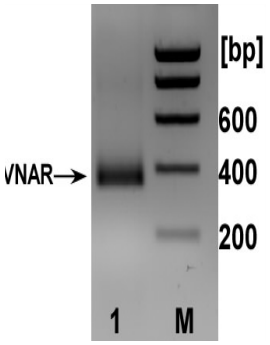

Figure 15. PCR amplification of vNAR fragments. A PCR reaction was performed using species-specific oligonucleotides to amplify vNAR fragments from *H. francisci.* As template cDNA obtained in section IV.1.2 was used, the product of the PCR reaction was analyzed in 2% agarose gel, loading 750 ng of it in lane 1 and compared it with standard molecular weight markers in lane M. The expected size of vNAR fragments is ~400 bp as it is shown by the black arrow.

IV.1.4 pHEN-2 vector purification and treatment for the ligation reaction

The phagemid vector pHEN-2 shown in **figure 16** and **17** was routinely used for phage display technique.

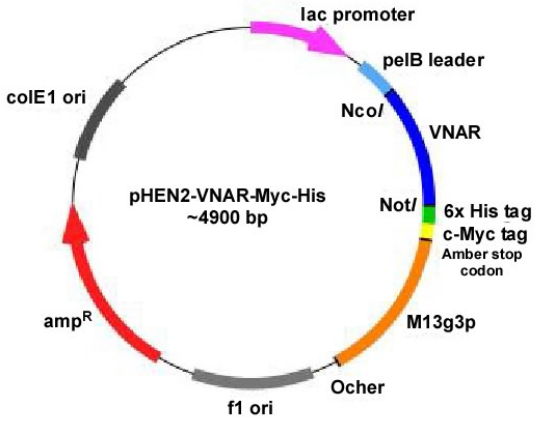

Figure 16. Schematic representation of pHEN2-vNAR vector. Important features of phagemid vector are shown, such as the *E. coli* and phagemid replicative origins, the *lac* promoter and the site of the pelB leader sequence. The vNAR fragments shown in dark blue cloned as fusion with the 6 histidine flag, the c-Myc tag and the pIII gene.

For constructing the vNAR library against mCD38, the vector was digested with Nco *I* and Not *I* enzymes and then treated with shrimp alkaline phosphatase to remove the phosphate groups at the ends, in order to avoid its religation. After both treatments the vector was purified by elution from an agarose gel as shown below. The concentration of the purified plasmid was 590 ng/µl.

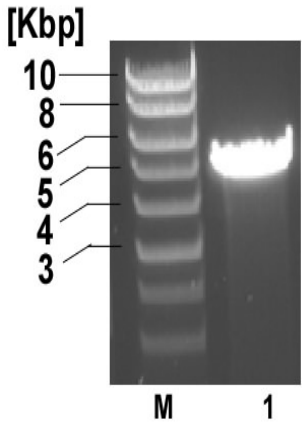

Figure 17. Enzymatic linearization of pHEN2 vector. In attempts for cloning the vNAR library, the vector pHEN-2 was linearized with Nco *I* and Not *I* enzymes and treated with shrimp alkaline phosphatase. Afterwards it was purified by agarose gel extraction and an aliquot of this purification was compared with molecular weight markers (lane M) in a 1% agarose gel. Linearized vector has an approximately size of 5Kbp as it is shown in lane 1.

IV.1.5. Ligation of the pHEN-2 vector and vNAR fragments

To determine the optimal conditions for generating the library, a test ligation was performed using 50 ng of vNAR-PCR product and 200 ng of digested pHEN-2 vector. The purified vector and insert used in the ligation were analyzed by agarose gel electrophoresis as shown in **figure 18**.

Fifteen individual bacterial colonies from the test library transformation were screened by colony PCR to find out if they contained a vNAR insert, showing that 80% of them had the correct insert size of 400 bp (data not shown). Subsequently, a final ligation was carried out with 400 ng of insert and 1.3 µg of vector. This was incubated overnight at 16°C and then transformed into electrocompetent cells. Individual colonies on agar plates were counted to calculate the size of the vNAR library, revealing a total size of $1.37x10^6$ c.f.u.

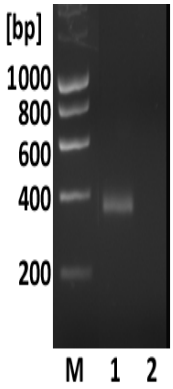

Figure 18. DNA products used for test ligation reaction. A small immune vNAR library was generated in attempts to screen the best conditions for ligation and if the diversity of the resulting library. To approach this, a ligation reaction was made according with the material and methods section. The DNA fragments used were analyzed in a 2% agarose gel comparing their size with molecular weight standards (lane M), lane 1 represents 50 ng of purified vNAR-PCR product digested with Nco *I* and Not *I* enzymes and lane 2 shows 200 ng of digested and dephosphorylated pHEN-2 vector. After incubation, the reaction was transformed by electroporation to competent *E. coli* cells.

IV.1.6 Colony PCR

To confirm the presence of the vNAR fragments cloned into the pHEN-2 vector, individual colonies from the vNAR library were taken and placed on new LB-Agar plates containing carbenicillin as selective antibiotic. From each colony, a PCR reaction was performed using the sequencing oligonucleotides LMB3 and seq1.

The expected size of vNAR fragments was approximately 600 bp. If the colonies contained only empty vectors, the expected size of PCR products was approximately 300 bp. The PCR reactions were checked in a 2% agarose gel electrophoresis as shown in **figure 19.**

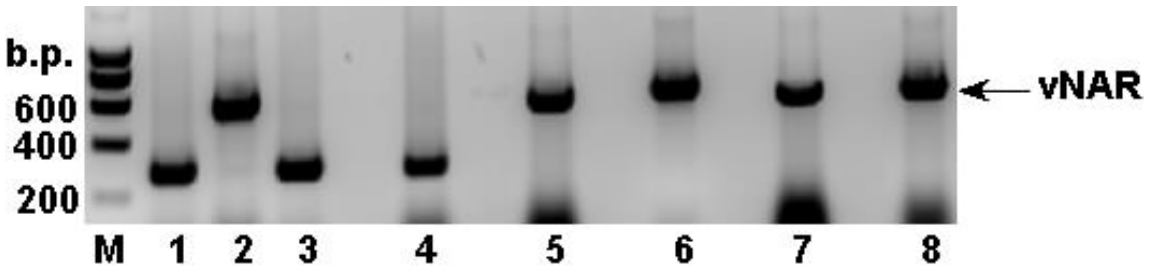

Figure 19. Colony PCR of initial vNAR library. To detect the presence of the cloned vNAR cassette in the pHEN2 vector, a PCR reaction of individual colonies from the initial vNAR library was performed using the sequencing primers LMB3 and fseq1. An aliquot of 10 µl of every reaction were loaded into a 2% agarose gel for analysis and comparison with molecular weight standards (lane M). As it is shown in lanes 1, 3 and 4, the primers amplified a 300 bp fragment corresponding to the empty vector, but if the vNAR fragments are cloned into the phagemid vector, a PCR product of ~600 pb appeared as shown in lanes 2, 5, 6, 7 and 8 (black arrow).

Colony PCR analysis was performed for every round of panning to determine the presence of vNARs. Those colonies selected as positive were cultivated on liquid LB-Carb media for further plasmid isolation and for sequencing of the cloned VNAR.

IV.2 Diversity of the initial vNAR immune library

After analysis by Colony-PCR, a representative sample of the clones that were scored positive for bearing a vNAR cassette were selected to be sequenced. This was used to estimate the variability of the library.

Twenty-nine colonies from the primary library were analyzed. Of these, 59% were identified as intact vNARs, 14% had stop codons inside the sequence, 17% had a

shift in the reading frame, and 10% were unreadable. Some of the sequences derived from the initial immune library are shown in **figure 20.**

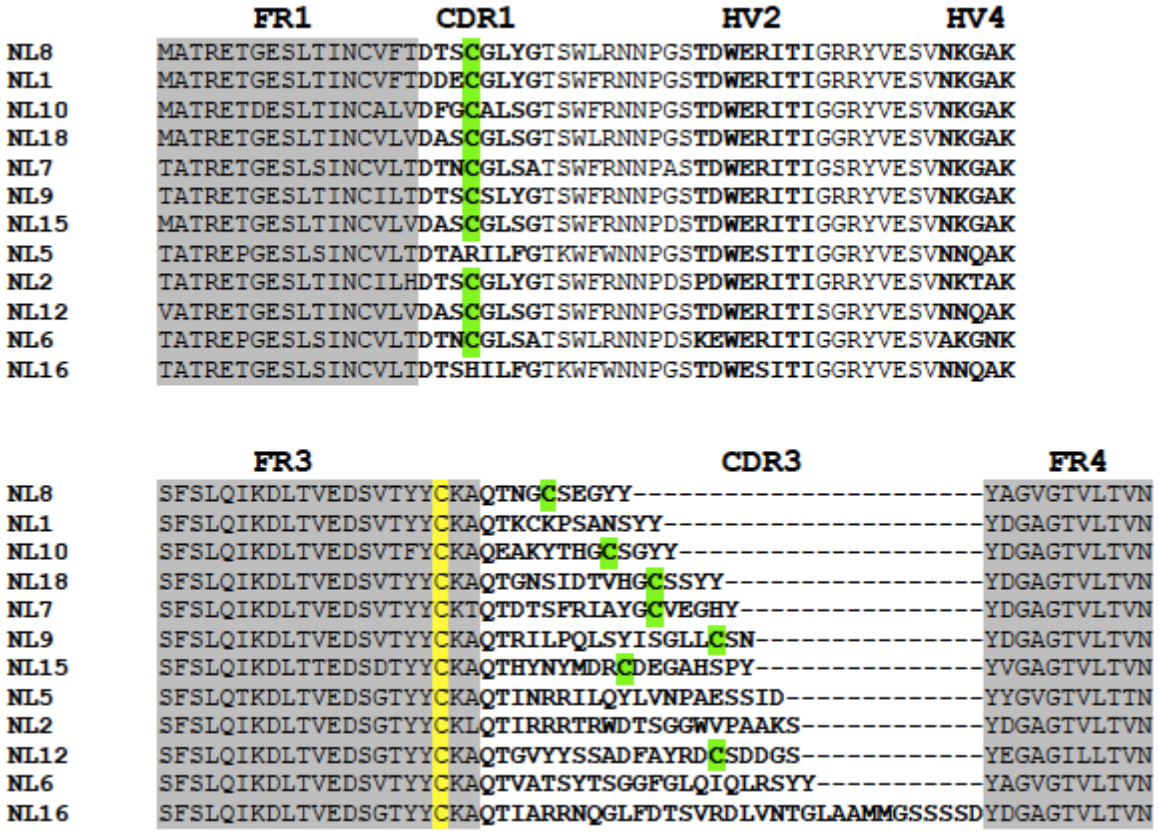

Figure 20. DNA sequences from initial immune vNAR library. Plasmid from isolated clones from the initial immune vNAR library were purified and used together with the LMB3 primer for sequencing reactions to define if the insert cloned corresponded to a vNAR fragment. DNA sequences were aligned and compared in terms of is CDR3 regions. The hypervariable regions of the vNARs such as CDR1, HV2 and HV4 and CDR3 are showing as bold letters, canonical cysteines are shown in yellow and additional cysteines in green. Framework regions (FR1, FR2, FR3 and FR4) are shadowed in gray.

Table IV summarizes the lengths of the CDR3 from all clones obtained from the initial immune library against murine CD38.

Table IV. CDR3 length of clones from initial immune-vNAR library against murine CD38

Clone Name	Lenght of CDR3	Number of cysteines		Clone Name	Lenght of CDR3	Number of cysteines
NL8	10	4		NL21	19	
NL1	12	4		NL5	21	2
NL10	13	4		NL6	22	3
NL18	16	4		NL2	23	3
NL7	17	4		NL12	23	4
NL9	18	4		NL25	25	
NL15	18	4		NL16	33	2

Importantly, sequence analysis of 29 randomly picked clones from the initial library did not reveal any repeated sequences, indicating that the library was essentially free of contaminating clones in large copy numbers. This finding allowed to proceed with the panning selection rounds.

IV.3 Selection of specific vNARs against murine CD38 by phage panning

To select phages encoding specific vNAR molecules against the murine CD38 protein, two different strategies of selection were used: solid-phase and biotinylated panning. For solid phase panning, an ELISA plate was coated with the antigen (recombinant CD38) and blocked with milk powder. After this step, phage suspension that had been pre-cleared with milk was incubated in the antigen-coated well for at least 30 min, followed by several stringency washes. The bound phages were eluted by competitive elution using increased amounts of soluble mCD38 to each well.

To implement the second strategy, the antigen was first biotinylated and bound to streptavidine agarose beads. The pre-cleared phage library was then incubated with the bead-bound mCD38, unspecific phages were washed away, and mCD38-specific phages bound to the pellet were eluted by competition with non-biotinylated CD38 soluble protein. This method is the best and cleanest way to

isolate specific phages, and yielded less aberrant sequences (sequences containing premature in-frame stop codons or frame-shifts) than solid-phase panning.

IV.3.1 Solid phase panning rounds

After each round of solid phase panning a titration of output phages was done by infecting 50 µl of TG1 log phase cells with 1 µl of output phages at a 10^8 dilution. Infected cells were plated out in LB-Carb agar plates, and colonies were counted to calculate the titer of each panning round, as shown in **figure 21.**

The titer is high at the beginning of the panning procedure, representing the complete phage population of the initial immune library. After each round of panning, the titer decreases in comparison to the initial one, but at the same time the content of specifically binding phages increases.

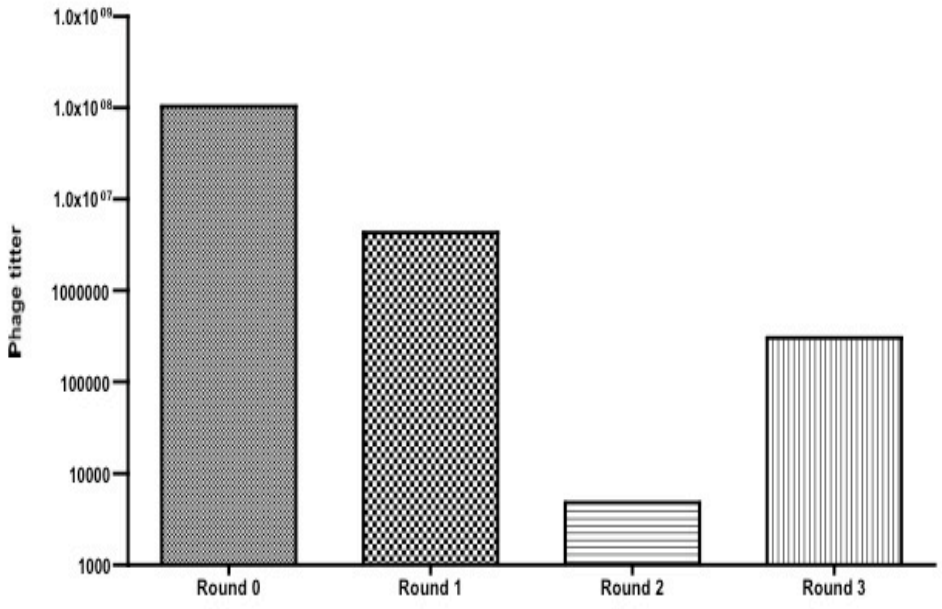

Figure 21. Output phages from selection rounds of solid phase panning. Phages produced in every round from solid panning selection against murine CD38 were titering by infecting TG1 *E. coli* cells. Round 0 represents the initial immune phage-vNAR library while round 1 to 3 corresponded to every single selection round performed in the panning.

To measure the recognition of mCD38 by the output phages from each panning round, a phage ELISA was performed. The wells of a 96-well plate were coated with 120 ng of antigen and blocked with milk powder, and then a solution of MPBS with 10^{12} phages was added to each well and incubated for 1hr. After washing away the unbound phages, bound phages were detected by an anti-M13g8p-peroxidase antibody. **Figure 22** shows the binding to mCD38 of output phages from each round of solid-phase panning. Recognition of mCD38 increased considerably from the initial library to the output of the first panning round, but there were no further increase in the following panning rounds. Milk powder was used as a control for irrelevant binding.

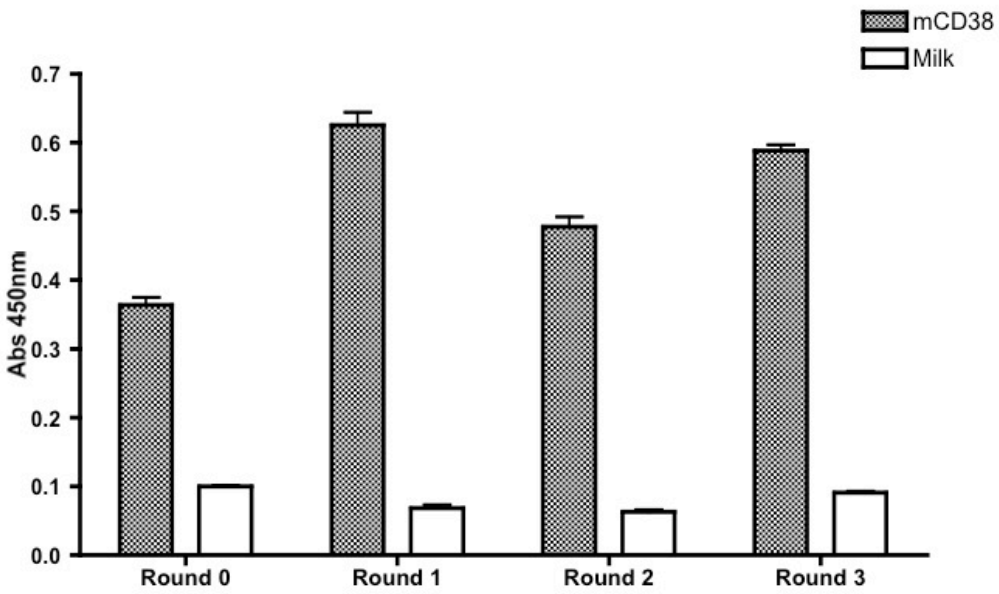

Figure 22. Specific reactivity of phages from panning rounds to murine CD38. An ELISA assay was performed to determine the capacity of phages from different rounds of solid panning to react with CD38 murine protein (gray bars) or milk (white bars). After blocking, phage solution was added to each well and inespecific particles were washed away and bound phages were detected with an anti-M13 peroxidase antibody according with the material an d methods. The graphic depicts the mean and the SD of a threefold independent experiment.

IV.3.2 Biotinylated panning

In order to reduce artifacts that might result from non-specific binding of phages to the plastic of the wells, a second panning procedure was performed on the library, this time allowing binding of the phages to the antigen in solution. To this end, recombinant CD38 protein was biotinylated, the success of this was checked by Western blot, using peroxidase-conjugated streptavidin for detection as shown in **figure 23**.

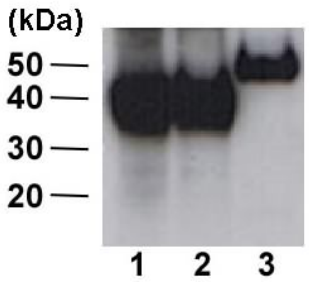

Figure 23. Biotinylation of murine CD38. Murine protein CD38 was biotinylated for using it in further panning selection of biotinylated antigen. According with the material and methods section the protein was labeled using the kit EZ-Link Sulfo-NHS-Biotin and the successful of this was analyzed by a western blot detection of biotinylated CD38 in a 12% SDS-PAGE. Lanes 1 and 2 represent 2 and 1 µg of biotinylated CD38, respectively. The observed bands correspond to the expected molecular weight of 38 KDa. In lane 3 200ng of a biotinylated VHH-Fc conjugated antibody was run as a positive control.

Three rounds of biotinylated panning were performed. From each round, 10 or more colonies were picked, and the obtained plasmids were sequenced. At the end of the panning procedure, many clones could be sorted into “families”, defined by similarities in their CDR3 regions. The families were named after signature sequence motifs found within their CDR3 regions. Of note, the repertoire of clone families isolated in the two different panning procedures was remarkably constant. An overview of the clone families isolated in each panning procedure is given in **table V.** Some individual clones were also isolated in most panning rounds.

Although similar or identical CDR3 regions characterized the families, some family members showed differences in their CDR1, HV2, or HV4 regions. Two examples are shown in **figure 24.**

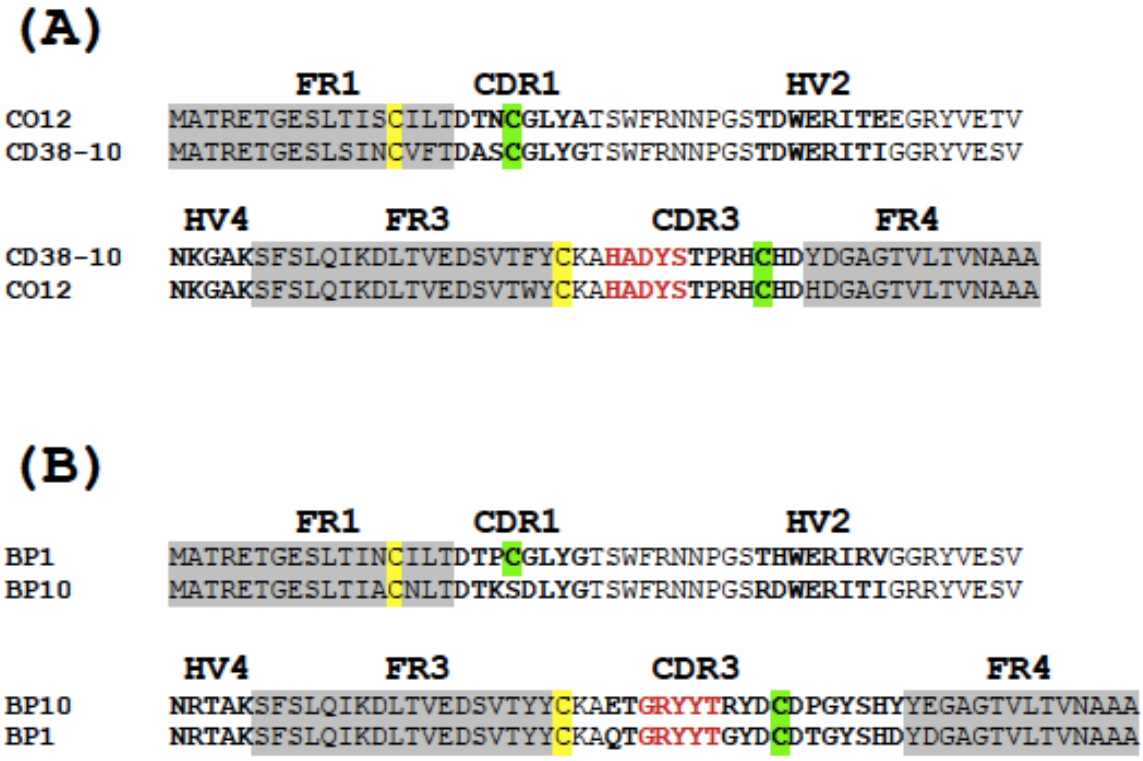

Figure 24. Comparison of amino acid sequences of isolated vNAR families. Some clones had identical (A) or divergent but definitely related (B) amino acid sequences in their CDR3 regions, but differed in other variable regions such as CDR1, HV2 or HV4. The two clones shown in (B) were derived from the second round of biotinylated panning. Note the similarity in the amino acid sequence in the CDR3 region between the two clones with the conserved amino acid motif (red letters) in (A) HADYS or in (B) GRYYT. Residues belonging to defined framework regions are labeled as FR1-4 (gray shadow), variable regions are indicated by bold letters, canonical cysteines are labeled in yellow while additional ones are in green.

Table V. Clone families isolated by panning against murine CD38

Family	Times found	Panning	CDR3 lenght	CDR3 sequence	Some members
HADYS	16	Solid	12	**HADYS**TFRHCHD **HADYS**TPRHCHD	**C12F** CO12,CD38-10,NF-11, Z8,C1-7,NL-8
KYTH	6	Solid, Biotin	12	QEAKYTHGCSGYY	Y1,B1,B7,B8,B9,B11,B12, F1,F12,NL-10, BP-11, CD38-1
DIEGO	25	Solid Biotin	15	QTSLR**DYAS**DSCDYY	NF3R2-2,9,12, Z1, NF-9, NF3RO-10
LYSHY	3	Solid	16	QTLLGMLCDGV**LYSH**	CO2, CD38-7, Y7
PIFQ	2	Solid	16	QV**PIFQ**GDLRCGYKKY	R1-1-, R0-1
FRITY	8	Solid Biotin	17	QTDTS**FRITY**GCVEGHY QTDTS**FRIAY**GCVEGHY	B4, B5, B6, F4, F6, F7, F11, BP-13, NL-7
KTG	2	Biotin	17	**KTG**QLYTGCSDGGSWHF	BP-7, BP17
SYIS	2	Solid	18	QTRIL**P**QL**SYIS**GLLCSN QTRIL**Q**QL**SYIS**GLLCSD	**NL-9** NF-6
TKIL	6	Solid Biotin	18	Q**TKIL**QQLTYISGLLCSD	BP3, NF3R
SAALAK	13	Solid	18	EVT**SAALAK**CECSCDSSY	Y2, CD38-2, C1-3

Table V. (Continue) Clone families isolated by panning against murine CD38

Family	Times found	Panning	CDR3 lenght	CDR3 sequence	Some members
GRYYT	2	Biotin	18	Q**T**T**GRYYTG**YDCD**T**GYSHD E**T**T**GRYYTR**YDCD**P**GYSH**Y**	BP1, BP10
PHIL	10	Solid	18	KTIGKRR**PIHL**IGGVMRS	K13
GRIL	2	Solid	19	QT**GRIL**QSPGMPCDGGSHY	F2-4, F2-10
SASARI	2	Solid	21	QGRYFSS**SASARI**CDEGSIF Y	C11, CO11
SAGTK	8	Solid	25,26	KGRYFS**SAGTK**PLFESSCD EGSS**S**YY	D1,D6, NF3R0-7, NF3R1-10, **NL25**

In total, 13 families were identified. Members of some of them were isolated more frequently than others. Families, whose members were isolated often from solid panning were HADYS (26 times), SAALAK (13x), and SAGTK (8x). Clones KTG and GRYYT were isolated by biotinylated panning and were found two times. However, some families were found in both types of panning (highlighted in blue in the table), which were also highly represented, such as DIEGO (25 times), FRITY (8x), KYTH (6x), and TKIL (6x).

Overall, more families were obtained from the solid phase than from the biotinylated panning.

IV.4 Expression of recombinant vNAR proteins in *E. coli*

Plasmids isolated by panning were used to transform *E. coli* HB2151 cells to express recombinant proteins. vNAR protein production was induced with IPTG, and after 4 hrs, the cells were recovered and lysed by osmotic shock to liberate proteins from the periplasmic space. Recombinant vNAR proteins were purified from the periplasmic lysate (PPL) by metal affinity chromatography, since the recombinant proteins carry a C-terminal tail of 6 histidines.

In **figure 25** depicts the protein expression of clones belonging to the families SAALAK (Y2), HADYS (C1-7), DIEGO (R2-9) and PIFQ (R1-1), all of them were efficiently produced and purified. Following metal affinity chromatography, the eluted fractions were analyzed by SDS-PAGE and Coomassie staining. Fractions that contained purified VNARs were pooled, and the elution buffer was exchanged for 1x PBS using PD10 columns (Amersham Biosciences Cat. 170851-01). The purified proteins were quantified using the BCA kit, obtaining yields of 4.5 mg for SAALAK, 1.7 mg for HADYS, 1.12 mg of DIEGO and 0.38 mg of PIFQ VNAR families, from a 100 ml induction culture in *E. coli* HB2151 cells.

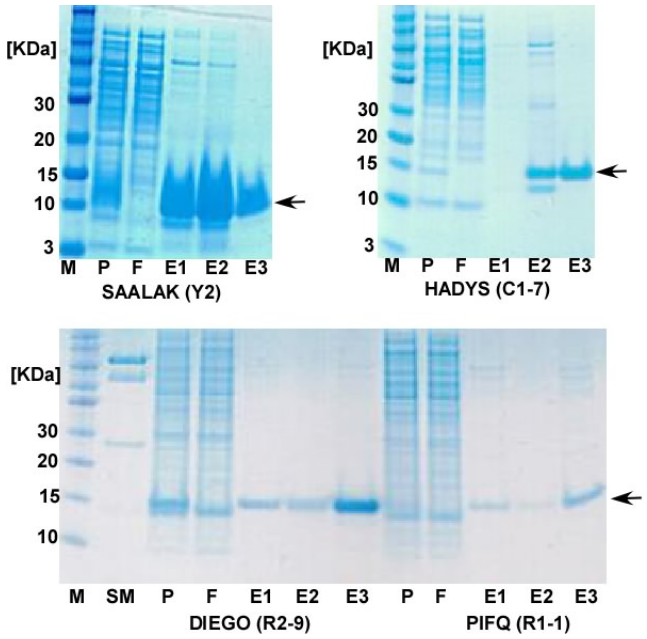

Figure 25. vNAR protein purification by nickel affinity chromatography. Recombinant vNARs were purified from PPL extracts, by means of metal affinity chromatography using its 6x histidine tail. After passing the PPL extract through the column and washing steps, the bound protein was eluted with a buffer containing 100 mM of imidazole, eluted fractions of 1ml each were collected and an aliquot of 10 µl of them were analyzed by an 12% SDS-PAGE and stained with coomassie blue. Eluted fractions from clones SAALAK, HADYS, DIEGO and PIFQ were analyzed and the size of the labeled protein bands was compared with molecular weight standards (lane M). Lane P, represents 10 µl of the total 10 ml volume of PPL extract, F corresponds to 10 µl of the flow-through obtained from the column and E1, E2 and E3 showed the eluted fractions containing recombinant proteins. Obtained vNARs are pointed by a black arrow.

However, protein expression problems arose with some of the vNARs. Even though the selected plasmids had correct reading frames, the expression levels were very low or undetectable. As an example, **figure 26** shows the amino acid sequences of some clones isolated from the second round of biotinylated panning against mCD38.

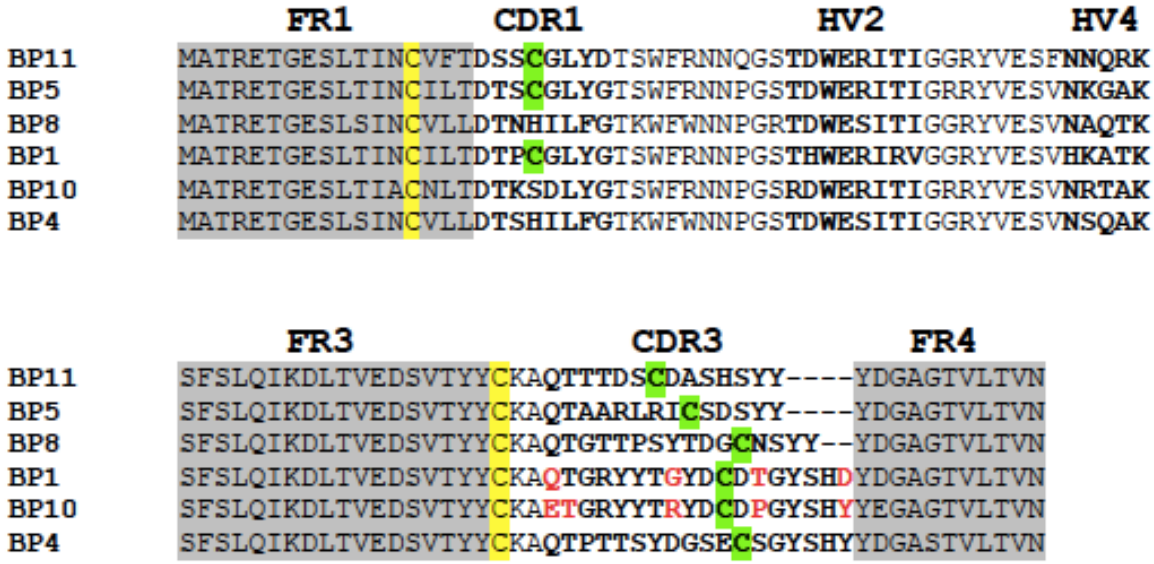

Figure 26. Amino acid sequences from clones of the second round of biotinylated panning against CD38. Regions in the sequence are labeled as in Figure 24. Canonical cysteins are highlighted in yellow, additional cysteines in green. Differences between amino acids in the CDR3 from two clones BP1 and BP10 belonging to the same family (GRYYT) are shown in red.

Clones BP1 and BP10 were both grouped into the GRYYT family because of their similar CDR3s. The clones differed only by a few amino acids (highlighted in red). Although both clones had correct reading frames, they differed greatly in expression levels. While expression of BP1 could be detected in a Coomassie–stained gel, expression of BP10 was undetectable even by Western blot using an antibody directed against the c-Myc tag present in the recombinant protein (**figure 27**). Other clones, such as BP4, BP5, BP8, and BP11, were not detectable in a Coomassie-stained gel, and only barely detectable by Western blot.

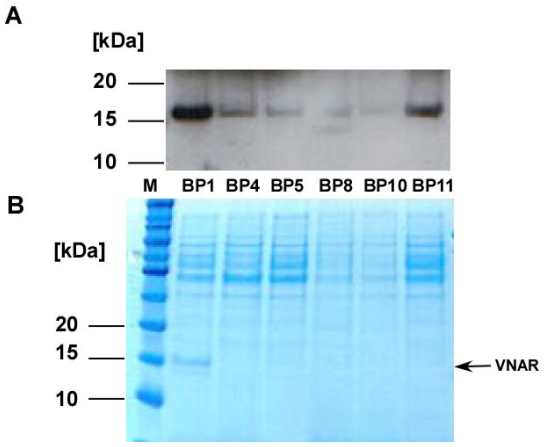

Figure 27. Protein expression of vNAR clones. Recombinant protein expression of some clones from the second round of selection from biotinylated panning was analyzed in a 12% SDS-PAGE gel. The figure depicts the low levels of protein expression, detectable by western blot using the c-Myc flag (A) but barely detectable by coomassie blue staining (B). Protein bands were compared with molecular weight standards in Lane M (labeled as kDa). Analyzed clones on lanes in (A) have their counterpart on (B). Sequences of clones presented here are listed on the **figure 26**.

Figure 28 shows the purified vNAR proteins used for further experiments on FACS analysis or in inhibitory enzymatic assays.

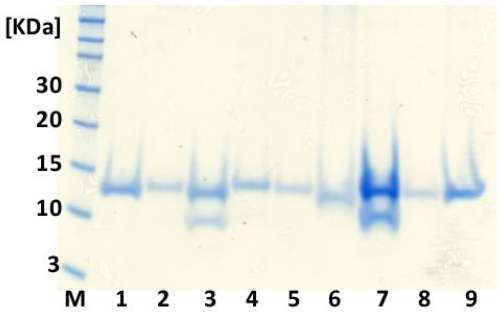

Figure 28. Recombinant purified vNARs proteins. Different families of vNAR were expressed as recombinant protein and purified by nickel metal affinity chromatography. Eluted proteins were then concentrated and quantified by a BCA assay; standardized amounts of purified proteins were chose and used to run a 12% SDS-PAGE, proteins were visualized by stain them with coomassie blue solution. As it is indicated in the gel M represents the standard molecular weight markers (kDa), and lanes 1 to 9 represented 200ng of different vNAR families as follows: 1) HDAYS, 2) DIEGO, 3) SAALAK, 4) GRIL, 5) PIFQ, 6) SAGTK, 7) PHIL (400 ng), 8) BP2R1-1 and 9) VHH heavy chain single domain antibody.

Further experiments were carried out to improve the expression of those vNARs that had a correct reading frame but did not express in *E. coli* HB2151. Other *E. coli* strains, such as SC1, stably transformed with the vector pSE111 were used for expression. Nevertheless, some clones remained without detectable expression. Therefore the *Pichia pastoris* yeast expression system was selected to see if the yield of problematic VNARs could be improved in this system.

IV.5 vNAR expression in *Pichia pastoris*

vNAR proteins expressed in the yeast system of *Pichia pastoris*, were purified from a 100 ml yeast culture induced with 2% of methanol for two days. After induction, supernatant of the media was collected and precipitated with acetone and trifluoroacetic acid, and further purified by a nickel metal affinity chromatography. Eluted proteins where then loaded into a SDS-PAGE and stained with coomassie blue, as it is shown on **figure 29**.

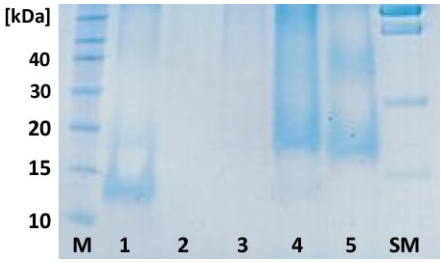

Figure 29. vNAR proteins expressed in the yeast *Pichia pastoris*. vNAR recombinant proteins were expressed in yeast by an induction with methanol during 2 days; expressed proteins were secreted to the media and were further purified by a nickel affinity chromatography. Eluted proteins were analyzed by a 12% SDS-PAGE and visualized by a coomassie blue staining. Recombinant molecules were loaded into the gel and compared with molecular weight markers on lane M and SM. vNAR families were loaded in lanes 1 to 5 as follows, 1) SAGTK (~2 µg), 2) SASARI, 3) SAGTK (NL25), 4) SAALAK (Y2) (~ 5µg) and 5) DIEGO (Z7) (~3.2 µg).

IV.6 Testing the recognition of murine CD38 by recombinant vNARs by ELISA

Using the purified vNAR proteins described in the previous sections, an enzyme-linked immunosorbent assay (ELISA) was performed to detect if the vNARs

recognized murine CD38. To accomplish this, an ELISA plate was coated with the antigen and blocked with milk powder. Subsequently, 100 ng of purified vNAR protein was added to each well, incubated for 1hr at room temperature, and then washed away. Bound molecules were detected using an anti-c-Myc antibody conjugated to peroxidase. Milk powder was used as negative control to control for unspecific binding.

As shown in **figure 30**, vNARs from the families DIEGO, HADYS, SAGTK, PHIL, and individual isolated vNARs such as Z13 and Z14 bound specifically to CD38, while SAALAK (Y2) bound weakly to murine CD38.

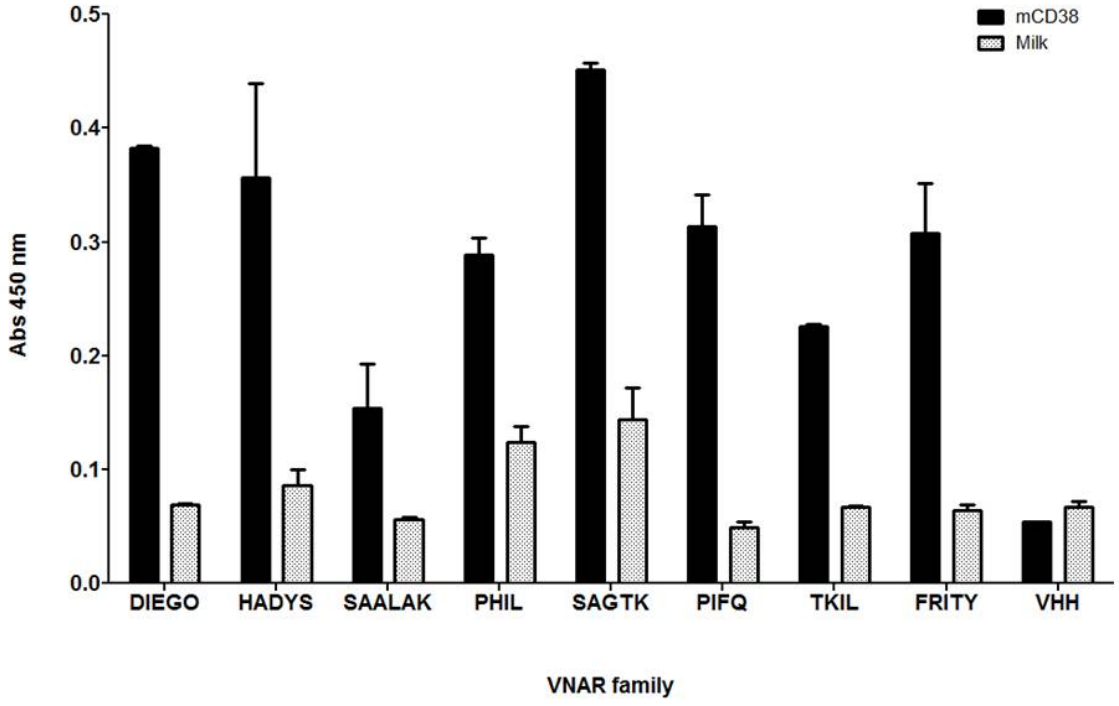

Figure 30. vNAR families react specifically with murine CD38. To test the capacity of isolated vNAR families to react with murine CD38 protein, an ELISA assay was performed using 100 ng of murine CD38 protein for coating the plate, after blocking step, 150 ng of recombinant vNAR protein per sample were added and detection was done by the c-Myc flag present in the C-terminal of recombinant proteins. As irrelevant control of binding the plate was coated with milk and as unrelated control protein a single domain llama antibody (VHH) was tested. The results show the mean and the SD of a threefold independent experiment.

IV.7 Phage ELISA to test the binding of vNARs to CD38

To corroborate the results obtained from the ELISA described above, a phage ELISA was performed following the protocol detailed in the material and method section. This procedure also offered the advantage that it was possible to test the binding of vNARs that could not be efficiently produced as purified recombinant proteins. The wells of an ELISA plate were coated with CD38 protein and blocked as described above. Subsequently, 10^{12} phages displaying a given vNAR were added and incubated for xyz minutes at room temperature. Unbound phages were washed away, and bound phages were detected using an antibody directed against the major coat protein of the phage. A phage displaying a single-domain antibody from llama (VHH) was employed as a negative control for unspecific binding of phages, and milk powder in PBS was used as an irrelevant antigen control. It is shown in **figure 31** that phage BP2R1-1 from biotinylated panning, binds unspecifically to mCD38 and milk, and that the binding of VHH-phage to mCD38 is not significant.

Seven of the eight phage-families tested bound to CD38 in the phage ELISA (**figure 31**). However, the binding of phage FRITY to the protein was weak in comparison to the other families, the binding of phage SAALAK (Y2) was not above that of the llama VHH used as a negative control, and was therefore not considered to be significant in this assay.

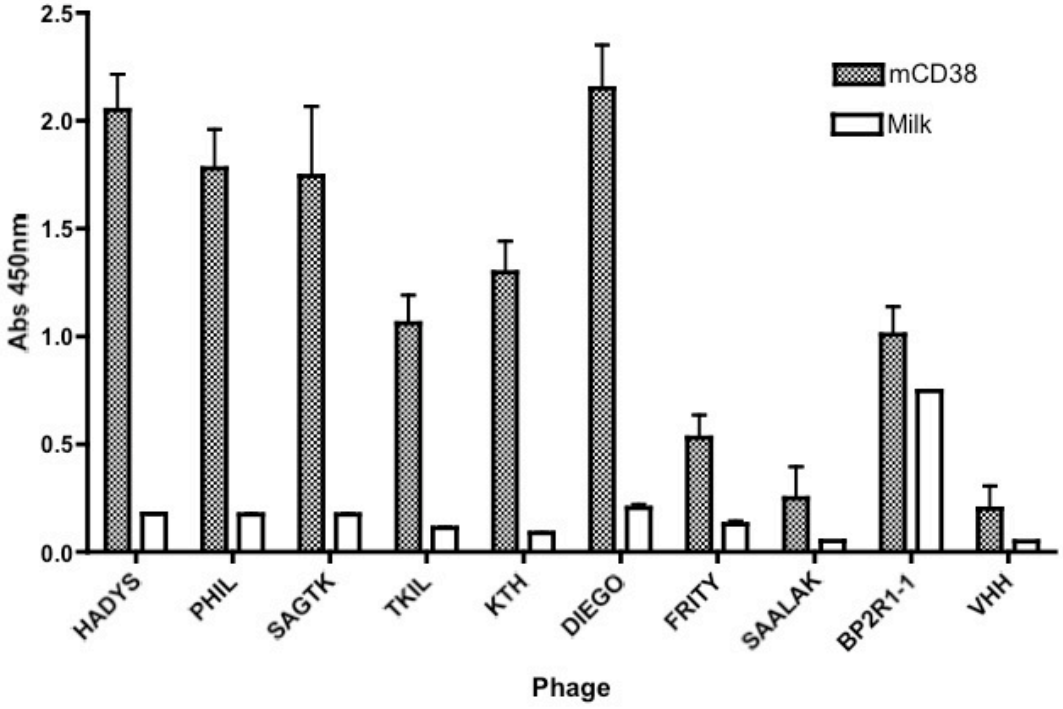

Figure 31. Reactivity of vNAR-phages to murine CD38 protein. To corroborate the binding of phage-vNAR derived from panning selection rounds, an ELISA assay was done using a mCD38 coated and blocked plate, afterwards 10^{12} phages were added per sample. As negative control of binding control of binding the plate was also coated with milk and as unspecific control of phage biding a VHH displayed phage was tested. All other phages belong to different families are listed in **Table V** or individual isolated phages such as BP2R1-1. The results presented in this figure represent the mean and SD of a threefold independent experiment.

IV.8 Development of an assay to test the inhibitory capacity of vNARs against CD38

After showing that the vNARs isolated by the panning procedures specifically recognized murine CD38 (see previous section), it was useful to test whether they were able to inhibit its enzymatic activity. To this end, an assay to measure the NADase activity of CD38 was developed as described in the methodology. Briefly, radioactive P^{32}-NAD was incubated with recombinant CD38 for 5 min, and the resulting product was analyzed by thin layer chromatography (TLC) to visualize the substrate (P^{32}-NAD) and the product (ADP ribose, ADPR) of the enzymatic

reaction. In a first step, titration of CD38 was accomplished using a fixed amount of radioactive substrate and different amounts of enzyme (**figure 32**). Uncleaved P^{32}-NAD migrates to the upper part of the membrane (lane 7), while ADPR migrates more slowly (as seen in lane 1).

In order to detect the inhibition of CD38 activity by vNARs, conditions were chosen so that hydrolysis of NAD by CD38 was not complete, as seen for example lanes 3 (12.5 ng CD38) and 4 (6.25 ng CD38) in **figure 32**. Therefore, the following experiments were carried out using 9 ng of CD38.

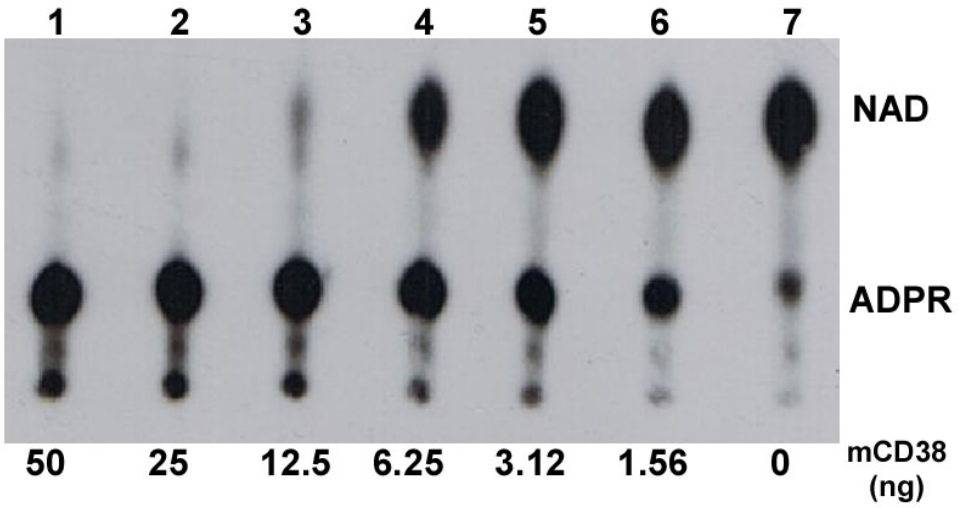

Figure 32. Titration of murine CD38 enzyme activity. Different amounts of murine CD38 ranging from 1.56 ng to 50 ng (lanes 1 to 6) were incubated with 100 µM of NAD containing 1µCi of radioactive P^{32}-NAD for 5 minutes, after stopped the reaction, 1µl of it was placed into a TLC membrane to visualize the migration pattern of the samples, the membrane was exposed to radiographic film at -80°C. Autoradiogram was developed after 2 hrs incubation as result, it is possible to visualize that P^{32}-NAD runs in the upper part of the membrane (labeled as NAD), whereas hydrolysis product ADPR migrates in the lower part of the TLC. Non treated P^{32}-NAD is shown in lane 7.

IV.9 Inhibitory assay of enzymatic activity of murine CD38 by vNARs

Selected recombinant vNAR proteins that had been shown to bind to CD38 were tested for their capacity to inhibit the enzymatic activity of CD38.

To test the inhibition of CD38 NADase activity, 9 ng of the enzyme were pre-incubated with 100 ng of selected vNARs at RT for 30 min, thereafter 100 µM of NAD containing 0.1 µCi of P^{32}-NAD was added, and the reaction products were analyzed by TLC. As described in the previous section, samples containing P^{32}-NAD alone and P^{32}-NAD plus enzyme were run as positive and negative controls.

As shown in **figure 33**, P^{32}-NAD (lane 8) migrates to the upper part of the TLC membrane, while the product ADP-ribose (ADPR) resulting from hydrolysis by CD38 migrates to the lower part (lane 7). None of the tested VNARs (lanes 1 to 6) inhibited CD38, since in all treated samples only a spot corresponding to ADPR was visible on the TLC membrane.

Although all the vNARs tested in this section except SAALAK (Y2) recognized the enzyme in a soluble form bound to an ELISA plate, none of them inhibited the enzymatic activity of CD38. We therefore concluded that the selected vNARs represented binders, but not inhibitors. In the next set of experiments we tested if they could bind to CD38 expressed on the cell surface.

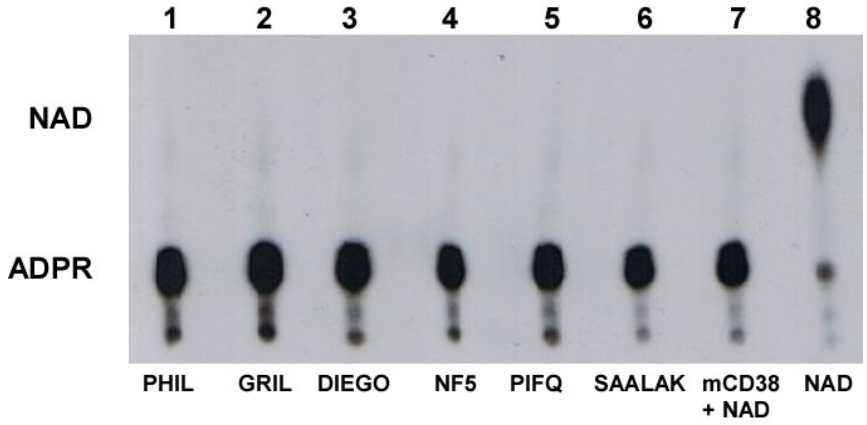

Figure 33. Inhibition of CD38 enzymatic activity. To test if the isolated vNAR families from the panning rounds were able to inhibit the NADase activity of murine CD38, 9ng of the enzyme were pre-incubated with 100 ng of purified vNARs for 30 min and then 100 µM of NAD containing 0.1 µCi of radioactuive P^{32}-NAD was added to all the samples. After 5 minutes of incubation, the reaction was stopped and 1 µl of them were run into a TLC membrane to visualize their migration pattern. Incubation and detection procedures are the same as the described on **figure 32.** As a result, P^{32}-NAD runs in the upper part of the membrane (lane 8), while hydrolysis product ADPR migrates in the lower part of the TLC (lane 7). Lanes 1 to 6 showed the activity of the vNAR families tested.

IV.10 FACS analysis with Phage-vNAR on spleenocytes from wild-type and CD38 knock-out mice

To find out if the vNARs also recognize CD38 on the surface of living cells, a FACS analysis was performed on mouse spleen cells, which express high levels of CD38 on their surface (**figure 34**). Whole vNAR-encoding phages were used for staining, since for some vNARs it proved difficult to produce sufficient quantities of recombinant protein. Splenocytes from CD38 knock-out (ko) mice were used as negative controls.

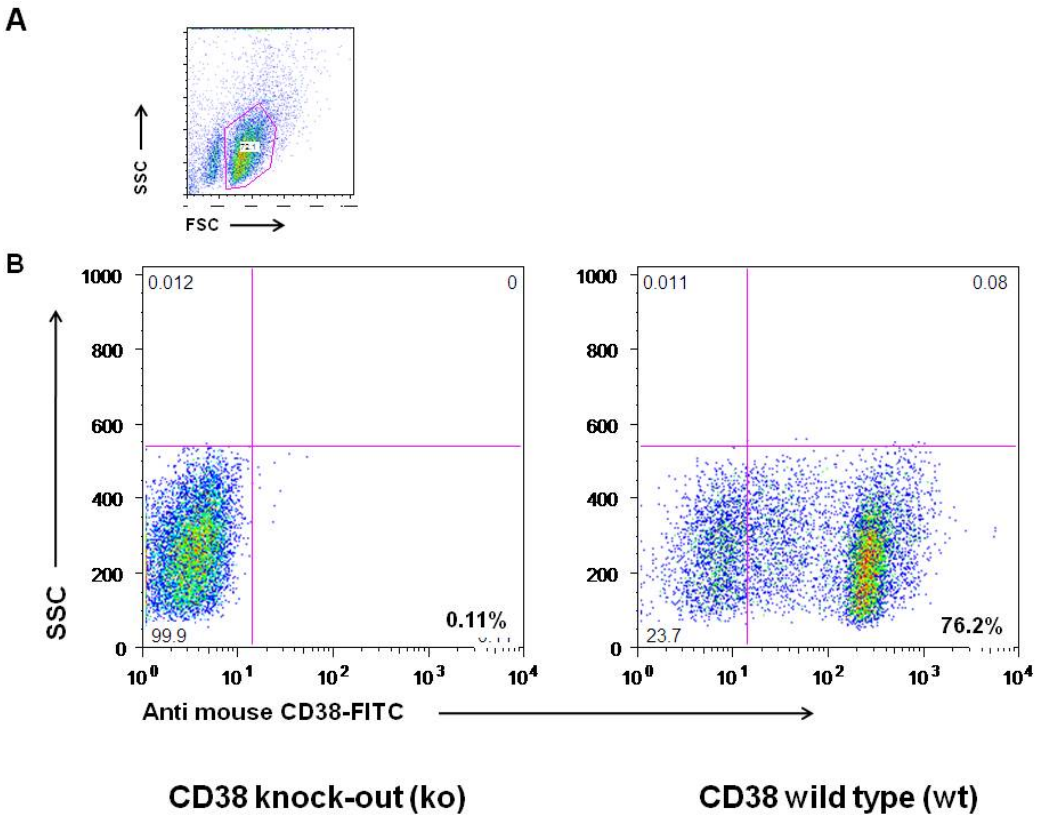

Figure 34. CD38 cell surface expression on mouse spleenocytes. To analyze the expression of mouse CD38 on the surface of spleen cells from a ko and a wt mouse, $1x10^6$ spleen cells per sample, were incubated with an anti-mouse CD38 antibody conjugated with FITC for 30 min at 4°C, after two washing steps cells were resuspended in PBS and the emitted fluorescence was measured by FACS. Panel (A) shows the gated of measured cells whereas panel (B) depicts the fluorescence emitted by the anti-mouse CD38-FITC antibody bound to the cell surface of spleen cells from a ko (left) and wt (right) CD38 mice.

FACS analysis was performed as described in the material and method section. Briefly, $1x10^6$ cells per sample were incubated with $1x10^{12}$ phage-vNAR. After washing, bound phages were detected by a FITC-conjugated antibody directed against the M13 phage major coat protein. The results are shown in **figure 35**

In the case of DIEGO no difference was observed in the binding between the wt and ko cells, indicating that this vNAR domain did not recognize CD38 on the cell surface. However, in the cases of SAGTK and SAALAK, differences in binding of

~4% and 1.8%, respectively, were observed, suggesting that these vNAR domains bound weakly to cell surface CD38.

The binding of all the phage-vNAR tested to cell-surface CD38 is summarized in **figure 36**. The graph shows the percentage of cells from wild-type *vs* CD38 ko mice stained with the indicated vNAR domains. The highest specific binding to CD38 was observed for phage SAGTK (D6), the remaining families of vNARs have no significant differences between the binding to CD38 negative and positive cells.

Since the binding of the vNARs to CD38-expressing spleen cells was low or undetectable, we tested the binding to other cell types that differentially express CD38 on their surfaces.

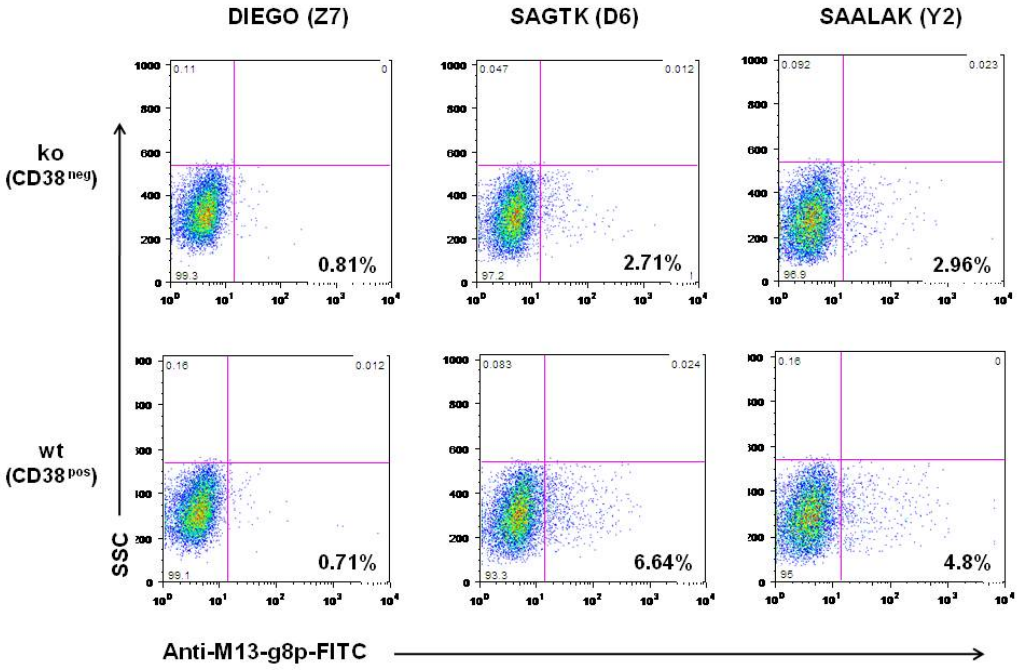

Figure 35. Binding of vNAR-phages to cell surface CD38 on mouse spleen cells. Spleen cells from a CD38 ko and wt mice were pre-incubated with vNAR displayed-phages, after washing steps the bound particles were detected by an anti-M13g8p-FITC conjugated antibody. Here it is shown the comparison of fluorescence from bound phages of three different families: DIEGO (Z7), SAGTK (D6) and SAALAK (Y2) to ko (upper panels) and wt CD38 cells (lower panels). The binding percentage of each family is shown in the lower right corner of each panel.

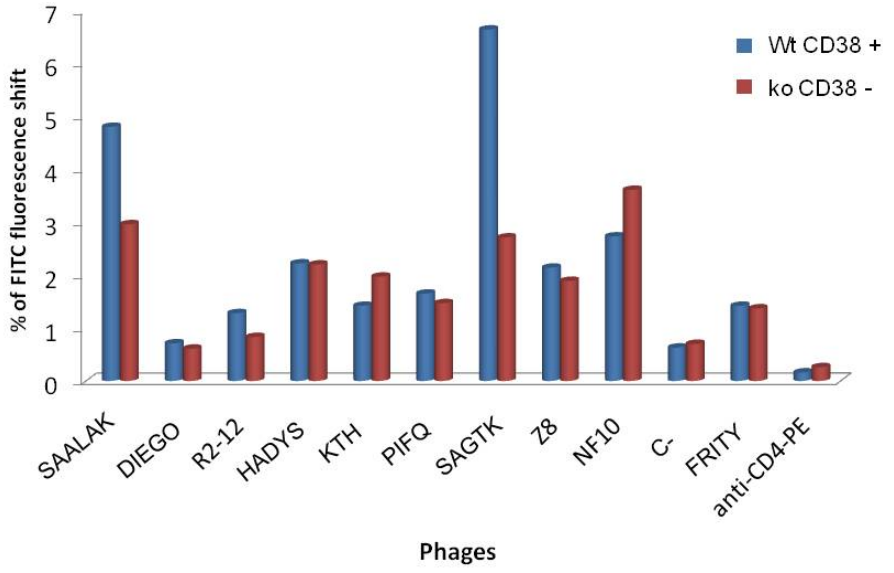

Figure 36. Reactivity of vNAR-phages to CD38 cell-surface spleenocytes. The capacity of seven families of vNAR-phages to specifically bind to mouse CD38 was tested by using spleen cells from a CD38 ko and wt mice as described in **figure 35**. Detection of bound particles were performed by an anti-M13-FITC antibody. The figure represents the percentage of emitted fluorescence by each tested family. Blue bars represent the phage binding to CD38 ^{pos} cells, whereas red bars shown the reactivity to CD38 ^{neg} cells. VHH was used as negative control of binding.

IV.11 FACS analysis with Phage-vNAR on lymphoma cell lines

For these experiments, two mouse lymphoma cell lines cell lines were used for FACS analysis: CTTL-2, which does not express CD38 on its surface ($CD38^{neg}$), and EL4-R is positive for CD38 cell-surface expression ($CD38^{pos}$).

FACS analysis was performed as described above, using $1x10^6$ cells and $1x10^{12}$ phages per sample. The differential cell surface expression of CD38 was verified with an anti-CD38-FITC antibody.

As shown in **figure 37** (**A**), EL4-R cells are positvie and CTTL-2 cells are negative for the expression of CD38. As a negative control for unspecific binding the VHH-S+16a-phage was used, this VHH molecule was raised against another target and did not bind to any of the cell lines tested. To confirm that the anti-M13g8p-FITC antibody did not react with any cell surface protein, a control staining in the

absence of any phages was performed for both cell lines, showing no recognition at all.

Nine vNAR families were tested. Eight of these (HADYS, PHIL, SAGTK, TKIL, KTH, DIEGO, FRITY and PIFQ) recognized soluble CD38 in ELISA assays (section xyz), while one (SAALAK) did not bind to soluble CD38.

The results are shown in **figure 37**. The black line represents the binding to CTLL-2 cells, the blue line the binding to EL4-R cells. All vNAR families recognize CD38-positive EL4-R cells, but do not react with cells lacking the enzyme. The results are summarized in **table VI**.

It is remarkable that phage SAALAK binds to CD38 positive cells in 10% meanwhile an individual isolated phage like R2-16 binds in 16% to CD38. The percentages of biding from remaining families are very similar in average ~ 20% which contrast with the almost null binding of VHH phage and the staining control used.

Table VI. Binding percentage of phage-vNAR families to mouse lymphoma EL4-R (CD38 positive) cell line

Family	Binding %
HADYS	24.13
PHIL	24.26
SAGKT	23.25
TKIL	20.10
DIEGO	20.61
PIFQ	20
FRITY	20
SAALAK	10
R2-16	16
VHH S+16a	0.09
Anti-M13	0.05

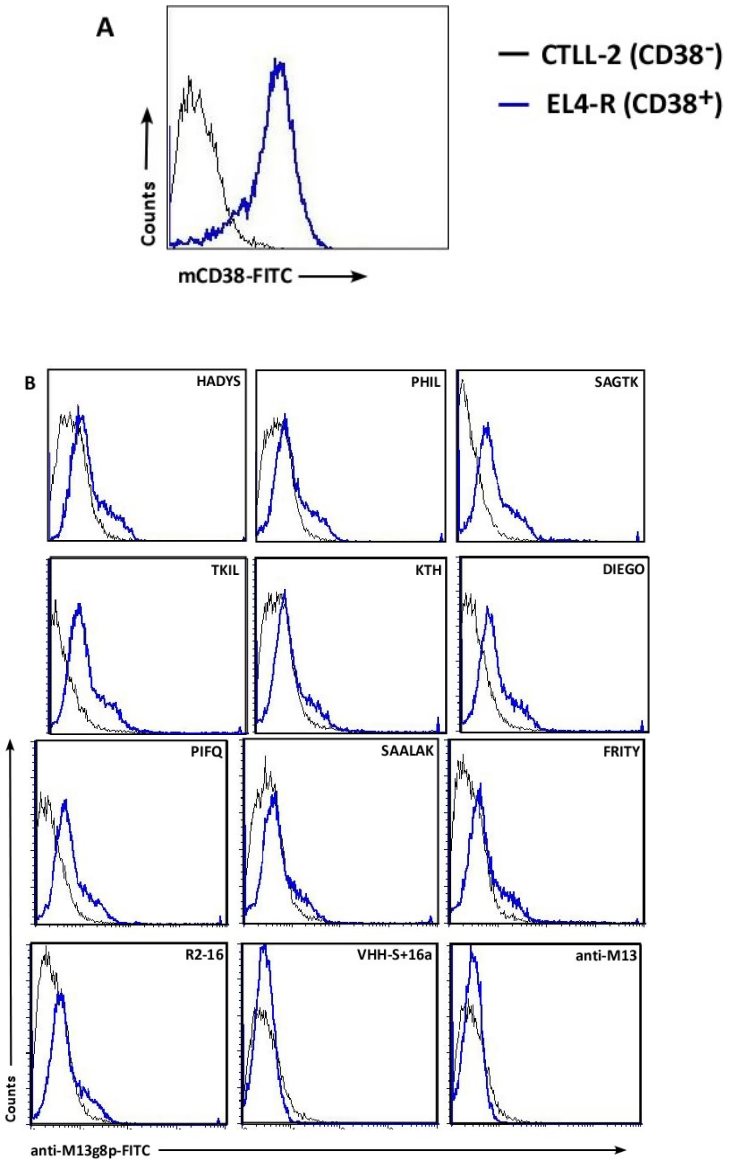

Figure 37. Reactivity of vNAR-phages on mouse lymphoma cells. The binding capacity of different vNAR families was tested by FACS analysis, using two mouse lymphoma cell lines: CTTL-2 and EL4-R. As shown in panel (**A**), CTLL-2 cells (blue black line) are negative for CD38 expression while EL4-R cells (blue line) are positive. Both cell lines were incubated with the vNAR-phages and bound particles were detected by an anti-M13g8p-FITC antibody. Panels in (**B**) represent the binding comparison of every family to CD38 negative (black lines) and CD38 positive (blue line) CD38 expressed cells. Names of each vNAR family tested are shown in the upper right corner of each panel.

Surprisingly most vNARs did not bind to mouse spleen cells, regardless of whether they were tested as phages or as purified proteins (not shown), even though the expression level of CD38 is higher on spleen cells than on the EL4-R lymphoma cells. So far, only clone SAGTK (D6) was able to discriminate between wt and CD38 ko splenocytes.

CHAPTER V

RESULTS SECTION II

Generation of secondary antibodies directed against vNARs

At present there is no way to detect the development of specific IgNAR levels during a shark's immune response. We therefore wanted to generate molecular tools for this purpose. To this end, three mice were immunized with a purified vNAR (SAALAK). The sera from these mice were tested for recognition of vNARs by ELISA using diverse vNARs and control proteins as antigens. All sera specifically reacted with different vNARs, but not VHHs or other proteins. In addition, all sera specifically recognized vNARs in immunoprecipitation, Western blot and dot blot assays. Therefore, monoclonal antibodies were generated against vNAR by fusing spleen cells from an immunized mouse with a myeloma cell line. Several clones were originally isolated from this fusion, but some of these lost their antibody-producing capacity during the early phase of cell culture. Finally, two vNAR-specific IgG1κ monoclonal antibodies (370 and 533) were retained after subcloning. In the following sections I will present more detailed results from these experiments.

V.1. Immunization of mice with purified vNAR

Three BALB/c mice were immunized for 3 months on every third week with 20 mg of endotoxin-free purified SAALAK-vNAR in an emulsion with the adjuvant Specol. Sera were obtained from the mice before beginning and after completing the immunization protocol. To confirm if the immunized mice (named 3535, 3536 and 3537) had developed an immune response against the vNAR SAALAK, an ELISA assay was performed, as shown in **figure 38**.

The ELISA plate was coated with vNAR, and with VHH, protamine, and ART 2.2 as specificity controls. All sera were used at a dilution of 1:1000. An immune serum

from a mouse immunized against ART 2.2 (i.s. ART 2.2) was utilized as a positive control. Binding was detected by an anti-mouse-IgG antibody conjugated with peroxidase.

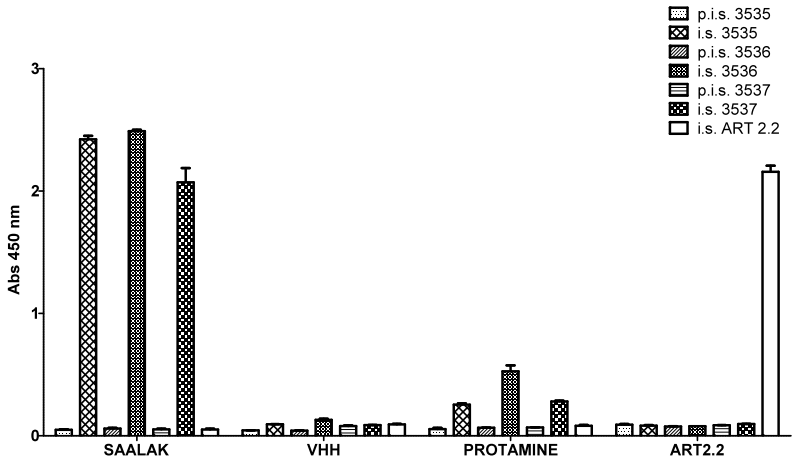

Figure 38. Specific antibody response of vNAR immunized mice. To detect the specific reactivity of mouse immune sera against vNARs an ELISA assay was performed using the pre-immune (p.i.s) and immune sera (i.s) from mice 3535, 3536 and 3537. It was used as controls for unspecific binding a VHH, protamine and ART2.2 protein while immune mouse serum raised against ART2.2 protein was used as internal positive control. The ELISA plate was coated with the antigens and after blocking, the mouse sera were added at 1:1000 dilution, after several washing steps, bound antibodies were detected using an anti-mouse-IgG peroxidase antibody. The graphic depicts the mean and SD of binding reactivity from each sera in a threefold independent experiments.

As shown in **figure 38**, the pre-immune sera did not react with any of the antigens. The three immune sera showed a slight reaction against protamine, but reacted strongly against the SAALAK-vNAR. It was also evident that the immune sera did not react either with VHH or ART2.2 proteins. The anti-ART2.2 positive control also gave a strong signal, indicating that the detection system was working. This experiment clearly showed that all the three mice had developed a specific immune response against the vNAR-Y2.

However, a main goal of these experiments was to generate secondary reagents with a broad specificity able to recognize many different vNARs. Thus it was tested if the antisera could recognize vNARs from different families.

For this purpose an ELISA plate was coated with 7 purified vNAR proteins (PHIL, DIEGO, Z14, Z16, GRIL, F2-6 and SAALAK) and one irrelevant antigen (VHH) as a negative control. An immune serum from a mouse immunized against the S+16a VHH was used as a positive control. As in the previous experiment, the sera were tested at a 1:1000 dilution.

The results are shown in **figure 39**. The mouse serum against VHH recognized its specific antigen, but did not react with any of the VNARs tested. By contrast, the immune sera from mice 3535, 3536 and 3537 specifically recognized VNAR molecules, but did not react with the VHH domain. Importantly, these results also show that none of the anti-vNAR immune sera recognize the c-Myc tag or the 6 histidines tail, which are present in both the vNAR and the VHH recombinant proteins.

Since all three immune sera cross-reacted with at least 7 different vNARs, one of the immunized mice (#3537) was chosen to make a fusion in order to generate monoclonal antibodies.

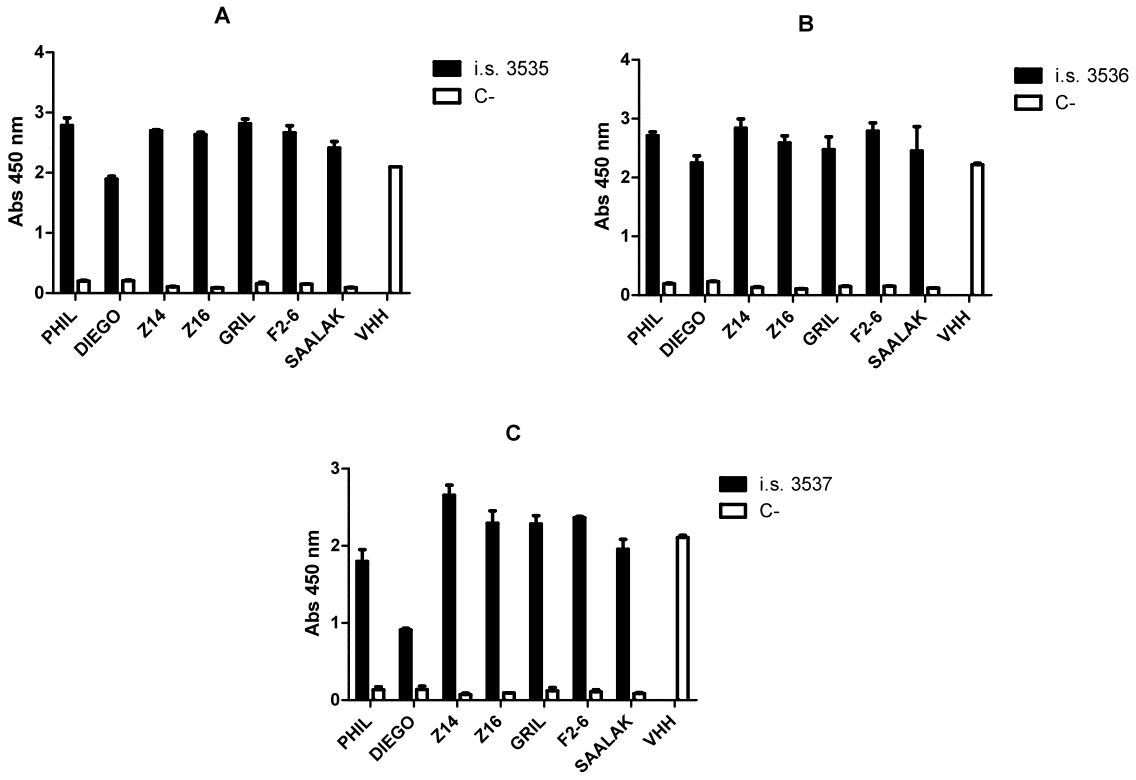

Figure 39. Immune mouse sera recognize different vNAR molecules. To test if the immune sera from mice 3535, 3536 and 3537 recognized not only the vNAR SAALAK, but different vNARs, an ELISA assay was performed by coating the plate with 120 ng of each vNAR. After blocking step the 100 µl immune mouse sera was added to each well at 1:1000 dilution, detection was achieved by an anti-mouse-IgG antibody conjugated with peroxidase. As negative control a mouse serum against VHH (white bars) was used. The reactivity of immune sera from mouse 3535 is shown in panel (**A**), sera from mouse 3536 in (**B**) and sera from mouse 3537 in (**C**). The graphics represent the mean and SD of triplicates from a threefold independent experiments.

In preparation for fusion, the immune serum from mouse 3537 was compared directly to a similar antiserum directed against VHH domains with regard to recognition of the specific antigens and to unspecific binding to a non-related protein such as protamine (**figure 40**). The plate was coated with the vNARs PHIL, DIEGO, GRIL, and SAALAK, as well as the VHH and protamine, and serum was added at a 1:1000 dilution. The results show that immune serum #3537 did not recognize either VHH or protamine, but did cross-react significantly with all 4

vNARs tested. The anti-VHH immune serum showed a slight reaction with the vNARs DIEGO and GRIL, possibly due to cross-reaction with the c-Myc or 6-histidines tags.

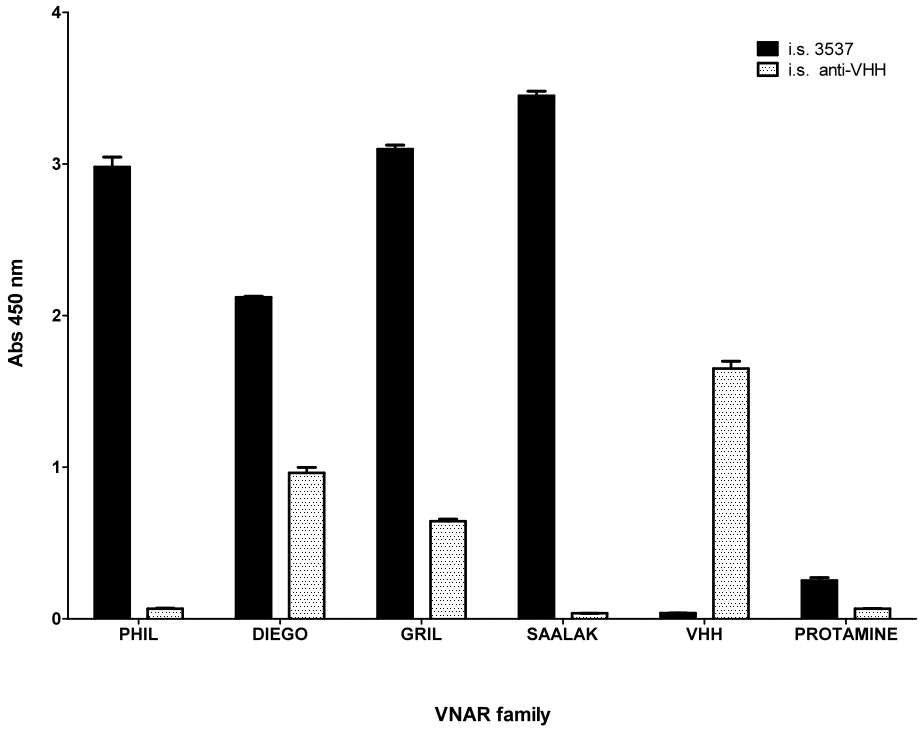

Figure 40. Immune serum from mice 3537 recognizes different vNARs. The cross reactivity of immune serum from mouse 3537 with different vNAR families was tested in an ELISA assays as described on previous figure. An anti-VHH serum was compared for binding to the indicated single-chain antibody domains, or to protamine as a non-specific control. The figure depicts the mean and SD of triplicates from three independent experiments.

V.2. Generation of monoclonal antibodies against vNAR

To generate monoclonal antibodies capable of binding to vNAR molecules, spleen and lymph node cells from the immunized mouse 3537 were fused with the myeloma cell line SP20. After the fusion, five cell aliquots of 1 ml were obtained and stored at -120°C.

Two aliquots, designated fusion 3537(1) and 3537(2), were diluted in RPMI-HAT media and plated out in twenty-four 96-well plates, and incubated at 37°C and 5% CO_2 for 14 days or until colonies were macroscopically visible in the bottom of the wells. Hybridoma clones that secreted specific anti-VNAR antibodies were subsequently selected by ELISA.

From fusion 3537(1), 595 colonies were picked and transferred to new 96-well plates containing HAT-media. After 3 days of incubation at 37°C, supernatants from these cells were tested if they recognized vNAR protein. To achieve this, ELISA plates were coated with 120 ng of purified vNAR per well. After blocking with MPBS, 100 µl from the supernatant from each picked clone was added to the wells, and, after incubation time and washes, detection was done by using an anti-mouse-IgG antibody conjugated with peroxidase.

Twenty-two clones from fusion 3537(1) were tested as positive in the screening procedure, these are compared directly in the same ELISA assay used for screening (**figure 41**). The cut off line was chosen at 0.2, which was the value of the negative control. In summary, 10 clones (79, 102, 179, 215, 332, 505, 515, 532, 533 and 535) were highly positive, while the remaining 12 clones showed a weak binding to vNAR.

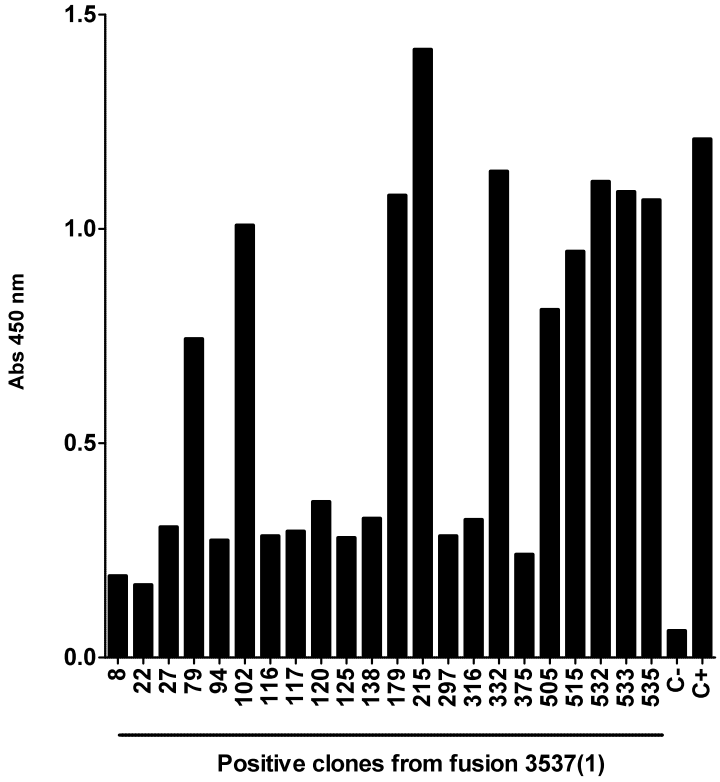

Figure 41. Reactivity of clones from hybridoma fusion 3537(1) with vNAR. To investigate the ability of the 22 selected clones from fusion 3537(1) to specifically recognize the vNAR DIEGO, an ELISA experiment was performed. Coating an ELISA plate with 120 ng of vNAR, after blocking the supernatant from fused cells were added to each well, detection of reactivity was done with an anti-mouse IgG-peroxidase antibody. The cut off for positivity was chosen at Abs $_{450\ nm}$ 0.2. The results graphed here represent the mean of triplicates from three independent experiments.

In addition, fusion 3537(2) was plated out, and 385 colonies were picked. From these, only 17 clones (4.4%) were identified as positive in a screening assay against vNAR (**figure 42**).

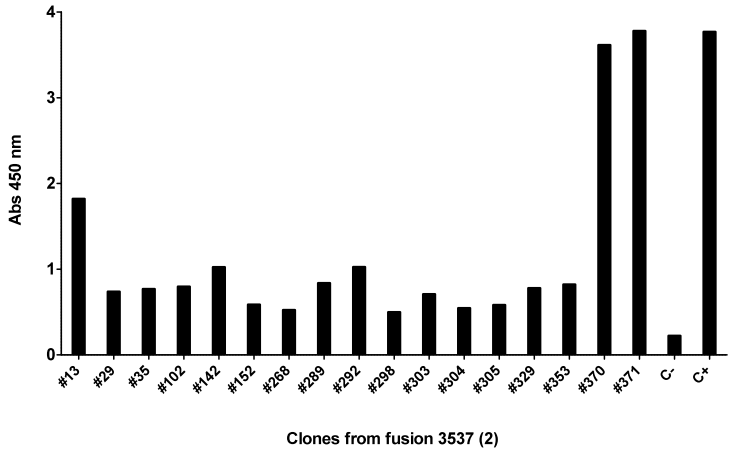

Figure 42. Recactivity of hybridoma cells from fusion 3537(2) with vNAR. As mentioned in figure 4, the reactivity of cells resulted from fusion 3537(2) was tested against the vNAR DIEGO as described in the previous figure. Threshold of positively was chosen to be 0.2 Abs 450 nm. The graphic depicts the mean of

All together, the 22 positive clones from fusion 3537(1) and the 17 clones from fusion 3537(2) were transferred to new 24-well plates and cultivated in the presence of HAT medium until macroscopically grown colonies were observed. At this point a second ELISA test was performed to determine whether the picked clones had remained positive for reactivity against the vNAR protein.

V.3. Second recognition test of selected clones from fusion 3537(1) and (2)

Positive hybridoma cells from both fusion aliquots 3537(1) and (2) were cultivated in 24-well plates in the presence of HAT medium to increase the chance that growing cells kept their ability to secrete specific immunoglobulins. After colonies were macroscopically visible, the supernatants were screened again by ELISA for reactivity against DIEGO vNAR protein; the results are shown in **figures 43** and **44**.

Figure 43 shows the second binding assay of cell fusion 3537(1). Positive clones are inidcated with black arrows. Only 6 clones (clones 8, 120, 125, 179, 533 and 535) from the original 22 remained positive.

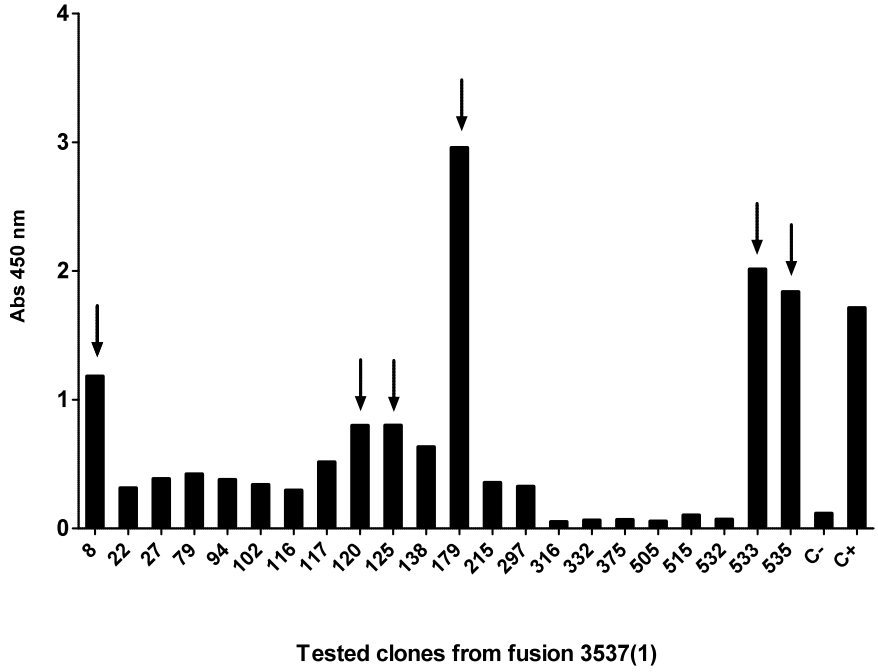

Figure 43. Second reactivity test of clones from fusion 3537(1). The capacity of selected clones from fusion 3537(1) to bind to purified DIEGO VNAR protein was tested using an ELISA plate as described previously. The threshold for positivity was set at an absorption value of 0.2 Abs 450nm, black arrows show the positive clones.

The clones from fusion 3537(2) that had been selected in the first round of screening were also tested if they had maintained their reactivity against vNAR. In addition, it was determined if they reacted to irrelevant proteins such as protamine, milk, and VHH (**figure 44**). Two clones, 370 and 371, reacted very strongly and specifically with the vNAR DIEGO and showed no unspecific reactions against other antigens.

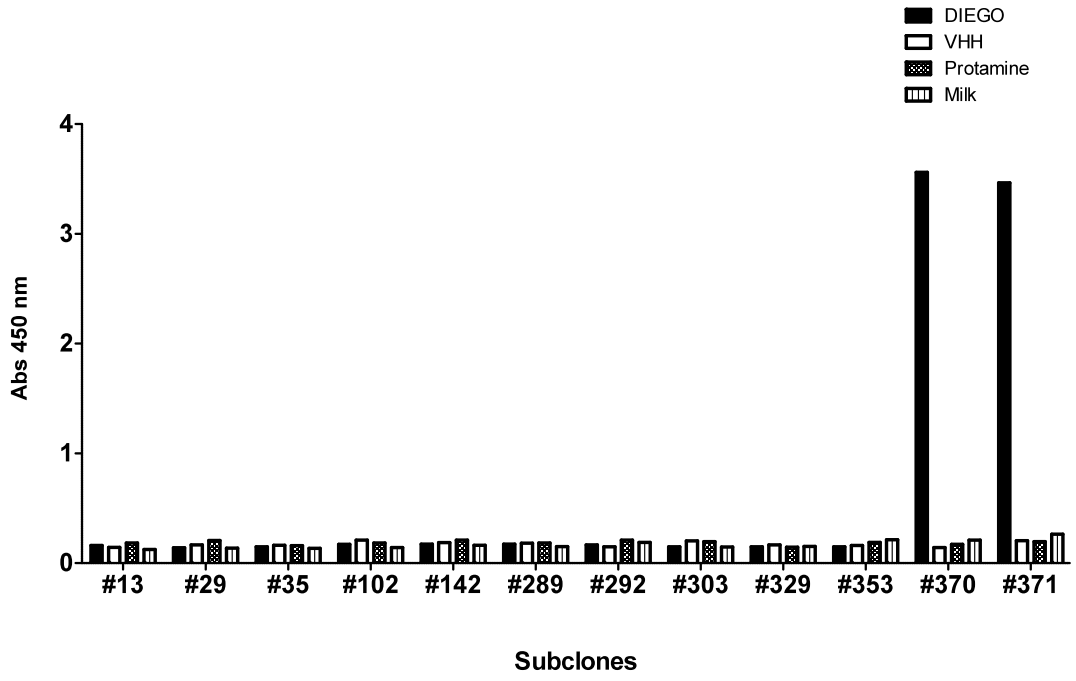

Figure 44. Second reactivity test of hybridoma clones from fusion 3537(2). Supernatants of clones from fusion 3537(2) were tested for binding to the vNAR DIEGO or to the irrelevant antigens like VHH, protamine and milk as decribed previously in material and method section. Threshold of positive clones was 0.2 Abs 450nm. The graphic shows that hybridoma supernatants from clones 370 and 371 bind strongly to vNAR, but do not react with any of the irrelevant proteins.

So far, the screening assays to identify vNAR-specific clones had only used a single species of vNAR (DIEGO) as antigen. Therefore, it was important to investigate if the isolated clones also recognized other vNARs besides DIEGO. To approach this, the supernatants from clones 8, 120, 125, 179, and 533 from fusion 3537(1) were tested for recognition of vNARs from 4 different families.

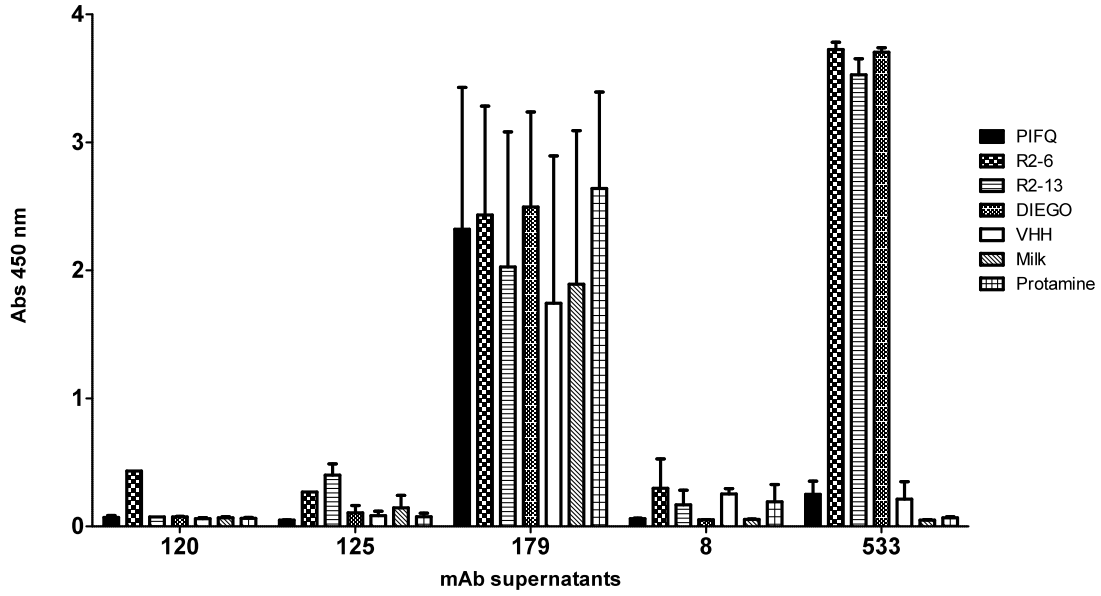

Figure 45. Cross reactivity of hybridoma cells with different vNARs. To assess the ability of selected clones from fusion cells 3537(1) to recognize a variety of vNAR molecules besides DIEGO, an ELISA assay was achieved by coating a plate with a variety of different vNAR molecules, after blocking it, cell supernatants were tested if they react with the indicated vNARs or if they have any unspecific recognition to irrelevant antigens like VHH, protamine or milk. Detection was done with an anti-mouse IgG peroxidase antibody. The results represent the mean of three different experiments made in triplicate.

The capacity of clones from fusion 3537(1) to recognize different vNAR species is shown in **figure 45.** Clone 120 recognized R2-6 but not the other vNAR families. Clones 125 and 8 showed only a low degree of reactivity, and were thus excluded from further use. Clone 179 showed a promiscuous binding behavior in that it reacted strongly also with the non-specific control antigens, and was discarded for this reason. The last clone of this series, 533, showed specific recognition of all vNARs tested, and was thus selected for further experiments. Interestingly, binding to the vNAR PIFQ was very weak compared to the binding of other vNAR species.
Up to this point three hybridoma clones (370, 371 and 533) had been identified that specifically recognized different vNAR molecules in an ELISA assay, and that did not have any apparent unspecific cross reactivity, either with other single domain

antibodies (VHH), with milk or protamine. These clones were thus chosen for further analysis.

V.4. Subcloning of hybridoma clones 370, 371 and 533

Hybridoma cells are genetically unstable in the early time after fusion and thus have a tendency to lose their capacity to secrete immunoglobulins during this period. In order to isolate genetically stable clones derived from a single cell, the clones were subcloned by limiting dilution.

To this end, each hybridoma clone was plated on a 96-well cell culture plate at three different dilutions of: 10 cells/well, 1 cell/well and 0.33 cells/well. In this case, the first dilution acted as a positive control for cell growth. Isolated subclones were taken preferentially from cells that had grown in the 1 or 0.33 dilution wells, assuming that they were from a single progenitor cell.

Hybridoma subclones were picked and transferred to 96-well plates with HT medium. After three days supernatants from all clones, were tested by ELISA for recognition of vNAR protein.

The results for the subcloning of #533 are shown in **figure 46.** Clone 125 was taken as a negative control because it had lost its ability to secrete vNAR-specific antibodies. As expected, none of the subclones of #125 recognized the DIEGO vNAR antigen. However, all three subclones picked from #533 bound to the vNARs. In further experiments, more subclones from #533 were tested, and most of them remained positive (data not shown).

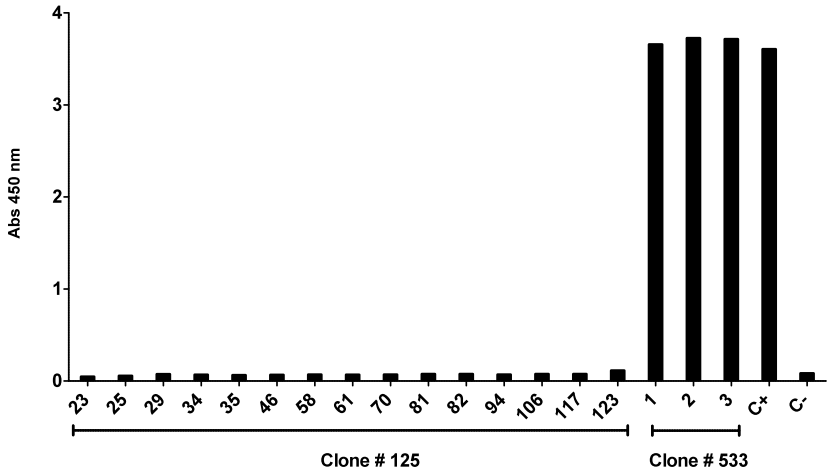

Figure 46. Reactivity of clones 125 and 533 with vNAR. Two clones from fusion 3537(1) were subcloned, isolated subclones were chosen and tested if they remained positive to react with the vNAR DIEGO. In this experiment an ELISA plate was coated with DIEGO-vNAR and supernatant from each subclone from hybridoma cells #125 and #533 were tested. Threshold of positivity was chose to be 0.2 Abs 450 nm. As it is shown, none of the selected cells from clone 125 bind to the antigen, but all the subclones from 533 were positive.

Clones 102, 129, 370 and 371 from fusion 3537(2) were subcloned, and their supernatants were tested as described above. Unrelated proteins such as VHH and protamine were used as negative controls. As shown in **figure 47**, one subclone of #102 specifically recognized the vNAR DIEGO, although only very weakly. None of the subclones of #129 recognized DIEGO, VHH or protamine. However, all the subclones tested from clones #370 and #371 strongly and specifically recognized the vNAR DIEGO, but did not react with the other antigens. These subclones were subsequently used in further experiments.

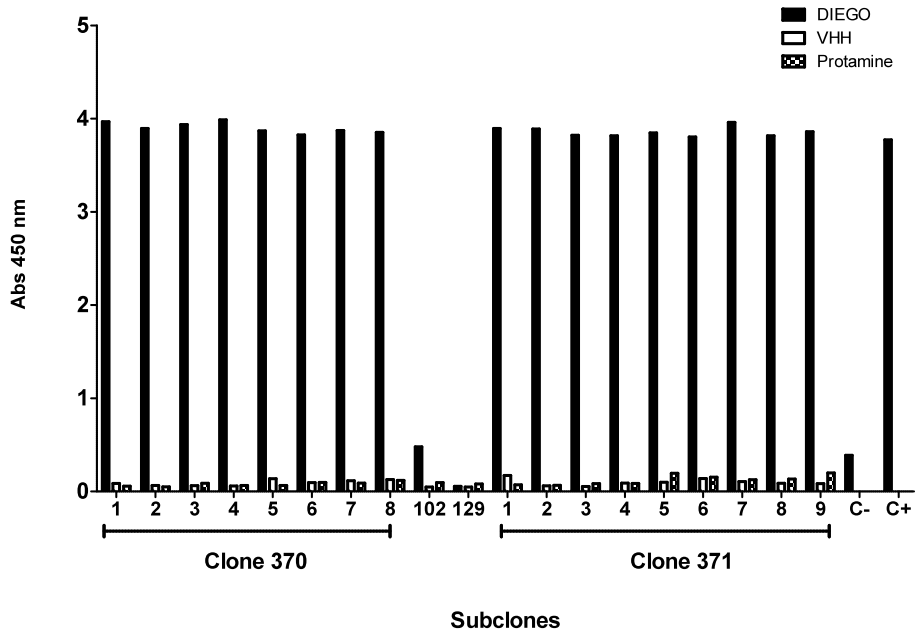

Figure 47. Subclones from #370 and #371 cells specifically recognize vNAR molecule. Subclones of hybridoma cells from fusion 3537(2) were tested if they react with the following molecules: vNAR DIEGO; VHH or protamine. An ELISA assay was performed coating a plate with the antigens and after blocking, cell supernatant from subclones were tested if they react with them. Detection was done using an anti-mouse IgG-peroxidase antibody. Subclones #370 and #371, but not 102 or 129, showed a binding reactivity to vNAR DIEGO and no cross reactivity with any other antigen.

Thus, at the end of the fusion procedure it was possible to say that I had identified three different hybridoma clones (#370, #371, and #533) that specifically recognized a broad range of vNARs, but that did not react with other single-domain antibodies or unspecific antigens. These clones were characterized in more detail in the following experiments.

V.5. Isotyping of hybridoma clones

Once it was established that subclones from hybridoma cells #370, #371 and #533 still had the capacity to produce immunoglobulins against vNARs, it was decided to determine the isotypes of the immunoglobulins secreted by these clones.

This was done using a sandwich ELISA, in which the plate was coated with antibodies against the different immunoglobulin isotypes. After blocking, supernatants from the different subclones were added to each well, and binding was detected by an anti-mouse-IgG antibody conjugated with peroxidase. **Figure 48** shows the isotyping of subclones from clones #370 (**A**), #371 (**B**) and #533 (**C**).

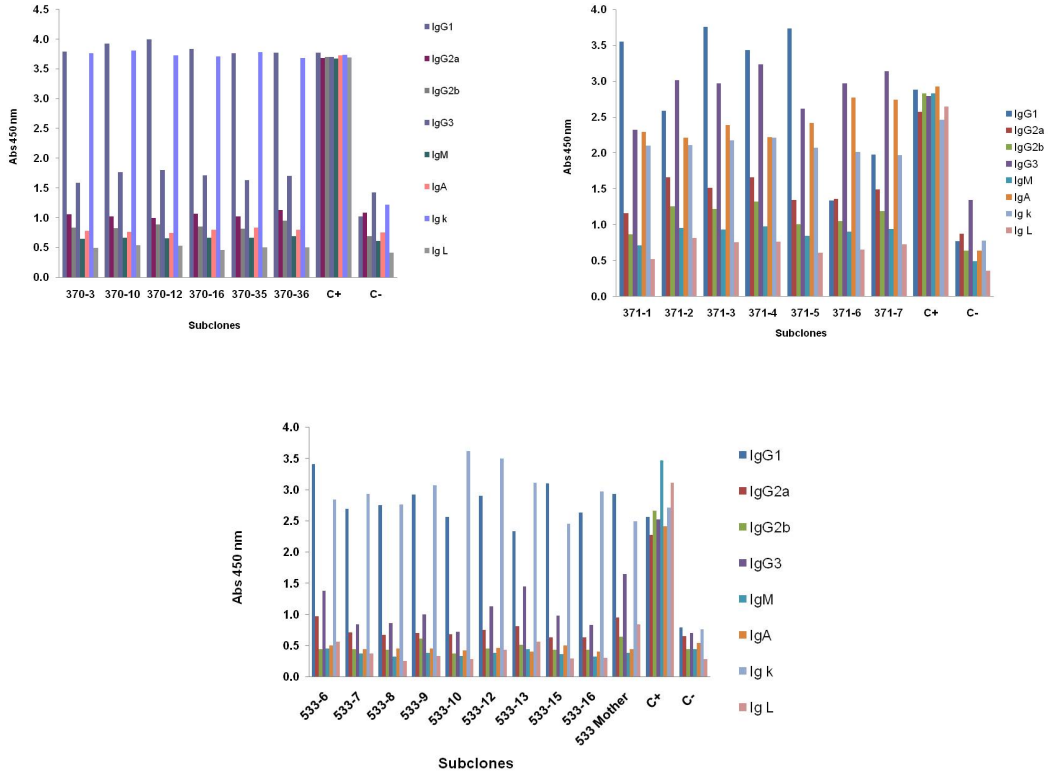

Figure 48. Isotyping of immunoglobulins secreted by hybridoma cells #370, #371 and #533. Supernatants from subclones of hybridoma cells 370 (A), 371 (B) and 533 (C) were tested to find out what kind of immunoglobulin isotype are secreted by these clones. C+ corresponds to the positive control and C- to negative control supplied in the kit. Legend on the right hand shows the color code of every isotype tested.

Figure 48 (A) shows that all the 6 subclones from clone 370 are of the IgG1k isotype, while (**B**) depicts that the 7 subclones tested from clone 371 have a mixture of different immunoglobulin isotypes. This experiment was repeated and

the results were confirmed. For this reason, clone 371 was not analyzed further for the time being. However, an aliquot of frozen cells was kept for future experiments. Finally, panel (**C**) represents the results of the isotype test of the 9 subclones from clone 533. In this case, all clones were IgG1κ.

Since clones #370 and #533 showed a high degree of specific recognition to vNAR proteins, and their isotypes could be easily determined, these clones were expanded for further analysis of their secreted antibodies. Frozen aliquots of both clones were kept as well.

V.6. Monoclonal antibody production from clones 370 and 533

In order to produce large amounts of purified monoclonal antibodies from the clones 370 and 533, one subclone of each clone was selected and grown in RPMI medium with 10% fetal calf serum (FCS). As soon as the cells had adapted to this medium, they were cultivated in RPMI with FCS that had been depleted of bovine IgG (RPMI/low IgG medium).

The use of low IgG medium allows the purification of immunoglobulins from hybridoma supernatants. It is important to slowly increase the percentage of low-IgG medium in the cell culture medium, starting at 100% complete RPMI medium and completely replacing this by RPMI/low IgG medium within a few weeks.

Subclones #370-12 and #533-10 were selected for the production of large amounts of hybridoma supernatant that were to be used for further antibody purification. Once the supernatants were obtained, it was important to test if they still contained adequate amounts of specific antibodies. To this end an ELISA was performed using seven different vNAR families (SAGTK, SAALAK, DIEGO, GRIL, HADYS, PHIL and PIFQ), as well as irrelevant proteins (VHH and milk) as antigens (**figure 49)**.

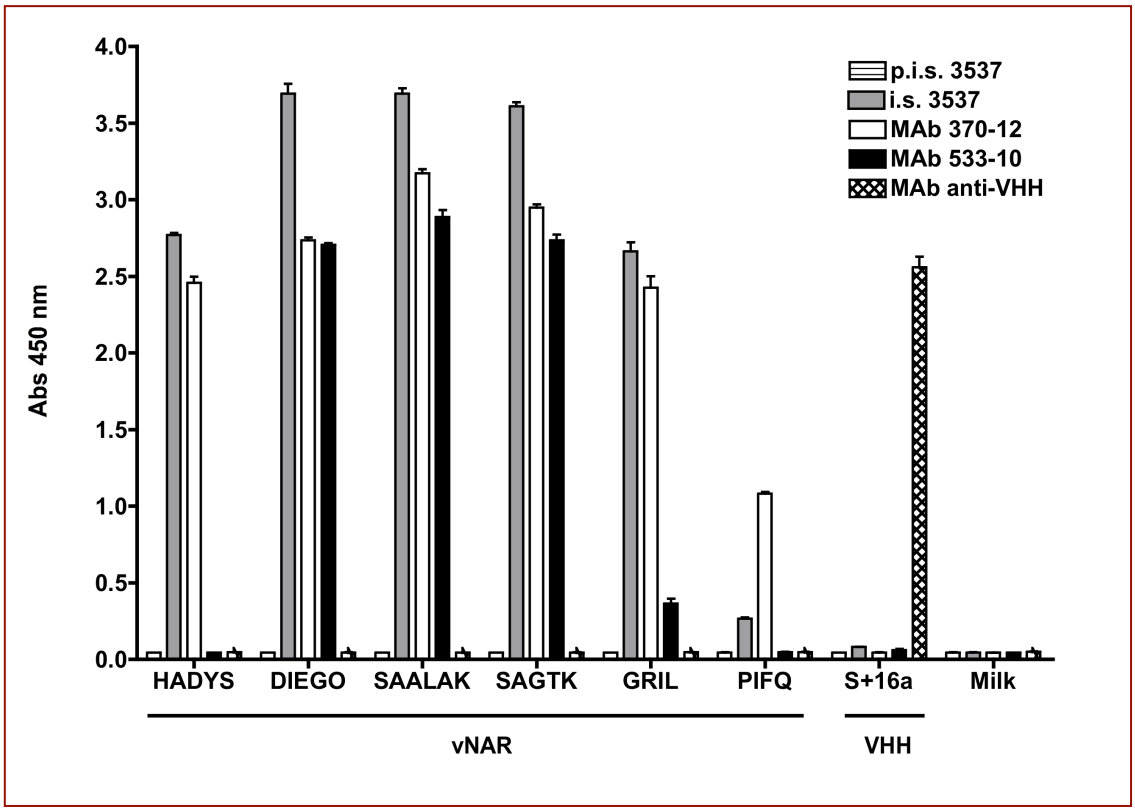

Figure 49. Hybridoma supernatants react with different vNAR families. Supernatants from subclones #370-12 and #533-10 were tested on an ELISA assays to investigate if they react with different vNAR families. An ELISA plate was coated with different antigens like vNARs, VHH or milk and after blocking the hybridoma supernatants from subclones #370-12 and #533-10 were added to each well, detection of reactivity was achieved by an anti-mouse IgG antibody conjugated with peroxidase. Pre-immune and immune sera from mouse 3537 were used as negative and positive controls respectively. A non-related hybridoma supernatant against VHH S+16a was used as negative control. The results represent the mean of triplicates from three independent experiments.

The ELISA assay shown in **figure 49** was performed using 120 ng of each vNAR family as antigens and 100 µl of each hybridoma supernatant for testing. Pre-immune and immune sera from mouse 3537 were used at 1:1000 dilution as negative and positive controls. A hybridoma supernatant derived from a non-related clone specific for VHH S+16a was used as a negative control of cross reactivity.

As shown in **figure 49**, the monoclonal antibody against VHH recognized its specific antigen, but did not react with any other molecule. None of the sera or hybridoma supernatants reacted with milk, which was used as a control for unspecific binding. As expected, pre-immune sera did not bind to any antigen, and immune sera specifically recognized all vNARs.

Interestingly, hybridoma supernatant from clone #370-12 interacted with all the vNARs tested, while the suprenatant from clone #533-10 did not react with HADYS, PHIL or PIFQ, and bound only weakly to GRIL. This result implies that, although both clones share the same IgGκ immunoglobulin isotype, they recognize different epitopes on the vNAR molecule. An alignment of amino acid sequences from the seven vNAR families is presented on **figure 50**, whereby is easy to compare the conservative regions (frameworks) and the variable regions (CDR1, HV2, HV4 and CDR3) between different vNAR molecules. As it is shown, frameworks have few differences between each other, whereas CDRs regions and the hypervariable regions 1 and 2, presented more changes in amino acids residues, being as expected the CDR3 the more variable region followed by the CDR1 and the HV2 and HV4 regions.

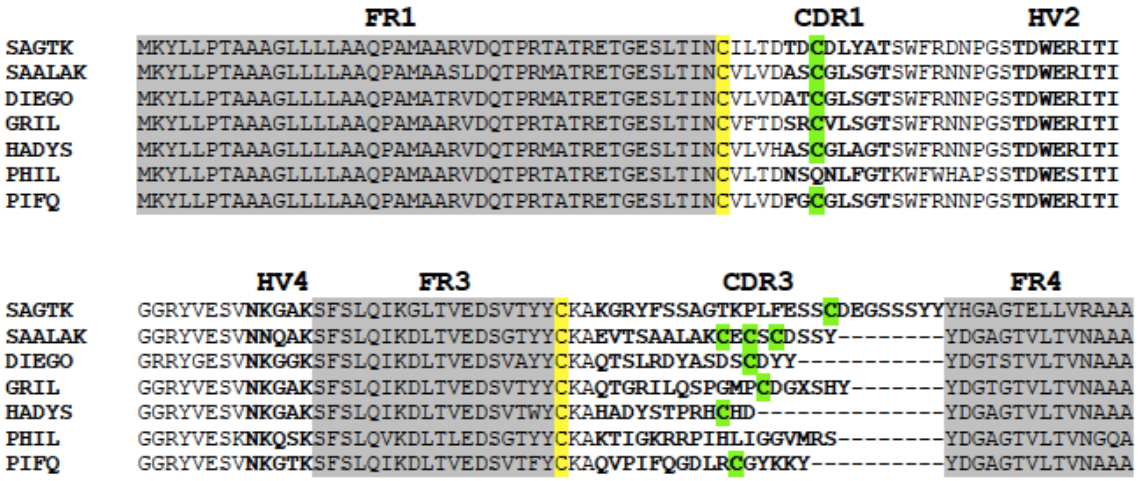

Figure 50. Amino acid sequence comparison of different vNAR families Seven families of vNAR molecules were used in this study as targets to test the reactivity of hybridoma cells supernatants #370 and #533. Here it is shown the comparison of amino acid sequences between all of them. Conservative amino acid sequence of the vNAR are represented by the framework regions (FR1- 4) whilst hypervaribale sequences are denoted bold letters (CDR1,HV2, HV4 and CDR3). Canonycal cysteines are labelled in yellow while additional cysteines are in green.

V.7. Immune mouse sera immonuprecipitated vNAR molecules

Since the mouse immune sera and monoclonal antibodies reacted with different vNARs in an ELISA assay, it was interesting to test if they could also detect vNAR proteins by other means.

Using the immune sera from mice 3535, 3536 and 3537, an immunoprecipitation assay was established. Briefly, the mouse sera were bound to protein G sepharose, unbound serum proteins were washed away, and the matrix was incubated with 1 µg of vNAR-SAALAK (Y2). The resin was washed several times with Triton X-100 1%/ 1x PBS, and samples were separated by electrophoresis in a 12% SDS gel and transferred onto a nitrocellulose membrane that was subjected to silver staining (**figure 51**).

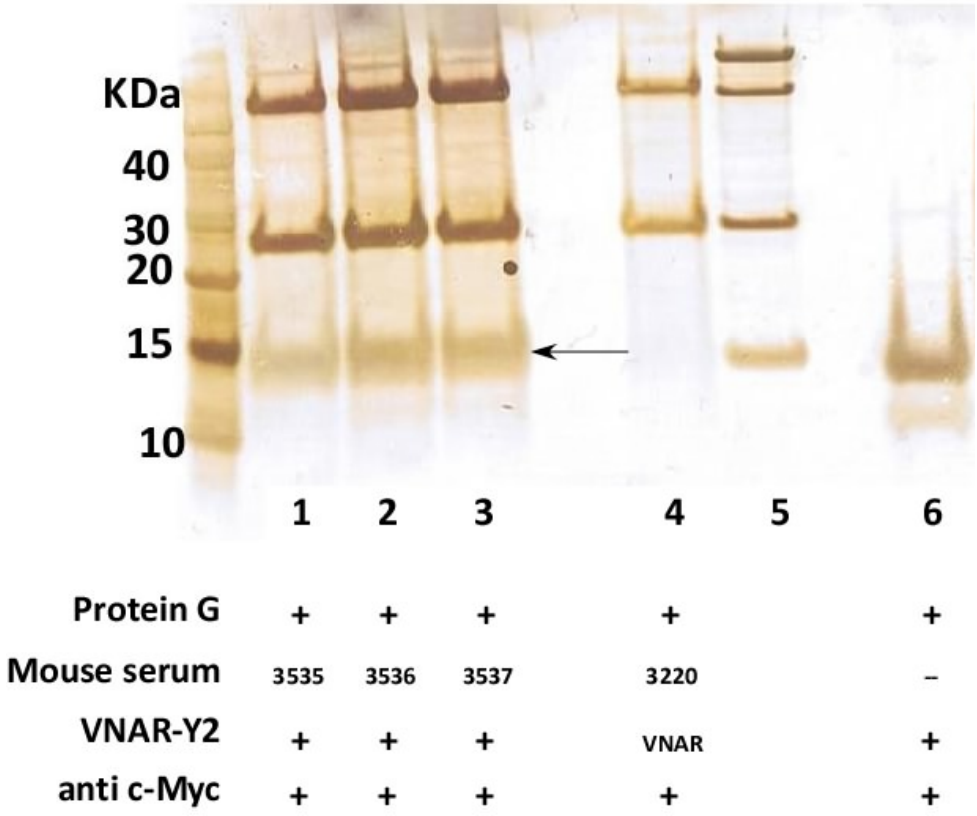

Figure 51. Immunoprecipitation of vNAR recombinant protein. To investigate the capacity of mouse immune sera 3535, 3536 and 3537 to react with purified vNAR protein, an immunoprecipitation assay was achieved. Immune sera were bound to protein G-sepharose and used to immunoprecipitate SAALAK (Y2)-vNAR protein which has an approximately molecular weight of 15 kDa (lane 6). As shown in lanes 1, 2 and 3, mouse immune sera specifically react with the vNAR denoted by the black arrow. As negative control of unspecific reactivity an immune mouse sera raised against VHH was used, as it is shown in lane 4 there was no reactivity with it. Lane 5 corresponds to a home-made molecular weight marker containing lysozyme (14 kDa), immunoglobulin light (25 kDa) and heavy (50 kDa) chains, and BSA (66 kDa).

All three mouse immune sera were able to immunoprecipitate vNAR-SAALAK (Figure 14, lanes 1 to 3). In all three samples three bands were detected. As shown in lane 4, the upper two corresponded to the heavy (50 kDa) and light (25 kDa) chains of mouse immmunoglobulin contained in the immune sera. The third

band of ~15 kDa corresponded to the purified vNAR-SAALAK (compare to an identical band in lane 6). Mouse immune serum 3220, specific for VHH was used as a negative control. As seen in lane 4, this serum did not bind the SAALAK protein, since just the two bands belonging to the immunoglobulins chains were present in this lane.

This experiment demonstrated that the immune sera not only recognized vNARs in ELISA assays but also were able to immunoprecipitate them. To confirm this, a further Western blot analysis was performed.

V.8. Western blot detection of immunoprecipitated vNARs

The vNAR proteins Z14 and PHIL were subjected to immunoprecipitation as described in the previous section. However, detection of the immunoprecipitated proteins was accomplished by Western blotting using an anti-c-myc antibody conjugated with peroxidase to detect the tag present in the carboxy terminal of recombinant proteins. In this experiment, the immune sera were also incubated with the S+16a VHH in order to ensure that they did not bind to any irrelevant proteins (**figure 52)**.

Both Z14 and PHIL proteins were precipitated by sera 3536 and 3537, and to a lesser degree by serum 3535. There was no detectable precipitation of the VHH molecule, confirming that the sera 3535, 3536, and 3537 specifically recognize vNAR molecules.

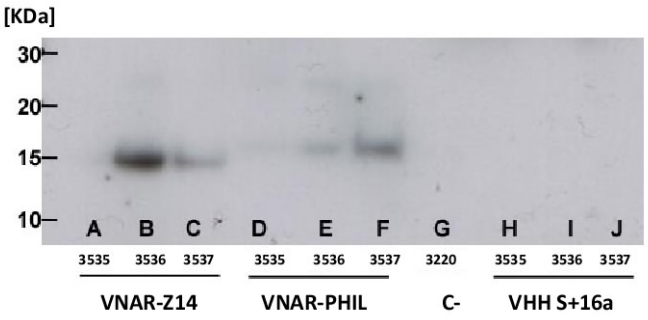

Figure 52. Western blot detection of immunoprecipitated vNARs. Immune mouse sera 3535, 3536 and 3537 were conjugated with protein G to immunoprecipitate two different vNAR molecules: Z14 and PHIL (lanes A to F) as described previously in figure 14. As negative controls it were used, a non-related immune mouse serum 3220 (lane G) and the VHH S+16a (lanes H to J). Detection of carried molecules was performed by western blot using an anti-c-myc antibody conjugated with peroxidase.

V.9. Using the monoclonal antibodies to detect vNARs and probe shark serum by dot blot

In the previous sections it was shown that the monoclonal antibodies 370 and 533 recognized a broad range of vNARs in ELISA assays and immunoprecipitation. The following experiments were designed to find out whether they could also recognize vNAR domains in the context of whole IgNAR molecules, and especially whether they could be used to specifically detect IgNAR in whole shark serum. As a first approach, a dot blot analysis was performed to test whether the antibodies could specifically recognize any antigens within shark serum.

V.10. Dot blot

In a first set of experiments it was investigated whether the mouse immune sera specifically recognized antigen in shark serum. A dot blot analysis was performed by placing 2 µl of each antigen directly on a nitrocellulose membrane and letting it dry at RT. After blocking, the membrane was incubated with a 1:1000 dilution of mouse sera. After washing, bound antibodies were detected with an anti-mouse-IgG antibody conjugated to peroxidase.

Llama serum and BSA were used as irrelevant antigens, an anti mouse VHH serum called 3220 was used to verify that there was no unspecific reactivity with the samples due to antibodies present in naive mouse serum. As illustrated in **figure 53**, the immune sera 3535, 3536, and 3537, raised against vNAR domains, strongly react to native shark serum, while the immune serum 3220, from a mouse immunized against S+16a VHH domain, shows no reaction. Surprisingly, this serum also did not react with llama serum; further results confirm that mAbs produced from fusion cells of mice 3220 do not show any cross reactivity instead all of them reacted specifically against the S+16a VHH domain.

The anti-vNAR sera showed reactivity with heat-inactivated shark serum, although the reactivity was weaker than against the untreated serum. Purified vNAR proteins DIEGO and HADYS were used as positive controls, since it was already established that the three sera recognized them in ELISA assays.

Since the anti-vNAR immune sera did not detect any antigens in llama serum, and serum from a non-immunized mouse did not detect any antigens in shark serum, it can be concluded that the three anti-vNAR sera 3535, 3536 and 3537 specifically recognize target antigens in shark serum.

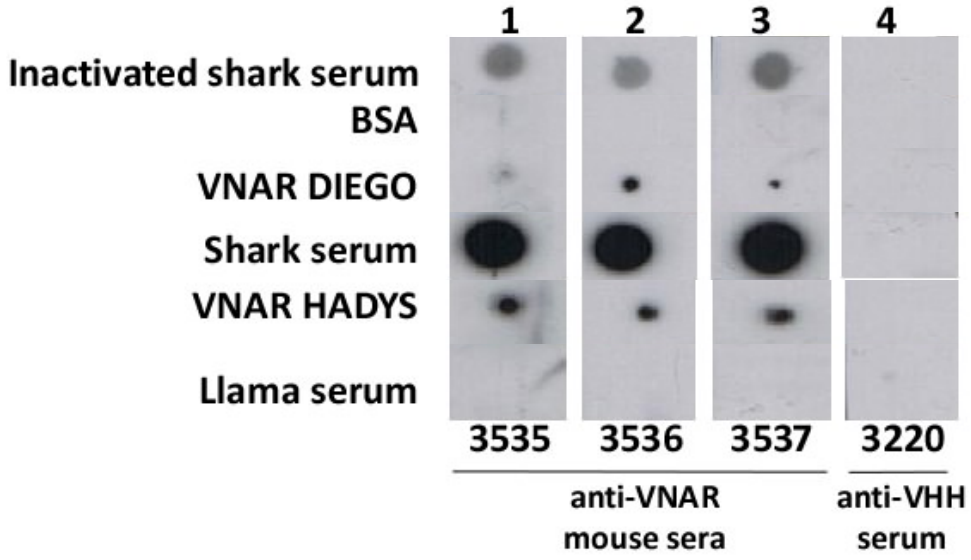

Figure 53. Detection of shark serum by dot blot analysis. Since it was previously established that immune sera from mice 3535, 3536 and 3537 specifically recognize vNAR molecules, it was interesting to investigate whether these sera react with native shark serum. To assess this a dot blot analysis was made, placing 2 µl of each antigens in a nitrocellulose membrane after blocking, the membrane was incubated with the specified mice sera at 1:1000 dilution (lanes 1 to 3), an anti-VHH immune serum 3220 was used as negative control of unspecific reactivity (lane 4). vNAR DIEGO and HADYS were used as positive controls whereas BSA and llama serum were used as unrelated antigens. Reactivity was detected by an anti-mouse IgG-peroxidase antibody.

V.10.1. Dot blot detection of shark and purified vNARs using hybridoma supernatant

The following experiments were designed to investigate whether the anti-vNAR monoclonal antibodies (MAbs) also recognized target antigens in shark serum. To test this, I performed dot blot analyses as described above, but using MAbs 370 and MAb 533, in place of mouse immune serum to detect the antigens (**figure 54**).

Figure 54 illustrates that hybridoma supernatants from clones #370 and #533 specifically reacted with shark serum (**A**) and vNARs (**B**), but not with llama or goat sera (A) or unrelated proteins like protamine, VHH or BSA (**B**). An anti-VHH (3320) and anti-vNAR (3537) mouse immune sera were used as negative and positive controls, respectively (panel **C**).

Taking these results together, it is possible to conclude that all three monoclonal antibodies 370, 371, and 533 specifically recognize different purified vNARs as well as antigens in shark serum in dot blot analyses.

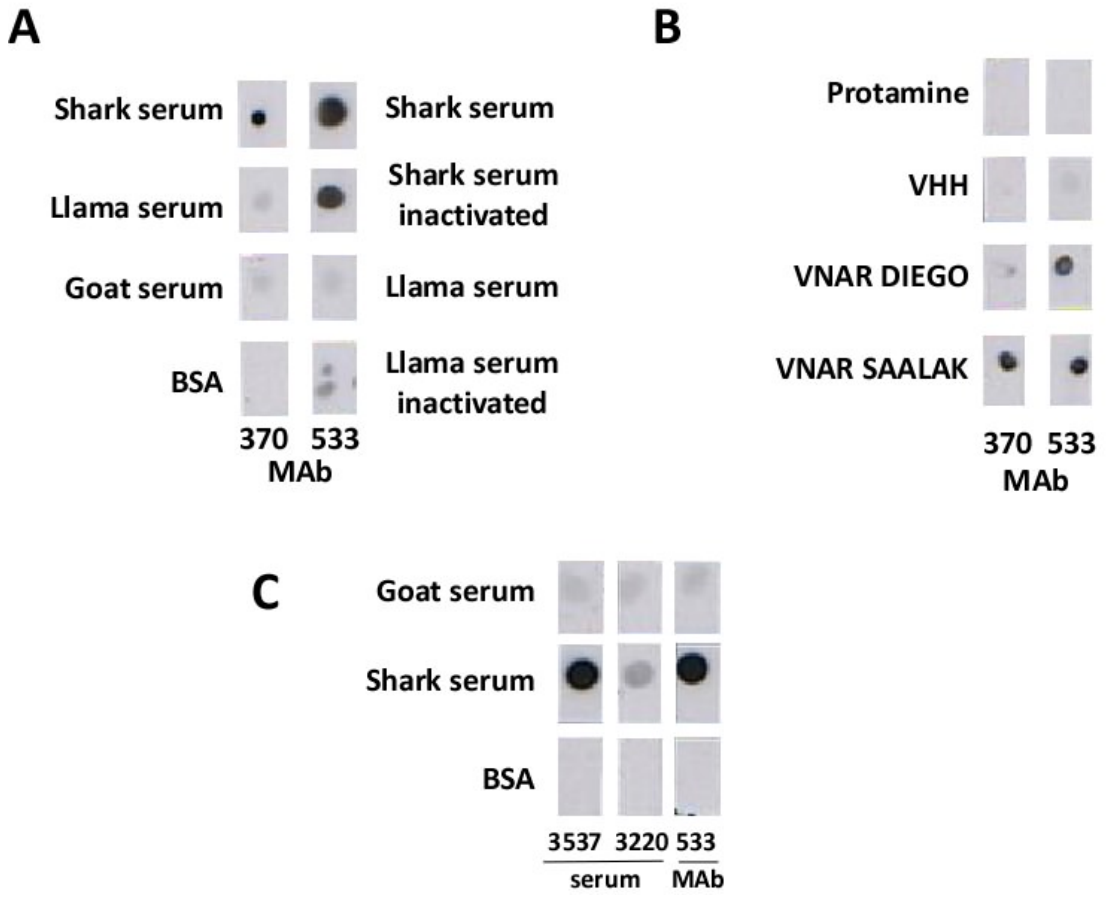

Figure 54. Monoclonal antibodies 370 and 533 specifically react with shark serum. A dot blot analysis was performed (as described in previous figure), to assess if monoclonal antibodies 370 and 533 specifically react with shark serum, as it is depicted on (**A**) as control of unspecific reactivity other sera were used such as llama and goat, BSA was used as control of irrelevant protein. As it is presnted on (**B**), MAbs 370 and 533 not only recognizes shark sera but vNAR purified proteins, such as DIEGO and SAALAK, unrelated proteins like protamine and VHH were used as controls. On (**C**) it is shown the comparison of reactivity of immune mouse sera 3537 with MAb 533 and unrelated immune mouse sera 3220. Detection was achieved with an anti-mouse IgG antibody peroxidase conjugated.

V.11. Using the monoclonal antibodies to immunoprecipitate antigens from shark serum

Since the monoclonal antibodies recognized purified vNAR domains in their native conformation, and also reacted with target antigens in shark serum in dot blot assays, it was interesting to determine if these MAbs could recognize IgNAR molecules present in the sera of the horn shark *H. francisci*. To approach this question, an immunoprecipitation assay was done. Briefly, hybridoma cell supernatant was bound to protein G sepharose. After washing, the matrix was incubated with 1 ml of shark serum, washed, and incubated with SDS samples buffer. The sample was subjected to SDS-PAGE gel electrophoresis and stained with coomassie blue. In some experiments, Western blot detection of immunoprecipitated molecules was achieved using MAb 370 as first antibody and for detection an anti-mouse IgG peroxidase conjugated antibody.

The immunoprecipitation of proteins from shark serum by MAb 533 is shown in **figure 55**. As expected, there was some direct binding of shark serum proteins to protein G even in the absence of MAb 533 (lane **A**). Two prominent bands of approximately 25 kDa and 70 kDa were identified, presumably corresponding to the heavy and light chains of IgM, the most prominent immunoglobulin in shark serum. The binding of MAb 533 to protein G in the absence of shark serum yielded three bands (lane **B).** The lower two presumably correspond to the heavy (50 kDa) and the light (25 kDa) chains of MAb-533. The upper band above 110 kDa could represent the unreduced form of IgG immunoglobulin. Several bands appeared in the lane corresponding to the immunoprecipitation of shark serum by MAb 533 (lane **C**). While some of these matched similar bands in the control lanes, two bands at 35 and 80 kDa were only found in this sample, and represent proteins specifically precipitated from shark serum by MAb 533. Furthermore, a purified vNAR domain (SAALAK) was used as a positive control for precipitation (lane **D**). Finally, untreated shark serum was loaded in the gel for comparison (lane **Sh**).

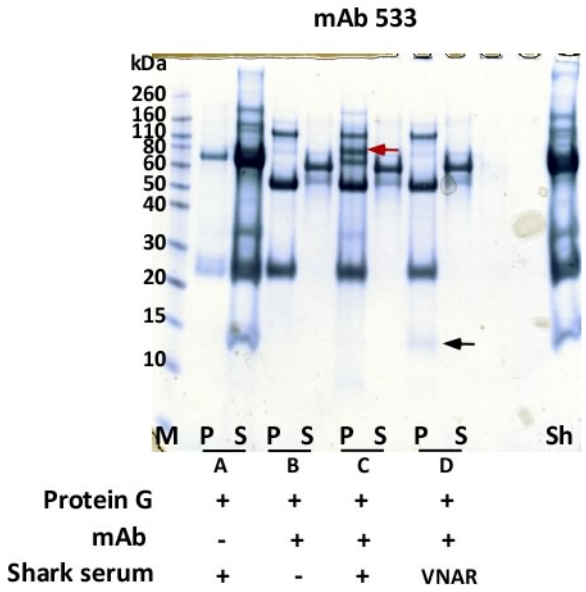

Figure 55. Shark sera immunoprecipitation by MAb 533. To address if MAb 533 was able to react with shark serum components, an immunoprecipitation assay was performed using protein G-sepharose. Naive shark serum was incubated directly with protein G sepharose (lane A), whilst protein G was incubated with hybridoma supernatant from clone #533 in the absence of shark serum (lane B), with shark serum (lane C) or in the presence of a purified vNAR-SAALAK protein (lane D). After several washing steps with 1% Triton X-100 in PBS, samples were resuspended in SDS-loading buffer to perform a further SDS-PAGE gel electrophoresis and a standard coomassie blue staining. Red arrow denoted the 80 kDa band precipitated from shark serum (lane C) and the black arrow shows the precipitated SAALAK-vNAR on control lane D.

A similar immuneprecipitation experiment was performed using mAb-370 (**figure 56)**.

The specificity controls (lanes **A** and **B**) contained the same bands as described in the previous section. However, in this experiment, three different proteins (with approximate molecular weights of 80, 160, between 160 and 260 kDa) were immunoprecipitated from shark serum by MAb 370.

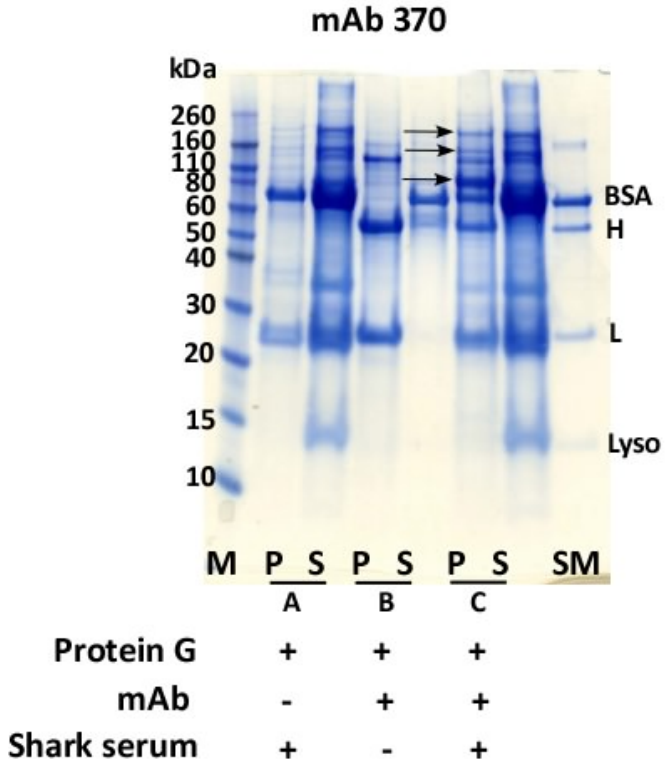

Figure 56. Shark sera immunoprecipitation by MAb-370. Immunoprecipitation of shark proteins with hybridoma supernatant #370 was performed as described previously in figure. Lane A depicts the binding of naive shark serum to protein G sepharose, lane B corresponds to the MAb bound to protein G-sepharose while lane C shows the specific immunoprecipitation of three components from naive shark serum by the MAb 370 (black arrows). SM corresponds to a home-made molecular weight standards consisting of lysozyme (14 kDa), light (25 kDa) and heavy (50 kDa) chains of an IgG antibody and BSA (66 kDa). Scheme of samples is depicted in the bottom of the figure.

The western blot detection of this blot using MAb-370 is shown in **figure 57**. The MAb did not react with any of the proteins precipitated directly from shark serum by protein G lane **A**. As was to be expected, the bands corresponding to the light and heavy chains of mouse IgG were detected by the secondary antibody used in the detection system (lane **B**). In the sample representing the immunoprecipitation of serum proteins by MAb 370, a single protein of approximately 80 kDa was detected by MAb 370 (marked by a red arrow in lane **C**), Finally, MAb-370 also detected the purified SAALAK-vNAR domain (lane **D,** black arrow).

Taking these results together, it can be concluded that the MAb370 is useful for precipitation and detection by western blot analysis, and it recognizes not only purified vNAR (Y2) but also a single protein of approx. 80 kDa in shark serum. Nevertheless the identity of this protein remains unknown. Mass spectrometry analysis is currently underway to attempt to identify this band and others immunoprecipitated by both MAb 370 and 533. However, it is possible that this analysis will remain inconclusive, given that the frequency of sequences of serum proteins from the horn shark *H. francisci* in the databases is really low.

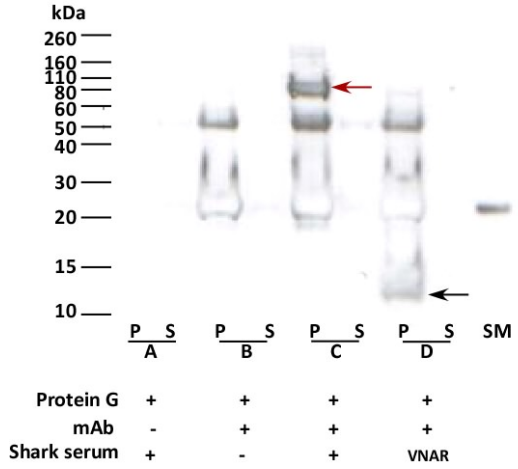

Figure 57. Western blot detection of immunoprecipitated shark sera proteins. Hybridoma supernatant #533 bound to protein G-sepharose was used to immunoprecipitate shark serum proteins as previously described previously Samples were run in a 12% SDS-PAGE electrophoresis gel and transferred into a PVDF membrane for a further Western blot analysis. Reactivity was detected using MAb #370 as first antibody and an anti-mouse IgG-peroxidase as a secondary detection molecule. Red arrow on line C shows a band of 80 kDa immunoprecipitated with MAb 533 from shark serum, whereas black arrow pointed the precipitated vNAR- SAALAK (lane D).

V.12. Using the monoclonal antibodies to follow the immune response of immunized sharks

A major reason for making monoclonal antibodies that recognize vNAR domains and IgNAR antibodies was to create tools to follow the specific immune response

of a horn shark during a course of immunization, in order to optimize immunization protocols or for basic research on the immune response in sharks.

To investigate whether the monoclonal antibodies could be used to monitor the immune response of sharks following a specific immunization, three horn sharks were independently immunized during a lapse of 149 days with different antigens: hen egg lysozyme, a synthetic peptide and human erythrocytes. Blood samples were taken before primary immunization and at the time of each boost.

The reactivity of the sera to the specific antigens was monitored by ELISA assays as follows: ELISA plates were coated with 1 µg of the individual antigens, and, after blocking and washing, shark sera obtained from every bleed were added to the plate in different dilutions. After incubation and washing steps, the wells were incubated with 100 µl of mAb-370 supernatant. The wells were washed again, and bound MAb-370 was detected with an anti-mouse-IgG peroxidase conjugated antibody.

Monitoring of the immunization against human erythrocytes and hen egg lysozyme is shown in **figure 58** (**A** and **B**). To monitor the response, ELISA assays were set up using the major human erythrocyte surface antigen, glycophorin, or lysozyme as antigens. The results of the ELISA clearly show the development of an immune response over time. While pre-immune sera showed only background reactivity to the antigens, a significant response was visible as soon as 10 days after immunization. The response peaked at 26 days. Between day 40 and day 121 there was a pause of almost 12 weeks between immunizations. During this time the antibody titers continued to decrease, but increased again when regular boosts were resumed (compare d133 to d121).

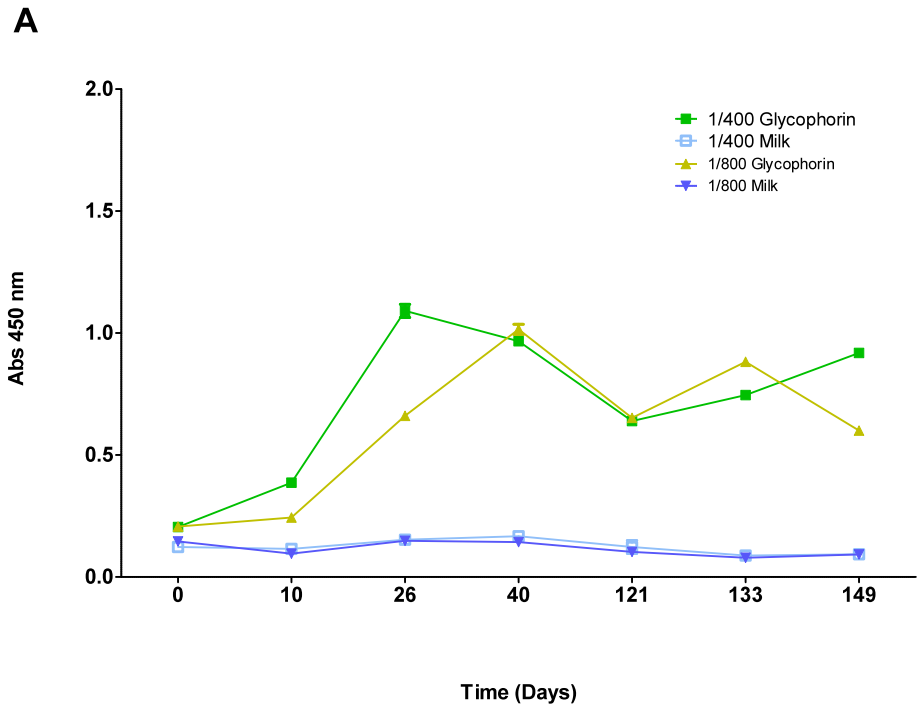

Figure 58. Specific reactivity of immune shark sera to different antigens. In attempts to investigate if the immunized sharks developed a specific response against the antigens which they were immunized with, an ELISA assay was performed coating the plate with 1 µg per well of antigen, after blocking the plate was incubated with 100µl of a twofold serial dilutions of immune shark sera and detection was achieved by adding hybridoma supernatants of MAb 370 and as secondary antibody an anti-mouse IgG-peroxidase conjugated antibody. The graphics depicted here show the reactivity of two serial dilutions of shark sera 1:400 and 1:800 (green lines) to a specific antigen like glycophorin (**A**), hen-egg lysozyme (**B**) and a synthethic peptide (**C**). Milk powder or BSA was used as negative control (blue lines). The results presented here are the mean of triplicates of a threefold independent experiments.

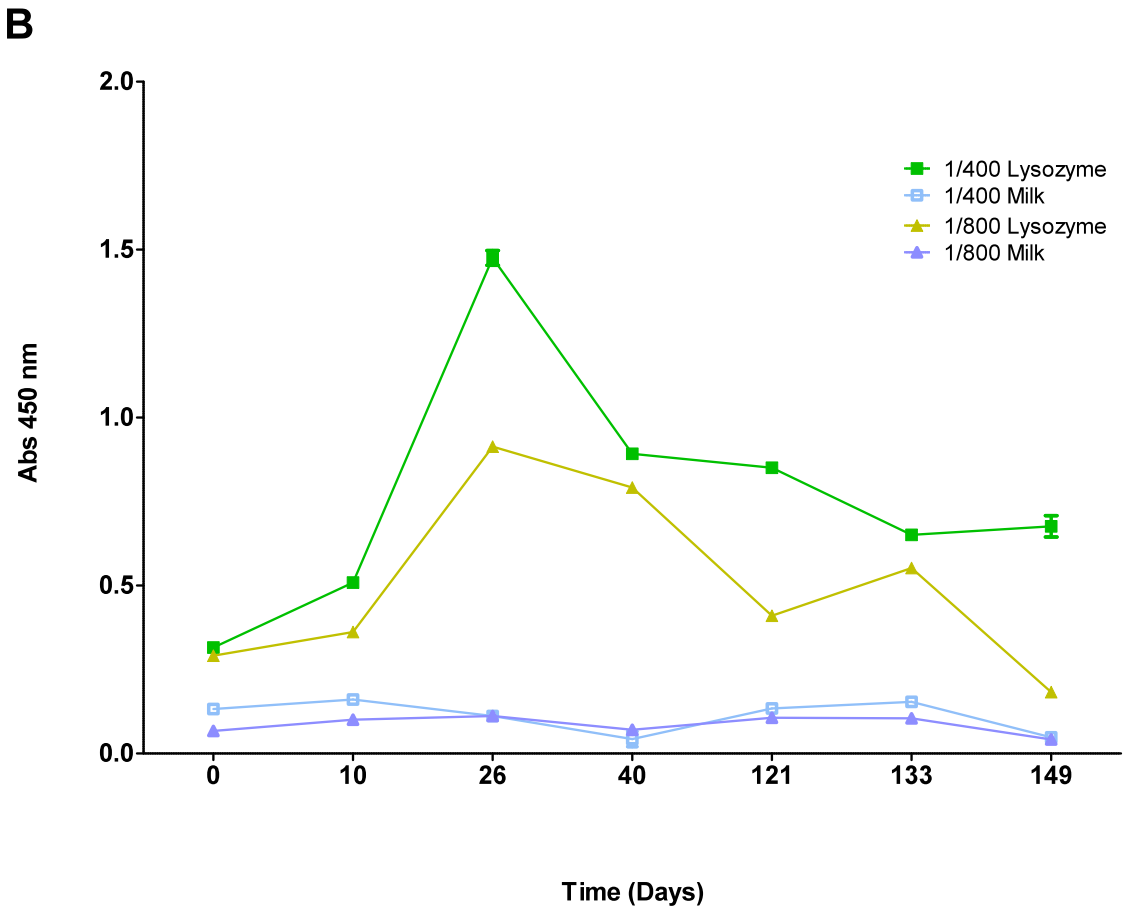

Figure 58 (Continue). Specific reactivity of immune shark sera to different antigens.

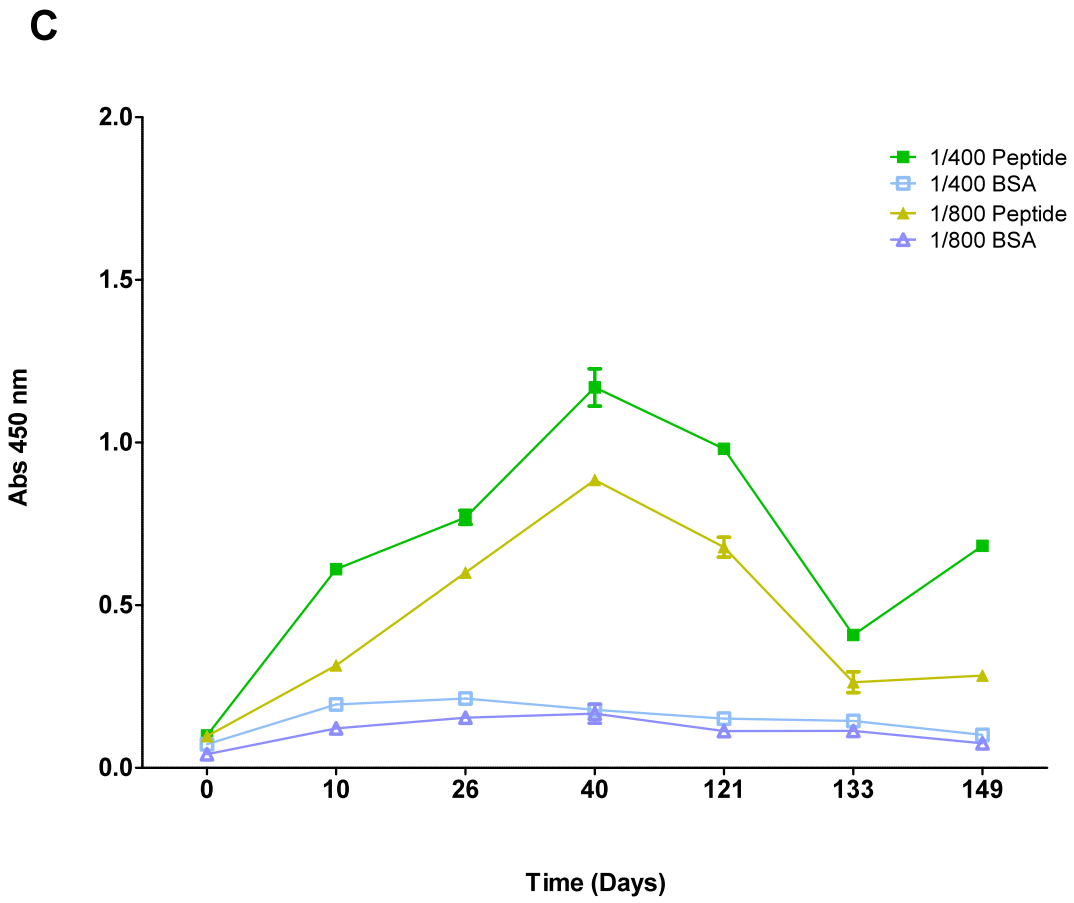

Figure 58 (Continue). Specific reactivity of immune shark sera to different antigens.

In the case of reactivity of shark serum immunized with a synthethic peptide **figure 58 (C)** it is possible to say that both dilutions have the same recognition pattern except at the very last point in which a detach is observed and dilution 1:400 tends to increase meanwhile the other tends to decrease. It is also evident that after 10 days post immunization there was already a recognition signal which increased gradually with the time until it reached the highest point at day 40, thereafter a slight decrease is observed until the last point in which a difference between both dilution is evident.

Interestingly, the binding of antibodies at low serum dilutions rose and fell more quickly in response to discontinuing or resuming booster immunizations than the binding of antibodies at high serum dilutions. In the former case, many antibodies that bind with low affinity may contribute to the signal, while in the latter case, the signal represents primarily the binding of antibodies of higher affinities. Thus, while the strength of the immune response against lysozyme, measured at high serum concentrations (e.g. dilution 1:50) fluctuates rapidly, the strength of the response measured at low serum concentrations (e.g. dilution 1:800) appears more stable (**figure 59**). This could possibly be an indication of affinity maturation of the antibody response in the course of immunization, but further experiments will be needed to address this question directly.

Further experiments using any of these monoclonal antibodies to investigate the immunological response of horn shark *H. francisci* immunized is useful. Altoguhg it is necessary to determine what kind of molecule both mAb are recognizing.

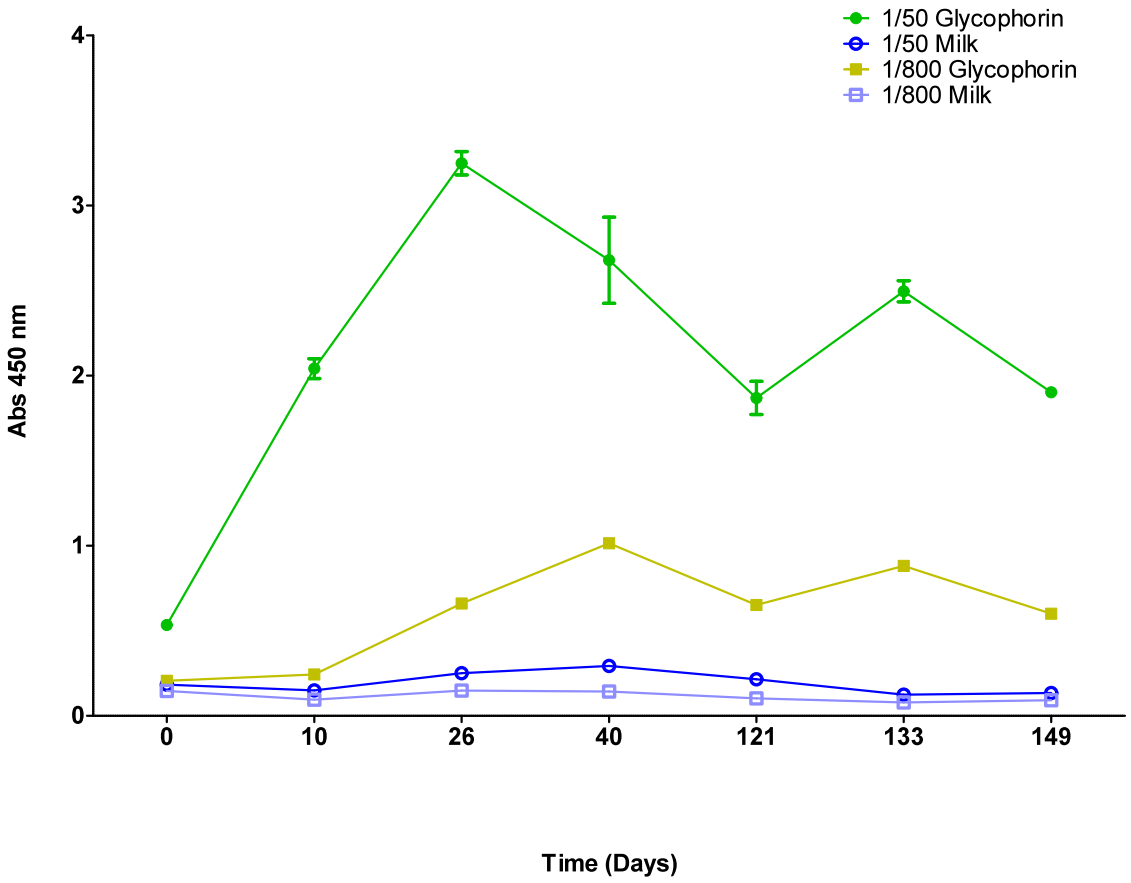

Figure 59. Immune shark sera react specifically with lysozyme. Immune shark sera in two different dilutions 1:50 and 1:800 were used to compare its reactivity to lysozyme (green lines) or to milk (blue lines). To approach this, an ELISA assay was perfomed as described in the previous figure. Supernatant of MAb-370 was used as first detection antibody and as secondary molecule an anti-mouse IgG-peroxidase antibody was used. The results presented here are the mean of triplicates from three independent experiments.

CHAPTER VI

DISCUSSION

Due to the relevance of CD38 protein as a prognostic marker in chronic lymphocytic leukemia (CLL) and to the lack of specific inhibitors for this enzyme, one of the main goals of this study was to isolate heavy chain single domain antibodies (vNAR) from the horn shark *Heterodontus francisci* that are able to bind to CD38 and to inhibit its intrinsic NADase activity.

To achieve this objective, one *H. francisci* shark was immunized for four months with CD38 recombinant protein produced in yeast, after the immunization protocol was completed, the spleen from the animal was obtained to generate an immune vNAR library.

Although the initial library was not extensive in size (1.37 $x10^6$ c.f.u.), it showed an acceptable degree of diversity. Sequencing analysis of randomly picked clones from the unselected library showed that 73% of the clones contained vNAR sequences, and from them, 59% had a correct open reading frame. The comparison of their CDR3 amino acid sequences revealed that all of them were different, confirming the variability of the starting immune library (**figure 20**).

The phage display technique was used to isolate bacteriophages that specifically bound to CD38 protein. Two kinds of panning selection were performed. The first consisted of immobilizing the antigen to a solid platform, in this case an ELISA plate, and was therefore denoted as solid-phase panning. The second method used a resin conjugated with streptavidin to immobilize the previously biotinylated antigen, and was therefore referred to as biotinylated panning. Both methods have been described for the successful isolation of phages (Hawkins et al, 1992, Tseng-Law et al, 1999).

Phages produced in every round of solid-phase panning were titered, and a classical behavior of enrichment of specific- bound phages was observed. The high titer observed in round zero reflected the unselected initial library, while in rounds 1 and 2 the titer of output phages diminished due to the specific selection of phages

able to bind to the antigen. Finally, in the last round, the titer increased because of the replication of specific phages isolated in previous rounds (**figure 21**). For both pannings three rounds of selection were performed, and the output phages from each round were tested to investigate if they specifically react with the protein CD38 on an ELISA assay. As shown in **figure 22**, an enrichment of specifically binding phages was observed through all rounds.

The clones isolated from each round of solid-phase or biotinylated panning were sequenced and analyzed, and it was seen that some clones partially overlapped in the sequences of their CDR3 regions. From the alignment of the amino acid sequences, it was possible to classify the clones into “families” on the basis of characteristic “signature” motifs within their CDR3 amino acid sequences.

Some clones had identical CDR3 regions, but showed differences in other regions. Examples are shown in **figure 24.** Clones CO12 and CD38-10 were grouped into the family HADYS, because they have an identical CDR3, although they differ in the sequences of their CDR1 and HV2 regions. Similarly, clones BP1 and BP10 were grouped into the GRYYT family, because they share this amino acid signature in the CDR3 region, although they differ in other regions. Altogether, it was possible to identify 16 families, 4 of which were isolated from both solid-phase and biotinylated pannings (**table V**). Some individual clones were also isolated by panning selections, but did not turn up again in subsequent panning rounds.

The repeated isolation of VNAR clones whose CDR3 are identical or similar in independent panning experiments most likely reflects the ability of these molecules to bind to major exposed immunodominant epitopes on CD38. So far, to my knowledge, the isolation of vNAR families has not been reported specifically by other authors. However, Dooley and coworkers (2006) have shown that IgNAR antibodies are subject to affinity maturation, and that this is accompanied by similar punctual changes of amino acids within the CDR3. In their work they identified the original germ-line sequences of vNAR molecules directed against hen-egg lysozyme, and demonstrated that point mutations in the CDR3 were associated with an increased binding affinity to the antigen.

Therefore it seems likely that the reiteratively found clones with similar CDR3 regions are the products of an affinity maturation process. Families of related VH-domains differing by point mutations in their CDR3 regions have also been isolated during antigen-specific panning of camelid heavy-chain single-domain antibody (VHH) libraries derived from immunized llamas (Mandy Unger, personal communication). Thus, the observation that antigen-specific vNARs are isolated in related families is in line with and supports the hypothesis proposed by Dooley et al. (2006) that IgNAR antibodies are subject to antigen-driven affinity maturation.

To investigate whether the vNAR families recognized CD38, it was necessary to express them as recombinant proteins. To do this some members of each family were randomly selected and induced for protein expression. However, there were some clones that did not express any protein, although their nucleotide sequences had the correct reading frame (**figure 26**). For instance, expression of vNAR protein from some clones isolated during the second round of biotinylated panning was only barely detected by Western blot, except for the clone BP1, which was strongly detected by Western blot and even visible in Coomassie blue staining (**figure 27**). Although both clones BP1 and BP10 belong to the same vNAR family, because they share the amino acid sequence GRYYT in the CDR3, their amino acid sequences differ in other regions of the vNAR. It is thus likely that these changes are responsible for the differences in protein expression levels observed between the clones. However, some other vNAR clones, such as SAALAK, HADYS and DIEGO, expressed proteins in a satisfactory level, permitting productions of 1 to 2mg per 100 ml of bacterial culture (**figure 25**).

To overcome the low protein production yields of the vNAR molecules, a yeast expression system in *Pichia pastoris* was used. With this it was possible to increase the yields of recombinant protein obtained to approx. 8 mg of total protein per 100 ml of yeast induction culture (**figure 29**). This revealed that the yeast expression system was efficient to increase the yield of shark single-domain antibody production. However, vNARs that were not possible to express in bacteria also did not express in the yeast system.

One possibility to explain the low expression of the vNAR molecules may be the differences in preferential codon usage between bacteria, yeast and sharks; it is possible that the differences in the use of tRNAs influences the expression of shark single domain antibodies. Another possibility could be the instability of vNARs as transcripts, or the toxicity that these sequences could have for the bacterial strain. However, the low yield in protein production is a phenomenon that has also been observed for other vNAR molecules (Dr. Alexei Licea, personal communication), making it evident that these antibodies are intrinsically hard to express. In this context the detailed analysis of the preferential codon usage in the shark, as well as the establishment of an optimal expression system is urgent.

Recombinant vNARs were tested for reactivity against CD38 by an ELISA assay. Most of the families reacted specifically with CD38, except for SAALAK (**figure 30**). These data were confirmed by a phage ELISA (**figure 31**), an assay that used vNAR displayed on phages instead of purified proteins as antigen. Again, all the families tested except for SAALAK bound specifically to CD38, and in this test the detection levels of reactivity were even higher than in the conventional ELISA.

Once it had been established that the vNAR families bound to CD38, their ability to block the enzymatic activity of CD38 was investigated. To approach this, an assay for NADase activity based on thin layer chromatography (TLC) and using radioactive NAD as a substrate was established. In this assay system, none of the tested vNAR families were able to block the activity of CD38 (**figure 33**).

So far we had shown that the isolated vNAR families bind CD38 as a soluble protein, but do not inhibit its enzymatic activity. In further experiments we investigated if the vNARs also were able to recognize CD38 as a cell surface molecule. To test this, spleen cells from a knock-out (ko) and wild type (wt) CD38 mouse were tested.

In a first experiment, the intrinsic cell surface expression of CD38 on mouse spleen cells was assessed (**figure 34**), and afterwards the binding reactivity of vNAR phages was analyzed on spleenocytes from a ko and a wt CD38 mouse. The results showed that, although the expression level of CD38 on mouse spleen cells

was high, most of the phage families did not show any binding to the cells. The highest level of binding was shown by the vNAR phage SAGTK (D6), which reacted with 4% of the spleen cells (**figure 35**). To further explore the reactivity of the vNAR families with cell surface CD38, a pair of mouse T lymphoma cell lines, which differ in the expression of CD38 on their surfaces, was used.: CTLL-2 (CD38 ^{neg}) and EL4-R (CD38 ^{pos}). In this assay, most of the families specifically recognized approximately 20-24% of CD38 ^{pos} cells, but did not react with CD38^{neg} cells (**table VI, figure 36**).

The vNAR family SAGTK reacted with 23% of the CD38 ^{pos} lymphoma cell line, whilst the SAALAK family, which had already failed to react strongly to soluble CD38 in ELISA, reacted with 10% of CD38 ^{pos} cells.

It is interesting to note that CD38 expression level is higher on spleenocytes than in the EL4-R lymphoma cell line. However, vNAR-phages reacted more strongly to CD38 in the latter. FACS analysis using vNARs as purified proteins has not been completely accomplished for all the families, because of the lack of expression of some of them; however, the results obtained so far show no particular differences to the phage-FACS analysis in the reactivity against CD38 positive or negative cells. This fact is surprising since it was expected that purified VNARs bind better than vNAR molecules displayed on phages. Nevertheless, it was reported that protein stability can be improved by displaying them on the coat of filamentous bacteriophage M13 (Kotz et al, 2004). This may explain the differences in reactivity observed in ELISA assays using CD38 as target, where purified vNAR proteins reacted less strongly than vNAR-phages.

A possible explanation for the low binding capacity of the vNAR molecules isolated in this study towards cell-surface CD38 may lie in differences in the structure of CD38 as a soluble or as a cell-surface expressed protein. The immunization and the panning procedures were performed using soluble CD38 as antigen. If CD38 adopts a different conformation on the cell surface, this may induce the hiding of epitopes that were previously accessible in the soluble form.

A possible difference in conformation between soluble CD38 may be due to different factors. CD38 has been reported to occur in the cell membrane both as a monomer and as a dimer (Deaglio et al. 2007). It is not clear if different cell types differ in their preference for expressing one form or the other, and to what relative proportions the two forms are expressed. It is conceivable that expression as a dimer could hide epitopes that would be accessible in the soluble form. It is also known that CD38 can be recruited into lipid rafts on activated B lymphocytes. This may also change the folding of the enzyme, resulting in a different structural conformation on resting and activated cells (Deaglio, et al. 2007). Finally, the lateral interactions of CD38 with other cell surface proteins may change the structure of the enzyme or lead to the masking of epitopes. For instance, when CD38 is recruited into lipid rafts of activated B lymphocytes, it interacts directly with CD19 and indirectly with CD81 (Deaglio et al. 2007).

A possible explanation for the failure to isolate a vNAR molecule able to inhibit the enzymatic activity of CD38 in this study may be that the most accessible epitopes on the surface of CD38 might be far away from the catalytic site at the carboxyl-terminus. Hence, none of the isolated vNARs would block the active site, even though they bound to CD38.

The vNAR molecules obtained in this work showed a specific reactivity to CD38 molecule as soluble protein and as a cell surface expressed protein on EL4-R cells. It is conceivable that antibodies with different or improved binding characteristics can be isolated by increasing the size of the immune vNAR library, as reported by Vaughan et al (1998). These authors increased the size of an antibody library by a threefold logarithmic scale, and thereby increased the affinity of the isolated antibodies from micromolar to nanomolar ranges. Therefore it seems worthwhile to increase the size of the immune library against CD38, and look for new and better binders and inhibitors. Another way in which the low binding capacity of the vNARs can be improved is by making the selection rounds directly on CD38 expressing cells. A possible approach would be to first do differential panning on CD38 positive and negative cells, such as CD38-ko and

wild-type spleenocytes. This could be further improved by changing to other CD38 expressing cell lines like EL4-R cells in subsequent panning rounds. Such a protocol has already been described as a method to isolate specific ligands on cell surfaces by direct panning on cells (de Kruif et al, 1995; Marks, 2004).

A new contribution of this work is the characterization of vNAR families isolated repeatedly by two different panning strategies, and the fact that different molecules within a family may share the same CDR3 sequence, or at least a common signature motif. This supports the notion that the CDR3 region is directly involved in the recognition of the antigen, and that punctual changes in this region may be due to an antigen-driven affinity maturation process, as shown previously by Dooley and Flajnik (2005, 2006). These results suggest that also in the horn shark *H. francisci,* affinity maturation takes place as an antigen-driven process.

A second major goal of this thesis, related with the previously discussed point, was to generate secondary reagents that enable the isolation, characterization and purification of IgNARs from *H. francisci*, as well as help to understand the role of those immunoglobulins in the development of an adaptive immune response in sharks.

To accomplish this goal, three mice were immunized with a purified vNAR protein (SAALAK), and the pre-immune and immune sera from mice were taken and analyzed by an ELISA assay. The results revealed that all immunized animals had developed a specific immune response against the vNAR SAALAK (**figure 38**).

Interestingly, when the capacity of the three immune sera to react with other vNARs was tested, (**figure 39**), the immune sera recognized not only SAALAK, but bound to members of all seven VNAR families tested. Moreover, it was shown that none of the immune sera recognized the c-myc tag or the 6-histidine tail present in the carboxyl-terminus of recombinant proteins, or any other unspecific antigen like VHH, protamine, milk or ART2.2 protein. Therefore, it was concluded that the immunized mice had developed a specific immune response against a vNAR protein that recognized a common epitope present on many different vNAR

molecules, a feature that is desirable for the generation of a monoclonal secondary antibody against vNARs from *H. francisci*.

To generate monoclonal antibodies able to react with vNAR molecules, the spleen and lymph nodes from mouse 3537 were taken and fused with the myeloma cell line SP20. Approximately 900 clones resulting from this fusion were analyzed by an ELISA assay to determine whether they specifically recognize the vNAR DIEGO, yielding 39 clones that were positive (**figure 40** and **41**). However, after one week in culture, only 8 clones remained positive in a second reactivity test (**figure 42** and **43**). Clones 8, 120, 125, 179, 370, 371, 533 and 535 were cultivated in HT medium, and tested for reactivity again after one week in culture (**figure 44**). The results indicated that clones 8, 120 and 125 had a low reactivity level and recognized just one specific type of VNAR, while clone 179 showed a promiscuous binding to all antigens used, including protamine and milk. Finally, clone 533 was the only one that retained its capacity to specifically react with many different vNAR molecules. Nevertheless, the recognition of the vNARs belonging to the PIFQ family was only moderate.

This experiment showed that hybridoma cells are genetically instable early after fusion, and have the tendency to lose their capacity to secrete specific immunoglobulins. Therefore it is necessary that, once the positive clones are identified, they immediately be sub-cloned in order to isolate individual clones that retain their secretory capacity, and to keep these in culture.

In the second reactivity test against different vNAR molecules, the best reactive clones were 370, 371 and 533. After sub-cloning, some subclones were picked randomly and tested if they reacted with the vNAR DIEGO. The results were positive for all subclones tested (**figure 45** and **46**). Subsequently, the isotypes of the secreted immunoglobulins were identified. Clones 370 and 533 had the same immunoglobulin isotype IgG1k, whilst clone 371 secreted a mixture of several isotypes. For this reason it was discarded, and further experiments were performed solely with clones 370 and 533 (**figure 47**).

The specificity of the hybridoma cell supernatants was confirmed by testing them on seven vNAR molecules, one VHH and an irrelevant antigen like milk. The results from this experiment (**figure 48**) showed that both monoclonals specifically recognized vNAR molecules, and that they did not bind to other antigens. Although both clones recognized multiple vNAR families, hybridoma supernatant 370 reacted with all the vNAR families tested, while the MAb #533 did not react with the families HADYS; PHIL and PIFQ. Therefore, it is clear that both MAbs bind to different epitopes, even though they have the same immunoglobulin isotype (IgG1k) .

The fact that MAb 370 reacts with all the vNAR families tested, and MAb 533 does not, suggests that MAb 533 recognizes an epitope defined by a region, in which the families HADYS, PHIL and PIFQ diverge from a common sequence shared by the other tested vNAR families. However, the alignment of those sequences (**figure 49**) shows that in all the vNAR clones the FR1 and FR4 regions show no differences, whilst FR2 and FR3 exhibit some specific changes in individual amino acids. It is proposed that MAb 370 binds to a conservative region present in all the vNARs, but until now it is not possible to determine which it is.

At this point, it was corroborated that both MAbs react with vNAR molecules in an ELISA, as well as in an immunoprecipitation assay (**figure 50**). Moreover, the identity of these molecules was confirmed by Western blot, using the c-myc tag present in the carboxyl-terminus of the recombinant vNARs for detection (**figure 51**).

Since I had shown that MAbs 370 and 533 bound specifically to isolated vNAR domains by recognizing epitopes that were shared between multiple vNAR families, it was interesting to find out whether any one of them could react with the variable domain in the context of the whole IgNAR molecule in the serum of the horn shark. To investigate this, a dot blot assay was performed to test the reactivity of both hybridoma supernatants with different antigens (**figures 52** and **53**). The dot blot assay was chosen because, in this assay, the antigen is accessible to the antibody in its native conformation, and we did not have any information yet

whether the MAbs would be able to recognize vNAR domains in the denatured form in which they are presented in the Western blot. The results showed that both, the immune sera as well as the MAbs 370 and 533, specifically recognized a component in the serum of the *H. francisci* horn shark. Importantly, they were able to discriminate between the serum of *H. francisci* and that of other species like llama and goat. As expected they also recognized different vNAR molecules in this assay and did not react with unrelated antigens like protamine, BSA and VHH. It has thus been shown that MAbs 370 and 533 specifically recognize shared epitopes on the variable regions of IgNAR molecules using different detection methods such as ELISA, dot blot, and immunoprecipitation.

So far I had shown that both hybridoma cell supernatants specifically recognized some component present in shark serum; however the identity or this component remained unknown. Thus, an immunoprecipitation assay was performed, in which both monoclonals were immobilized on protein G-sepharose and incubated with naive shark serum according with the protocol previously described in Material and Methods. The precipitated proteins were then analyzed by SDS-PAGE electrophoresis and detected by a standard Coomassie blue staining or by Western blot, using the MAb 370 as first antibody.

The results of this experiment showed two proteins of 25 and 70 kDa present in the shark serum that bound directly to protein G. These may correspond to the heavy and light chains of IgM, the major immunoglobulin present in shark serum (**lane A, figure 55 and 56**). Both MAbs 370 and 533 specifically precipitated a protein of approx 80 kDa from shark serum (**figure 55 and 56, lane C**). Additionally, MAb 370 immunoprecipitated two more bands from the shark serum, one with a molecular weight of 160 kDa and the other one between 160 and 260 kDa (**figure 56**, **lane C, black arrows**). When the MAb 370 was used to detect these immunoprecipitated bands by Western blot, the 80 kDa band was specifically recognized (**red arrow, figure 57**). In this experiment the vNAR SAALAK was used as positive control.

To determine their identity, the immunoprecipitated bands were cut out of the gel for analysis by mass spectrometry. In these analyses partial sequences from several peptide fragments were obtained (data not shown), but due to the poor representation of sequences from *H. francisci* proteins in the databases, it has not been possible to assign them to a definite molecule yet.

Future experiments may involve the usage of these MAb antibodies for affinity chromatography to isolate the target molecules from shark serum in quantities to subject them to direct protein sequencing.

An important goal in raising the monoclonal antibodies was to generate reagents that would make it possible to follow the development of IgNAR immune responses in immunized sharks. To determine whether the MAbs could be used for this purpose, three *H. francisci* sharks were independently immunized with human erythrocytes, hen-egg-lysozyme and a synthetic peptide, and serum samples during an immunization protocol of 149 days were obtained and analyzed with the help of the monoclonals. The sharks were immunized three times at two-week intervals, then rested for approximately 3 months, and then immunized twice more at two-week intervals.

To assess the reactivity of the sera from the immunized sharks, ELISA tests were set up, using the corresponding antigens to coat 96-well plates. The shark sera were added at different dilutions, and bound IgNAR molecules were detected using the hybridoma supernatant of MAb 370. In this way the presence of antigen-specific IgNAR molecules contained in the pre-immune or immune shark sera were evaluated (**figure 58**). The results clearly show that it is possible to follow the IgNAR response of sharks in the course of an immune response, e.g. after immunization. In all cases it was possible to detect a specific immune response against the antigen 10 days post immunization. However, the point of highest reactivity was between days 26 and 40. It was shown that in the absence of antigen administration (during the rest between days 40 and 121) the levels of reactivity decreased, and after a second application of the antigen (day 133) the

levels reach a higher value. The reactivity that the pre-immune sera showed was null and in no case any unspecificity was observed.

So far it is not possible to say with absolute certainty that the reactivity observed in the ELISA tests to monitor the immune response in shark sera is due to a specific IgNAR response, because we have not established conclusively the MAB 370 recognizes IgNAR immunoglobulins. However, this seems very likely, and this conclusion is supported by several lines of evidence. First of all, MAb 370 was raised by immunization of a mouse with purified vNAR protein, and reacts specifically with purified vNAR domains. Secondly, MAb 370 clearly recognizes an antigen-specific component present in the shark serum that increases in the course of the immune response. Apart from IgNAR, this response could also be due to IgM and IgW, the other immunoglobulin isotypes described in shark serum. However, MAb 370 precipitates a protein from shark serum that migrates as a single band and is distinct from IgM, which is precipitated from shark serum by protein G alone, and is made up of a heavy and a light chain. Little is known about antigen-specific responses of IgW, but this isotype is present in serum in only very low amounts. Taken together, these observations strongly suggest that MAb 370 can detect the development of an IgNAR immune response in shark serum.

The results of the ELISA also showed that low dilutions of shark sera (<1:50) have a comparatively high degree of unspecific reactivity (**figure 59**), while at higher dilutions (1:400 to 1:800) the response is more specific. This may be because in the former case some antibodies of low affinity contribute to the response, while as the dilution increases, the reactivity of low-affinity binding molecules also diminishes. These results agree with those obtained by Dooley and Flajnik (2006), where they suggested that pentameric IgM molecules are highly unspecific and constitute the first line of defense of sharks in the immune response, but that secondary immune response are mainly based on monomeric IgM and IgNAR immunoglobulins. In addition, the same authors suggested in another paper that IgNAR antibodies pass through an affinity maturation process mediated by the antigen (Dooley and Flajnik, 2005), a mechanism that until that time had not been

reported for any shark species. However, the presence of the recombinase enzymatic machinery in secondary lymphoid organs (Dooley and Flajnik, 2005; Flajnik 2002) and the variability of the IgNARs observed in the course of an immune response support this theory. The monoclonal antibodies generated and characterized in this study will permit more studies concerning the role of the IgNARs in the development of the horn shark adaptive immune response, and will facilitate the establishment of optimal immunization protocols for the generation of antigen-specific vNARs in the future.

CHAPTER VII

CONCLUSIONS

The results obtained in this work allow us to conclude that:

1. An immune vNAR library specific against the murine protein CD38 was generated
2. It was possible to identified the presence of CD38-binding vNAR families
3. Fifthteen vNAR families that specifically recognize CD38 on ELISA assay were identified and isolated
4. It was possible to isolate at least one vNAR named SAGTK which binds specifically to CD38 expressing cells on mouse lymphoma cell lines EL4-R

Concerning with the second part of the thesis work we can conclude that:

1. Two monoclonal antibodies #370 and #533 were generated that specifically recognize recombinant vNAR proteins and shark serum
2. A protein of approximately 80 kDa in the shark serum was immnodetected by the Mab 370
3. Two other bands in the shark serum were immunoprecipitated with the MAb 370
4. An immune specific response during an immunization protocol was determine in the shark *Heterodontus francisci* using the MAb 370 as detection molecule

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APPENDIX I

III. 1 *Pichia pastoris* medium

III.1.1. Yeast Extract Peptone Dextrose (YPD) medium

For 1 liter, dissolve 10 g of yeast extract and 20 g of peptone in 900 ml of distilled water. Autoclaved for 20 min on liquid cycle. Add 100 ml of 20% glucose solution, the liquid medium can be stored at room temperature.

III.1.2. Low Salt LB agar media

For 1 liter, dissolve the following in 950 ml of deionized water:
10g tryptone
5g yeast extract
5g NaCl
15 g Agar
Adjust pH of the solution to 7.5 with NaOH and bring the volume up to 1 liter
Autoclaved for 20 min at 15lb/sq.in, let cool to 55°C and add Zeocin (100 µg/ml) as antibiotic. Mix well and pour out on Petri dishes, store at 4°C in darkness.

III.1.3. Yeast Nitrogen Base (YNB) with ammonium sulfate without amino acids media

For a 13.4% solution, dissolve 134g of yeast nitrogen base (YNB) with ammonium sulfate and without amino acids in 1 l of distilled water, autoclave.

III.1.4. Minimal Glycerol Medium + Biotin (MGYB)

For 1 liter, combine the following components
800 ml of autoclaved water
100 ml of 13.4% YNB media solution
100 ml of 10% Glycerol solution

2 ml of 0.02% Biotin solution

III.1.5 Buffered Methanol-complex medium (BMMY)

For 1 liter dissolve in 700 ml of distilled water

10 g of yeast extract

20g of peptone

Autoclave for 20 min on liquid cycle, cool to RT and add the following

100 ml 1M potassium phosphate buffer, pH 6.0

100 ml of 13.4% YNB solution

2 ml of 0.02% Biotin solution

Methanol at established concentration, in this case 2% (v/v)

III.2 Cell media

III.2.1. RPMI 1640 media supplemented with IL-2

For 500 ml of RPMI 1640 (Gibco Cat. 21875)

Add 50 ml of Fetal Bovine serum (PAA laboratories Cat. A15101)

5ml Sodium Pyruvate MEM (Gibco Cat. 11360039)

5ml L-Glutamine 200mM (Gibco Cat. 25030024)

560 µl 2-Mercaptoethanol, 50mM (Gibco Cat. 31350010)

560 µl human Interleukine-2 (Invitrogen Cat. PHC0023)

Mix and sterilize with a Sterilecup of 0.22 µm pore filter size, store at 4°C.

III.2.2. Complete RPMI 1640 media

For 500 ml of RPMI 1640 (Gibco Cat. 21875)

Add 50 ml of Fetal Bovine serum (PAA laboratories Cat. A15101)

5ml Sodium Pyruvate MEM (Gibco Cat. 11360039)

5ml L-Glutamine 200mM (Gibco Cat. 25030024)

Mix and sterilize with a Sterilecup of 0.22 µm pore filter size, store at 4°C.

III. 2.3 HAT media

For 500 ml of media add the following components:

10 ml of HAT supplement (GIBCO, Cat. 21060017)

5 ml of Sodium Pyruvate

5 ml of L-Glutamine

25 ml of Bovine serum

2.5 ml of Gentamicine (GIBCO, Cat. 15750)

III.2.4. Frozen media for hybridoma cell lines

For 50 ml of frozen cell media add the following components:

25 ml ml of Fetal Bovine serum (PAA laboratories Cat. A15101)

5 ml of DMSO (Sigma Cat. D2650)

20 ml of HAT media

Mix and sterilize with a Sterilecup of 0.22 µm pore filter size, store at 4°C.

III.2.3. Frozen media

For 50 ml of frozen cell media add the following components:

25 ml ml of Fetal Bovine serum (PAA laboratories Cat. A15101)

5 ml of DMSO (Sigma Cat. D2650)

20 ml of complete RPMI 1640 (Gibco Cat. 21875)

Mix and sterilize with a Sterilecup of 0.22 µm pore filter size, store at 4°C.

III. 4 Cell isolation

III.4.1. Lysis buffer for isolation of spleenocytes

For 1 liter, dissolve the following components Autoclave for 20 min in liquid cycle and store at 4°C, open and keep in sterile conditions

8.24 g NH_4

1g $NHCO_3$

0.037g EDTA